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Methanogens in SFR

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Abstract

In the Swedish repository SFR, low level radioactive waste (LLW) and intermediate level radioactive waste (ILW) consists of a variety of natural and anthropogenic polymers such as ion exchange resins (IER), organic material such as bitumen and miscellaneous other waste. In addition to various materials, the waste contains radioactive substances. ^{14}C is one of the radioactive substances that contributes most to future doses from the repository. Initially, ^{14}C occurs either as inorganic carbon (CO_3^{2-}) and as unspecified organic substances. The risk is that methanogens can use this ^{14}C as a nutrient source and convert it to $^{14}CH_4$ (g) after the repository is closed and reducing conditions established. $^{14}CH_4$ may have different transport properties compared to with the initial ^{14}C -containing compounds.

Methanogens are ubiquitous found in anaerobic environments and could also generate methane in the repository. However, the conditions in the repository are not ideal for microbial life because the cementitious materials used for waste management will lead to a hyperalkaline environment. Therefore, research was conducted to investigate the pH dependence on methanogenesis.

Previous work has confirmed that methanogens (Archaea) are present in SFR-groundwater. These methanogens were able to use C_1 and C_2 organic acids for methanogenesis in cultivation studies. Furthermore, the cultivable methanogens were active up to pH 10 but their activity rapidly decreases after pH 9.5. It was observed that waste simulant experiments with IER generated minor amounts of methane including an experiment buffered at pH 12.5 by cement. This should be taken into consideration because IER will be the dominating waste and has ^{14}C-compounds bound to it. Therefore, the generation of $^{14}CH_4$ through methanogenesis is possible in SFR. Finally, the investigation of microbial methane development in containers that simulated the conditions of the SFR showed that autotrophic methane production competed successfully with acetoclastic production. This process reduces the molar amount of gas from 5 to 1 which reduces the risk for detrimental high gas pressure in the repository.

Sammanfattning

I slutförvaret för kortlivat låg– och medelaktivt avfall (SFR) slutförvaras driftavfall från det svenska kärntekniska programmet. En del av avfallet utgörs av olika naturliga och antropogena polymerer, såsom jonbytare (IER), organiskt material som bitumen och diverse sopor och skrot från de svenska kärnkraftverken. Förutom olika material innehåller avfallet radioaktiva ämnen. ^{14}C är ett av de radioaktiva ämnen som bidrar mest till framtida doser från förvaret. Inledningsvis förekommer ^{14}C antingen som oorganiskt kol (CO_3^{2-}) och som ospecificerade organiska ämnen. Risken finns att metonogener kan använda detta ^{14}C som näringskälla och omvandla det till $^{14}CH_4$ (g) efter det att förvaret förslutits och reducerande förhållanden etablerats. $^{14}CH_4$ kan ha annorlunda transport-egenskaper jämfört med de initiala ^{14}C-innehållande föreningarna.

Metanogener finns överallt i anaeroba miljöer och kan också bilda metan i förvaret. Dock är förhållandena i förvaret inte ideala för mikrobiellt liv, eftersom de cementmaterial som används för avfallshantering kommer att leda till en hyperalkalin miljö. Därför genomfördes en under-sökning av hur pH påverkar metanogenes.

Tidigare studier har bekräftat att det finns metanogener (Archaea) i SFRs grundvatten. Dessa metano-gener kunde använda C_1 och C_2 organiska syror för metanogenes i odlingar på lab. Vidare var de odlingsbara metanogenerna aktiva upp till pH 10 men deras aktivitet minskar snabbt efter pH 9.5.

Resultaten visade att avfallssimuleringsexperiment med IER bildade mindre mängder metan även i ett experiment buffrat till pH 12.5 med cement. Detta bör beaktas eftersom IER kommer att vara det dominerande avfallet som har ^{14}C-föreningar bundna till sig. Därför är bildning av $^{14}CH_4$ genom metanogenes möjlig i SFR. Slutligen visade undersökningen av mikrobiell metanutveckling i behållare som simulerade förhållandena i SFR att autotrof metanproduktion till stor del konkurrerade ut aceto-klastisk metanproduktion. Denna process reducerar molmängden gas från 5 till 1 vilket minskar risken för att ett skadligt högt gastryck uppstår i förvaret.

Contents

Abbreviations used in the report

Abbreviation	Meaning
LLW	Low level radioactive waste
ILW	Intermediate level radioactive waste
IER	Ion exchange resins
FC	Flow cell
Eq	Equation
FID	Flame ionisation detector
PVDF	Polyvinyl difluoride
mcrA	Methyl coenzyme M reductase
PEEK	Poly-ether-ether-keton
PDHID	Pulsed discharge helium ionization detector

1 Introduction

The general storage concepts for LLW and ILW are underground in near surface or deep geological repositories. These wastes contain a variety of natural and anthropogenic polymers such as ion exchange resins (IER), organic material such as bitumen and miscellaneous other waste. In addition to various materials, the waste contains radioactive substances. ^{14}C is one of the radioactive substances that contributes most to future doses from the repository. Initially, ^{14}C occurs either as inorganic carbon (CO_3^{2-}) and as unspecified organic substances. The risk is that methanogens can use this ^{14}C as a nutrient source and convert it to $^{14}CH_4$ (g) after the repository is closed and reducing conditions established. $^{14}CH_4$ may have different transport properties compared to with the initial ^{14}C-containing compounds. Therefore, the general objective was to investigate the presence of methanogens in the SFR groundwater and their potential of producing methane under repository conditions.

In Sweden, low and intermediate level waste will be stored at the SFR facility. The SFR1 facility comprises 90 000 m^3 and will have a total expected radioactivity of 10^{16} Bq. The site is situated 50 m below the Baltic Sea and is divided into four rock vaults and a main silo (Figure 1-1). The Silo will contain the main part of the radioactive waste. It is approximately 50 m in height and has a diameter of 30 m. Voids between waste packages will be filled with porous concrete. Bentonite clay will be used as backfill between the Silo walls and the surrounding rock. The dominating wastes will be IER and other organics (plastics, rubber, cables). Figure 1-2 shows the distribution of organic materials in the SFR1 silo. The waste will be either encapsulated in cement or bitumen (SKB 2015a). The waste in the rock vaults consists of IER, scrap metal and trash in concrete or bitumen matrix. The vaults are 160 m long and approximately 15 m in width. The vaults will either have concrete structures for the waste storage or steel drums on a concrete floor.

The scope of this work was to consider only processes in the Silo.

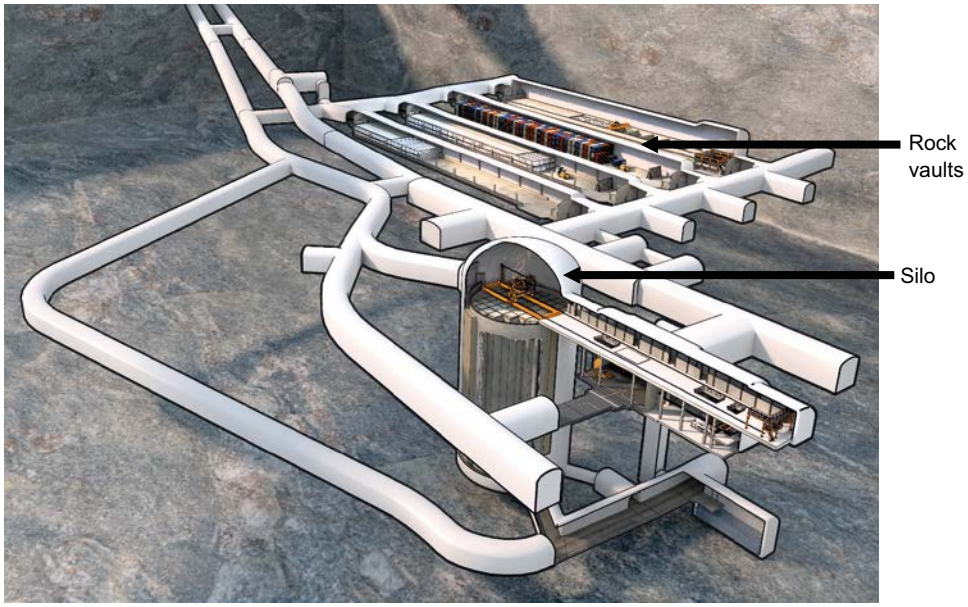

Figure 1-1. *SFR repository at Forsmark (skb.se).*

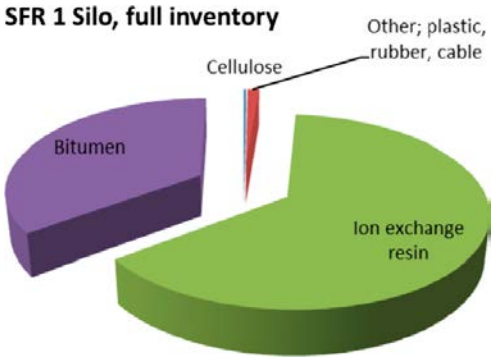

Figure 1-2. *Distribution of organic materials (by mass) estimated for the SFR1 Silo (Abrahamsen et al. 2015).*

1.1 Organic ILW materials

The organic wastes, bitumen and IER have the potential to fuel anaerobic microbial processes including C^{12}/C^{14} methane generation from consumption of hydrogen gas from anaerobic corrosion of metal and C^{12}/C^{14} carbon dioxide.

IER are comprised of an aromatic structure with functional sulfonyl or amine groups. The process of degradation has not been studied in detail, however resins used in nuclear industry are strongly resistant to radiation due to their aromatic nature (Warthmann et al. 2013, Van Loon and Hummel 1995). Biodegradation affects only the functional groups like methylamine from basic IER which are readily available and can be a substrate for methanogenesis (Purwantini et al. 2014).

The composition of bitumen depends on the source of crude oil used to produce the bitumen. However, high molecular weight hydrocarbons groups such as asphaltenes, saturates, resins and aromatics are typical. In contrast to IER the biodegradation of bitumen is well studied. Bitumen provides a carbon sources to microorganisms through leaching of water soluble fractions as well as chemical and radiolytic degradation (Walczak 2000, Eschrich 1980). However, the biodegradation is dependent on the chemical composition of the bitumen, availability of molecular oxygen and the microbial consortium (Ait-Langomazino et al. 1991, Heider and Schühle 2013, Rosenberg 2013). The rate of biodegradation is much slower under anaerobic conditions (Wolf and Bachofen 1991). CO_2, N_2 and linear carboxylic acids will be the main products from microbial bitumen degradation. Radiological degradation will form highly active free radicals from the organic compounds and generate H_2 which can be utilised by microorganisms.

1.2 pH conditions

The cementitious materials used for waste management will lead to a hyperalkaline environment which is important regarding corrosion, chemical degradation and microbial viability. Alkaline pH passivates steel corrosion which affects the amount of generated H_2 by steel corrosion (Gouda 1970). The evolution of pH in the repository is complex and will vary in space and time. pH will eventually decrease over time, but the rate is dependent on various factors like the amount of cement used.

Another important factor is radiological and chemical degradation of IER which may neutralise the pH by generation of organic acids and CO_2. Furthermore, fatty acids that are abundantly present in bitumen will be decarboxylated through abiotic processes and contribute as well to a decrease in pH (Valcke et al. 2000).

1.3 $^{14}CH_4$

An important safety aspect is the formation of $^{14}CH_4$. The largest source in the repository will be ^{14}C-compunds bound to IER from nuclear power plants (Riggare and Johansson 2001). Figure 1-3 shows the possible processes involved in $^{14}CH_4$ generation. Before microorganisms can transform the ^{14}C into $^{14}CH_4$, this radioactive isotope needs to be released from the waste and enter the water (Lindgren et al. 2001). $^{14}C_{org}$ encapsulated in bitumen is very resilient to degradation and will probably not escape (Neretnieks and Moreno 2015). However, $^{14}C_{org}$ encapsulated in cement will escape and dissolve in pore water. The water chemistry will be influenced by the cement present in the repository which creates a high pH at which organic acids in the pore water will dissociate, not to volatile but to mobile phases. Microorganisms can generate $^{14}CH_4$ from the organic acids together with the H_2 from metal corrosion. Figure 1-3 shows that cellulose can be degrade to carbon dioxide and non-radioactive volatile methane. The generated non-radioactive CH_4 considerably dilutes the radioactive CH_4 because there is totally about 250 g of ^{14}C in the entire SFR1 but the Silo contains 7 950 kg cellulose that potentially can be transformed to $^{14}CH_4$ (Almkvist and Gordon 2007, Neretnieks and Moreno 2015). Finally, gas bubbles from corroding metals or gas streams can carry formed $^{14}CH_4$ to the biosphere (Walczak 2000, Pedersen 2001). However as mentioned above, the $^{14}CH_4$ generation is dependent on possible microbial activity under alkaline pH.

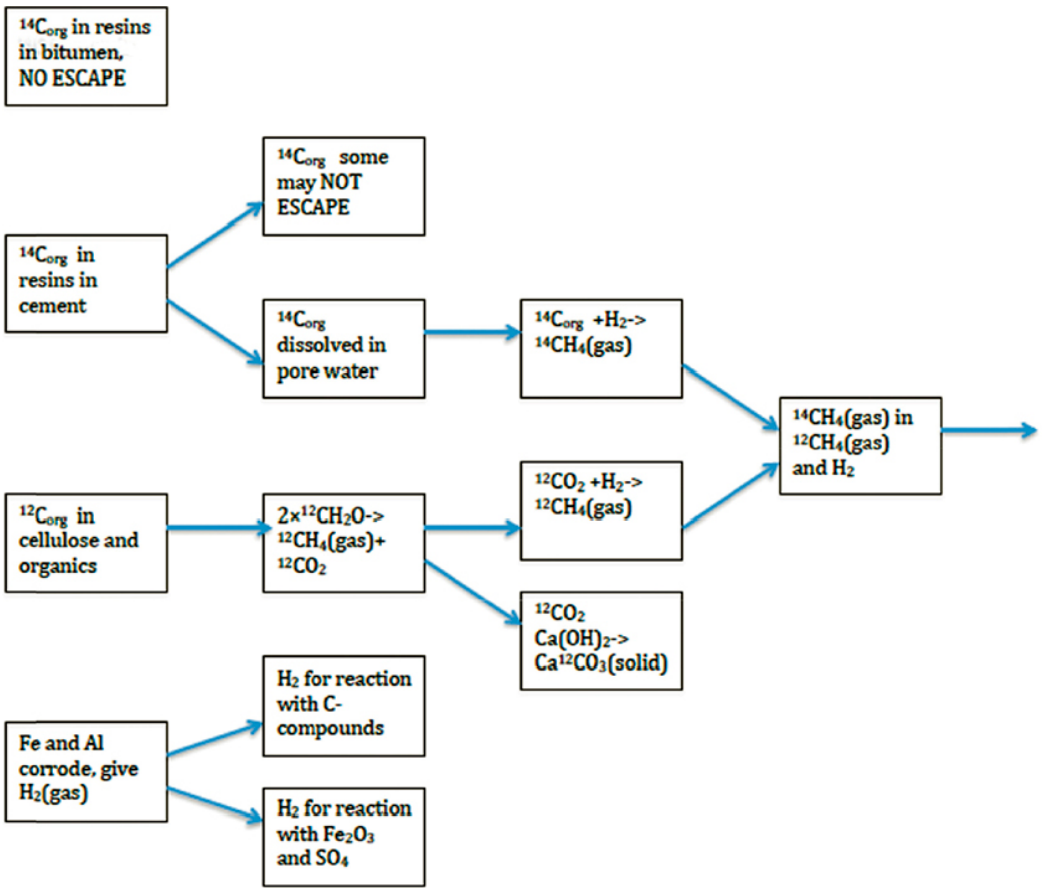

Figure 1-3. *Main sources and fates of ^{14}C compounds (Neretnieks and Moreno 2015).*

1.4 Microbial activity

Microorganisms are ubiquitous and will be either present in the wastes or brought into the repository during construction. They will face several stress factors in the repository such as salt, radiation and pH. Salt stress will be probably less relevant because bacteria are shown to adapt fast to osmotic stress (Krämer 2010, Harris et al. 2009, Parisi and Antoine 1974).

The radiation stress during the storage in SFR will not be strong enough to inhibit microorganisms. The pH will likely be the biggest stress factor for microbial viability because it is expected to be around pH 12.5 (Rizoulis et al. 2012, Sorokin 2005).

However, as mentioned before the pH will slowly decrease over time because of buffering by CO_2 and organic acids (Berner 1992). Microbes may also contribute to decreasing the pH by producing organic and inorganic acids.

Methanogens are ubiquitous, found in anaerobic environment and are active in the pH range from 4 to 10 (Zinder 1993). The hyperalkaline pH in SFR is assumed to inhibit the activity of methanogens (SKB 2015b). However, IER and bitumen in the waste provide a major source of organic carbon that has the potential to fuel microbial methane generation in the repository. CO_2 from waste degradation and H_2 from anaerobic corrosion and radiolysis are also substrates for methanogenesis (Abrahamsen et al. 2015, Zinder 1993).

Previous work has confirmed the presence of Archaea in groundwater at the position KFR105 at the SFR (Svensson et al. 2011). There is a possibility that these Archaea are methanogens with potential to form CH_4. A recent study observed increased cell amounts up to pH 12.6 and showed microbial activity in hyperalkaline environments is possible (Brazelton et al. 2013). Therefore, further research was conducted to investigate the pH dependence of methanogenesis in SFR.

1.5 Experiment approach and objectives

1.5.1 Methanogens in SFR-groundwater and biofilms

In the first approach, methanogens from SFR-groundwater were enriched via cultivation and identified using DNA methodology. Groundwater was collected from different position and used for cultivation of methanogens. Series of different enrichment media for methanogens were prepared and analysed for production of methane as an indicator for growth in the cultures. The cultivation approach is based on previous work with enrichment and cultivation of methanogens at Äspö HRL (Kotelnikova and Pedersen 1997, 1998, Kotelnikova et al. 1998, Kotelnikova 2002). The theory behind the media formulations is illustrated in Figure 1-4. Media were prepared in serum bottles to mimic different stages in the degradation of organic material present in SFR. It was expected that fastest growth with methane production would appear with C_1 and C_2 organic acids, at the bottom of the scheme, because methanogens cannot directly use more complex carbons. Over time, consortia of fermenters may produce C_1 and C_2 organic acids in the cultures from longer chained organic acids.

Flow cells (FC) were used to enrich microbes from the groundwater and allow them to form biofilms on garnet grains. Afterwards the DNA was extracted from the biofilms and analysed for the presence of methanogens in the groundwater using quantitative polymerase chain reaction (qPCR).

1.5.2 Methanogens at alkaline pH

In the second investigation growth of methanogens at alkaline pH (10 – 12.5) was examined to evaluate if there is a pH threshold for methanogenesis in SFR. The enriched unknown consortium of microbes from the previous investigation and *Methanobacterium subterraneum* (DSM 11074) were incubated in enrichment medium under various pH for 8 weeks. Additionally, to induce biofilm growth, either IER or cellulose were added to cultivations.

The cultivation approach was based on previous work with enrichment and cultivation of methanogens at Äspö HRL (Kotelnikova and Pedersen 1997, 1998, Kotelnikova et al. 1998, Kotelnikova 2002). Production of methane was analysed with gas chromatography and pH of the cultures was analysed to observe possible change caused by microbial activity.

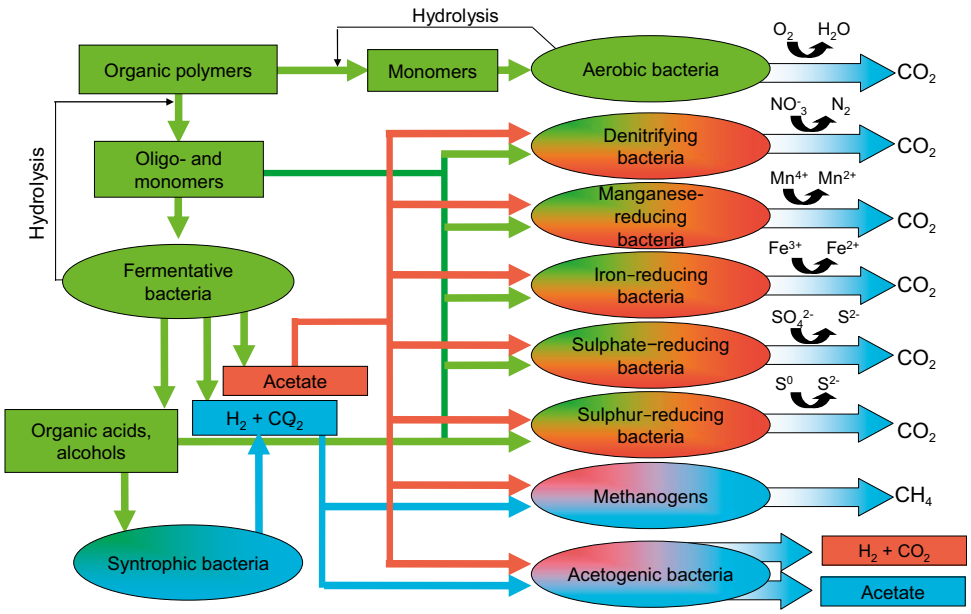

Figure 1-4. *Possible pathways for the flow of carbon in the subterranean environment. Organic carbon is respired with oxygen, if present, or else fermentation and anaerobic respiration occur with an array of different electron acceptors (Pedersen 2000).*

1.5.3 Microbial methane production in LLW and ILW

In the last investigation microbial methane development in containers that simulated the conditions of the SFR was studied.

Four air tight carbon steel containers were used with the following parameters:

1. IER and SFR-groundwater at pH 12.5
2. Cement, IER and SFR-groundwater
3. Bitumen IB55 (40 % w/w), IER (60 % w/w) and SFR-groundwater
4. Positive Control: IER and SFR-groundwater at pH 7.0

Sodium acetate with ^{13}C label on the methyl group ($^{13}CH_3CO_2Na$) was used to attribute analysed methane from acetate according to Equation 1-1 to microbial activity in the steel containers. The steel containers had a nitrogen atmosphere with a total pressure of 1.5 bar. Samples for pH and methane analysis were taken after 6, 7 and 9 months of incubation.

$$^{13}CH_3COOH \rightarrow {}^{13}CH_4 + CO_2 \qquad \text{Equation 1-1}$$

Table 1-1. Objectives of each experiment.

Experiment	Objective(s)
Methanogens in SFR-groundwater and biofilms	• Enrichment of methanogens from SFR-ground water. • Identification of methanogens through molecular analyses.
Methanogens at alkaline pH	• Examine the growth of methanogens at alkaline pH. • Estimate a pH threshold for methanogenesis.
Microbial methane production in LLW and ILW	• Investigate microbial methane development in containers that simulated the conditions of the SFR.

2 Materials and methods

2.1 Methanogens in SFR-groundwater

2.1.1 Addition of tissue paper or filter paper

Serum bottles (120 mL) were supplied with tissue paper or filter paper and left in an anaerobic box with an atmosphere consisting of 97 % N_2 and 3 % H_2, O_2 < 1 ppm (COY Laboratory Products, Grass Lake, MI, USA) for 24 h to remove all traces of O_2. The exact weight of added tissue paper or filter paper can be found in Table 6-1. The bottles were thereafter sealed with butyl rubber stoppers, removed from the anaerobic box and repeatedly evacuated and flushed with an 80/20 % mixture of N_2 and CO_2. A total of 64 serum bottles were prepared, in which 32 bottles contained tissue paper or filter paper.

2.1.2 Cultivation medium

The cultivation medium was made up of (g L^{-1}): NaCl, 7.0; $CaCl_2 \times 2H_2O$, 1.0; KCl, 0.67; NH_4Cl, 1.0; KH_2PO_4, 0.15; and $MgCl_2 \times 6H_2O$, 0.5. After sterilization, oxygen was removed from the medium by purging it with an 80/20 % mixture of N_2 and CO_2. Anaerobic solutions consisting of essential trace elements, vitamins, and organic acids (see 2.1.3) were added to the medium. Approximately 50 mL of medium was added to the prepared serum bottles using syringes (Pedersen et al. 2008).

2.1.3 Media combinations

Different organic carbon sources were added as outlined in Table 6-1, resulting in four media combinations. Medium 1 contained 20 mM sodium acetate, 30 mM formate and 37 mM methanol. Medium 2 contained 10 mM valeric acid, 10 mM propionic acid, 10 mM butyric acid and tissue paper. Medium 3 contained 20 mM glucose and filter paper. Medium 4 contained 1 mM valeric acid, 1 mM propionic acid, 1 mM butyric acid and 10 mM microcrystalline cellulose. pH in the serum bottles was adjusted to 7.0–8.0.

2.1.4 Sources of microorganisms and cultivation

Groundwater was collected from six positions in the SFR repository. The six positions were KFR02, KFR03:4, KFR105:1, KFR04:2, KFR08:1, and KFR7A:1. Approximately 5 mL of groundwater was added to serum bottles as described in Table 6-2. Two positive and negative medium control bottles were prepared for each media combination. Negative medium controls were not inoculated while positive controls were inoculated with 0.5 mL of *M. subterraneum* culture (Table 6-2). The bottles were incubated for 4 months at 18 °C.

2.1.5 Methane analysis

To confirm growth of methanogens in the serum bottles, methane was analysed on Varian CP-3800 gas chromatograph (Agilent Technologies Inc., CA, USA) with a carboxen column (2 m × 1/8 in. diameter) and detected with the flame ionisation detector (FID) and N_2 as the carrier gas. The detection limit was 0.04 µmol for the analysed gas phase. Methane was measured after 4 months of incubation. The methane in the gas phase was calculated with Equation 2-2. Furthermore, for the calculation the pressure inside the serum bottles and the volume of the gas phase are given in Table 6-3.

$$Methane_{G1} = \frac{Methane_A \times V_G \times P_1}{P_2}$$ Equation 2-1

$Methane_{G1} = Methane\ in\ gas\ phase\ (\mu L)$
$Methane_A = Methane\ analysed\ with\ GC\ (\mu L \times L^{-1})$
$V_G = Volume\ of\ the\ gas\ phase\ (L)$
$P_1 = Pressure\ measured\ in\ the\ serum\ bottle\ (bar)$
$P_2 = Atmospheric\ pressure\ (bar)$

$$Methane_{G2} = \frac{Eq.1}{V_m}$$ Equation 2-2

$Methane_{G2} = Methane\ in\ gas\ phase\ (\mu mol)$
$V_m = Molar\ volume\ (\mu mol \times L^{-1})$

2.1.6 Enrichment of methanogens

Six serum bottles with only medium, described in 2.1.2 were prepared for enrichment of methanogens after the initial incubation. The bottles were incubated with 1 mL of culture from six different bottles with confirmed methane production. The bottles chosen for further cultivation was determined to include different groundwater and different media compositions.

2.1.7 Sampling

Sampling was performed with flow cells and high-pressure filtration. Both methods have been previously verified to work well in groundwater (Eriksson et al. 2016).

Flow cells

Three flow cells (FC) were used for biofilm enrichment. The FCs had a stainless-steel shell (length 300 mm, diameter 65 mm) and were lined with polyvinyl difluoride (PVDF) plastic (Eriksson et al. 2016). Each FC had a 120-mm-long PVDF insert with a 22 × 32 mm opening that supported ~100 g of garnet grains (0.70 mm, Bulk 500 g, order no. 13123-05, Immuno Diagnostic Oy, Finland.) and glass beads (1 mm Hecht Assistant, art.no 201-0276 VWR, Sweden), per FC for microbial adhesion and biofilm growth. The garnet grains were of molecular grade meaning they were sterile, DNA-free and RNAse/DNAse-free. The glass beads were sterilized at 450 °C for 5 hours in a muffle furnace.

The different FCs were connected to flowing groundwater from borehole KFR02:3, KFR03:4 and KFR105:1 in the SFR. The groundwater was flowed through each connected FC for five weeks allowing microbes to form biofilms on the surface materials.

After enrichment in the field, the FCs were detached from the boreholes and transported back to the laboratory for further analysis. Excess groundwater was removed from the FC, leaving only the surface material with attached microorganisms. The glass beads and garnets were washed with phosphate-buffered saline to remove any planktonic microbes before proceeding with DNA extraction.

High-pressure filtration

Groundwater from KFR02:3, KFR03:4 and KFR105 was pressure filtrated using a high-pressure, stainless steel 47 mm filter holder (X4504700, Millipore AB, Solna, Sweden) with water filters from MO BIO Power Water kit filter units (Figure 2-1). The filter holder was equipped with a pressure relief valve (Swagelok SS-RL3S6MM; SWAFAB, Sollentuna, Sweden) and a manometer that enabled the adjustment of a pressure drop over the filter between 200 and 400 kPa relative to the ambient aquifer pressure. Groundwater was pressure filtrated for approximately 24 hours before the system was disassembled. The filters from the high-pressure filtration equipment were removed from the filter holder with sterile equipment and placed in provided sample tubes from the MO BIO Power Water kit and transported in coolers back to the laboratory for DNA extraction.

2.1.8 DNA extraction and Quantification of extracted dsDNA

From each FC, ten replicates with 4 to 7 g garnet grains were collected using sterile equipment and placed directly into DNA extraction vessels provided by the manufacturer. After DNA extraction the replicates were pooled together.

Genomic DNA from biofilms in the FCs and filters from high-pressure filtration was extracted using PowerWater® DNA Isolation Kit (cat. no. 14900) from MO BIO Laboratories, Carlsbad, CA, USA. (no. 14900-100; Immuno Diagnostics Oy, Finland) according to the manufacturer's protocol. The extracted DNA samples were thereafter stored at –20 °C until further processing commenced.

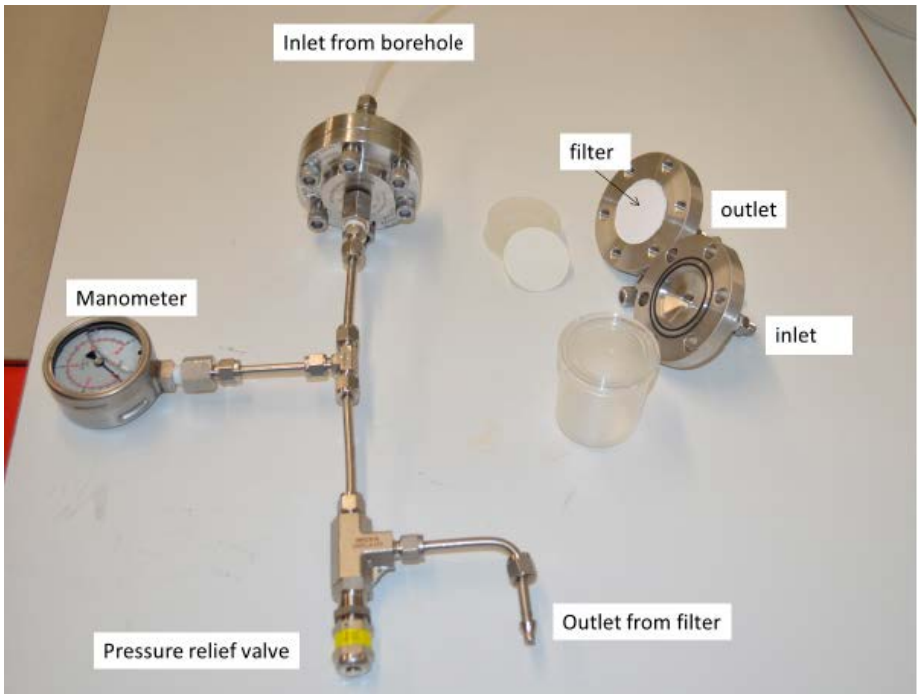

Figure 2-1. *Pressure filtration unit for sampling of bacteria in groundwater. Fully mounted unit is shown to the left; an opened filter holder with filter is shown to the right.*

Double stranded (ds)DNA concentrations were measured fluorometrically using the Stratagene MX3005p fluorometer with MXPro software (Agilent Technologies Inc., Santa Clara, CA, USA) and the Quant-it™ Picogreen reagent kit from Molecular Probes (cat. no. P7589; Invitrogen, San Diego, CA, USA), according to the manufacturer's specifications.

2.1.9 PCR analysis

The gene sequence for methyl coenzyme M reductase (mcrA) was targeted for PCR amplification. This gene is exclusive to methanogens, with methane-oxidizing Archaea being the only exception (Steinberg and Regan 2009) making it ideal to use for PCR detection of methanogens. Reagents used were iProof HF MasterMix, forward primer mlas (5'-GGTGGTGTMGGDTTCACMCARTA-3') (Steinberg and Regan 2008), reversed primer mcrA_rev1530R (5'-TTCATTGCRTAGTTWGGRTAGTT-3') (Luton et al. 2002) and molecular grade water. The PCR protocol was as follows: 98 °C for 3 min, 98 °C for 40 s then 54 °C for 1 min and 72 °C for 90 s with a 30-cycle repeat followed by 72 °C for 10 min. The methanogens *Methanosarcina barkeri* and *Methanobacterium subterraneum* were used as positive controls and *Acetobacterium carbinolicum*, *Desulfovibrio aespoeensis* and *Pseudomonas fluorescens* as negative controls.

2.2 Methanogens at alkaline pH

2.2.1 Preparation of serum bottles

Serum bottles (120 mL) were prepared in three settings. The IER Amberlite® IRA-400 (cat no. 247669-500G, Sigma-Aldrich. Stockholm, Sweden) and Dowex® 50WX2 (cat no. 44462-100G, Sigma-Aldrich. Stockholm, Sweden) were mixed in a ratio of 1:1.

1. Cellulose: tissue filter paper, 2 × 2 cm
2. IER: 1 g
3. Batch: no material.

Then the serum bottles were left in an anaerobic box with an atmosphere consisting of 97 % N_2 and 3 % H_2, O_2 < 1 ppm for 24 h to remove all traces of O_2. The bottles were thereafter sealed with sterile butyl rubber stoppers, removed from the anaerobic box and repeatedly evacuated and flushed with an 80/20 % mixture of N_2 and CO_2.

2.2.2 Cultivation medium

The enrichment medium was prepared as described in 2.1.2 with 20 mM sodium acetate and 30 mM formate. 50 mL was added to each serum bottle.

2.2.3 Microorganisms

The cultivation from 2.1 was analysed for methane with gas chromatography as described in 2.1.5. Cultivations from the positions KFR02, KFR4 and KFR105 had the highest methane values and were thus pooled in a new sterile serum bottle (120 mL). This mixture is referred to as "KFR". *M. subterraneum* (DSM 11074) was obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) and prior to the experiment cultivated in the same enrichment medium (2.1.2).

2.2.4 Inoculation and pH adjustment

Table 2-1 lists the different combinations of materials for biofilm formation, pH and microorganism used to investigate the pH dependence of methanogenesis. 1 mL of the respective bacterial culture was added to the prepared serum bottles with medium. Additionally, 1mL of *M. subterraneum* culture was added to the KFR series. Afterwards the pH was adjusted with 1 M anaerobe sterile KOH to the expected pH 10, 11 or 12.5. Series at pH 7 functioned as positive controls. Negative controls with either IER or cellulose were prepared as described and not inoculated. Finally, all serum bottles were infused with H_2 to 2 bar.

Table 2-1. Experimental matrix. Triplicates of all combinations.

Bacteria	IER [a)]	Cellulose [a)]	Batch [a)]
KFR	X	X	X
M. subterraneum	X	X	X
No inoculate (negative control)	X	X	

[a)] at pH 12.5, 11, 10 and 7

2.2.5 Methane and pH analysis

Methane was analysed as described in 2.1.5, 8 weeks after incubation. The methane in the gas phase was calculated as in 2.1.5. The volume of the gas phase for the M. subterraneum-series was 0.049 L and for the KFR-series 0.048 L. pH values were analysed on withdrawn aliquots with a pH meter (Scott, mod. CG 843P, VWR International AB, Stockholm, Sweden) equipped with a Hamilton electrode (Polilyte lab temp DIN, product no 242058/01, Genetec, Sweden).

2.3 Microbial methane production in LLW and ILW

2.3.1 Steel containers

The containers and lids were made from carbon steel. They had two inlets for gas and water sampling. One inlet had a tube made of poly-ether-ether-keton (PEEK) which reached into the water phase and was connected to a valve (Swagelok, Solltentuna, Sweden) on the outside (Figure 2-2). The other inlet was connected to a two-way valve which had on one side a manometer (AB Svenska Industri Instrument, Göteborg, Sweden) to be able to monitor the pressure inside the steel containers. The steel containers were tested for leaks with helium before the start of the experiment.

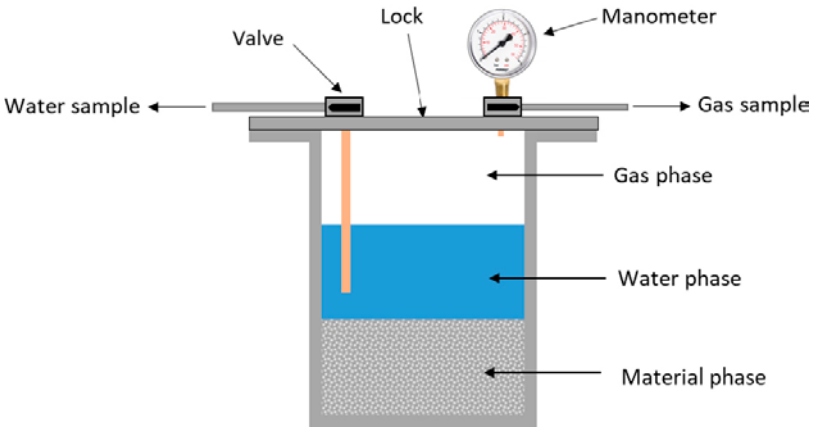

Figure 2-2. *Scheme of a steel container.*

2.3.2 Material

The materials used to simulate the conditions in the SFR are listed in Table 2-2. Amberlite® IRA-400 and Dowex® 50WX2 were mixed in a ratio of 1:1.

Table 2-2. Materials.

Material	
IER-mixture	Amberlite® IRA-400 anion exchange Dowex® 50WX2 cation exchange
Cement	Bascement Skövde (Cementa Heidelberg Cement group, Göteborg, Sweden): Total alkali, 1.3 % Sulphate (SO_4), 3.3 % Chloride, 0.08 % Water-soluble Cr^{6+}, < 2 ppm
Bitumen	Nybit 55 (Bitumen Technology Nordic, Nynäshamn, Sweden)

2.3.3 Microorganism

The cultivation KFR105:1 from 2.1 was used in this project and enriched in medium (2.1.2) before start of the incubation in the steel containers.

2.3.4 Preparation of the steel containers

First the steel containers were filled with the respective materials (Table 2-3). Steel container 1 contained 298 g IER-mixture and 0.5 g $Ca(OH)_2$ (cat. No 2495.1000, Th. Greyer, Renningen, Germany) to adjust the pH to 12.5. Steel container 2 contained 105 g of cement to adjust the pH to 12.5 and 214 g of the IER-mixture. Steel container 3 was filled with 180 g IER-mixture and then the bitumen was heated up until it was liquid and approximately 160 mL was poured over the IER-mixture and mixed to a bitumen-IER slurry. Steel container 4 contained 298 g IER-mixture. Thereafter each container was filled with 400 mL SFR-groundwater with 20 mmol L^{-1} Sodium acetate-2-^{13}C (cat no. 279315-1G, Sigma-Aldrich, Stockholm, Sweden). All steel containers were then transferred to an anaerobic box with an atmosphere consisting of 97 % N_2 and 3 % H_2, O_2 < 1 ppm. After 24 hours incubation the steel containers were closed and transferred out. Then they were evacuated for 10 minutes and filled with 1.2 bar of nitrogen. This was repeated 3 times to remove any remaining oxygen. Then 4 mL of 50 g L^{-1}cystein hydrochloride (cat no. 17769.03, SERVA, Heidelberg, Germany) and 4 mL of 48 g L^{-1} sodium sulphide (cat no. 424425000, Thermo Fisher Scientific, Göteborg, Sweden) were added through the water sampling inlet (Figure 2-3). Afterwards the inlet was flushed with 5 mL anaerobe SFR-groundwater. Then the steel containers were inoculated with 5 mL of methanogen enrichment culture (2.3.3), and the inlet was again flushed with 5 mL anaerobe SFR-groundwater. Finally, the pressure was increased to 1.5 bar with an 80/20 % mixture of N_2 and CO_2.

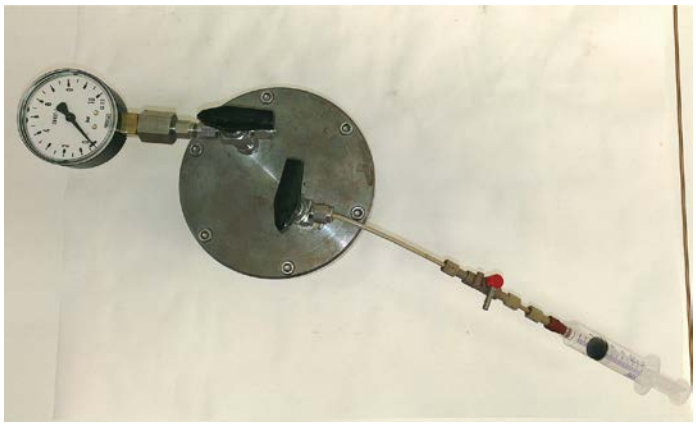

Figure 2-3. *Procedure of adding solutions.*

Table 2-3. Materials in steel containers.

Steel container	Material
1	IER-mixture $Ca(OH)_2$
2	Cement IER-mixture
3	Bitumen (40 % w/w) IER-mixture (60 % w/w)
4	IER-mixture

2.3.5 Sampling of gas and water samples

Gas samples were taken from the two-way valve trough a PEEK-tube with a valve and injection needle into evacuated 12 mL Exetainer (cat no. 538W, Labco, Lampeter, United Kingdom) (Figure 2-4).

Water samples were taken from the other valve trough a PEEK-tube with a valve and injection needle into evacuated 15 mL Falcon tubes (cat no. 62.554.502, SARSTEDT AG & Co. KG, Nümbrecht, Germany) (Figure 2-5).

Figure 2-4. *Procedure of gas sampling.*

Figure 2-5. *Procedure of water sampling.*

2.3.6 Analysis of gases and pH

$\delta^{13}CH_4$

Isotope analyses were performed at the Stable Isotope Service Lab, Department of Biology, Lund University. The gas samples were analysed with a Thermo GasBench for $\delta^{13}CH_4$.

CH_4, CO_2 and H_2

The gas samples were quantitatively analysed for CH_4, CO_2 and H_2 by injecting 100 µL of gas sample at atmospheric pressure into a Bruker 450 (Bruker Daltonics Scandinavia AB, Solna, Sweden) gas chromatograph equipped with a Pulsed Discharge Helium Ionization DETECTOR (PDHID) and following colons: CP7355 PoraBOND Q (50 m × 0.53 mm, ID) and CP7536 MOLSIEVE 5A PLOT (25 m × 0.32 mm, ID). Results were reported in µL L^{-1}. The detection limit for CH_4 was 0.0004 µL L^{-1}, for CO_2 0.0004 µL L^{-1} and for H_2 0.0003 µL L^{-1}.

pH

The pH was analysed as described in 2.2.5.

3 Results

3.1 Methanogens in SFR-groundwater and biofilms

3.1.1 Methane analysis

Methane values from cultivations with groundwater from KFR08 and KFR7A were too low, in either of the four media and were neglected (Table 6-3). Methane was detected in cultivation with groundwater from KFR02, KFR03, KFR105 and KFR04. Each showed positive cultivation in two different media. Cultures that were used for enrichment of methanogens are marked in Table 3-1.

Table 3-1. Analysed methane in cultivation bottles containing different SFR-groundwater and with different media compositions.

Sample name	Methane detected (µmol)	Further cultivation
KFR02 Medium 1:1	1.30	Yes
KFR02 Medium 2:1	0.06	
KFR02 Medium 3:1	0.85	Yes
KFR02 Medium 4:1	0.02	
KFR03 Medium 1:1	0.02	
KFR03 Medium 2:1	0.27	Yes
KFR03 Medium 3:1	0.11	
KFR03 Medium 4:1	0.33	Yes
KFR105 Medium 1:1	190	Yes
KFR105 Medium 2:1	0.14	
KFR105 Medium 3:1	0.25	
KFR105 Medium 4:1	0.37	
KFR04 Medium 1:1	7.46	Yes
KFR04 Medium 2:1	0.02	
KFR04 Medium 3:1	0.04	
KFR04 Medium 4:1	0.18	
KFR08 Medium 1:1	0.01	
KFR08 Medium 2:1	0.03	
KFR08 Medium 3:1	0.04	
KFR08 Medium 4:1	0.04	
KFR7A Medium 1:1	0.07	
KFR7A Medium 2:1	0.04	
KFR7A Medium 3:1	0.03	
KFR7A Medium 4:1	0.02	

3.1.2 Molecular analysis

DNA was extracted from flow cells and high-pressure filters, with water from KFR02:3, KFR03:4 and KFR105:1. From groundwater KFR02:3 and KFR03:4 the amount of DNA was higher in the flow cells than the high-pressure filters and in KFR105:1 the opposite was observed. The amounts of DNA in the samples were low as shown in Table 3-2.

Table 3-2. Concentration of dsDNA in flow cells and high-pressure filters.

Sampling method	Groundwater	dsDNA concentration (ng μL^{-1})
Flow cell	KFR02:3	1.03
Flow cell	KFR03:4	5.63
Flow cell	KFR105:1	1.04
High-pressure filter	KFR02:3	0.05
High-pressure filter	KFR03:4	0.29
High-pressure filter	KFR105:1	3.39

After quantification of dsDNA the samples were analysed with PCR to determine the presence of methanogens. Positive and negative controls were included to verify the set-up. Positive amplification for methanogen-specific mcrA gene was not observed in any of the samples.

3.2 Methanogens at alkaline pH

3.2.1 pH analysis

After 8 weeks of incubation the serum bottles were analysed for pH as described in 2.1.5. It was observed that the analysed pH deviated from the expected pH (Table 6-3 to Table 6-6). Serum bottles with expected pH 10 and 11 decreased on average to pH 9.4. Serum bottles with expected pH 12.5 decreased on average to pH 9.9. The pH decreased also in the medium controls and this decrease was, therefore, not caused by microbial activity. Furthermore, precipitation of probably $CaCO_3$ was observed at pH 10, 11 and 12.5 after adding KOH. The precipitation stayed visible at pH 12.5 during the incubation period (Figure 3-1).

3.2.2 Methane analysis

After 8 weeks of incubation the serum bottles were analysed for produced methane. The negative controls were excluded from the figures because methane was not detected.

Figure 3-2 shows the analysed methane plotted against the analysed pH for the serum bottles inoculated with *M. subterraneum*. In the series IER and Cellulose the methane production decreased with increase of the expected pH. In contrast in the series Batch the average amounts of methane were higher at expected pH 10 (23.9 ± 8.6 µmol CH_4) than at expected pH 7 (4.75 ± 2.6 µmol CH_4) (Table 6-4).

Figure 3-3 shows the analysed methane plotted against the analysed pH for the serum bottles inoculated with KFR. In the series Batch and IER the average amounts of methane were higher at expected pH 10 than at expected pH 7 (Table 6-5). In the series Cellulose the difference was less conclusive between expected pH 7 (25.3 ± 4.25 µmol CH_4) and expected pH 10 (36.2 ± 1.22 µmol CH_4).

M. subterraneum-series with IER showed on average higher amounts of methane at the analysed pH than the other two series (Table 6-5). Furthermore, it was noticed that serum bottles with expected pH 11 showed lower amounts of methane than those with expected pH 10. However, the analysed pH was on average 9.4 for both series. Methane production was observed in the range of pH 6.9–10.2 for *M. subterraneum* and in the range of pH 7.2–10.1 for KFR.

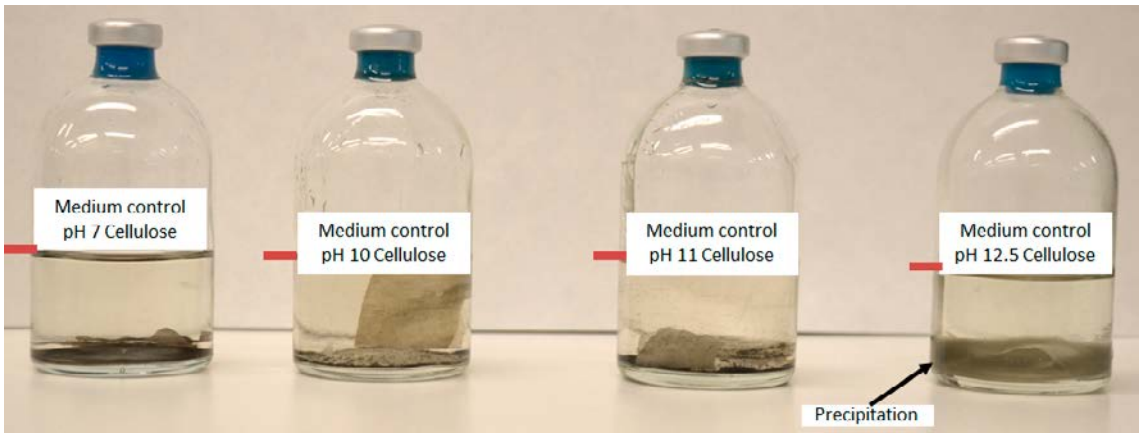

Figure 3-1. *Serum bottles with cellulose and enrichment medium at different pH. The arrow indicates the precipitation and the red line indicates the media level.*

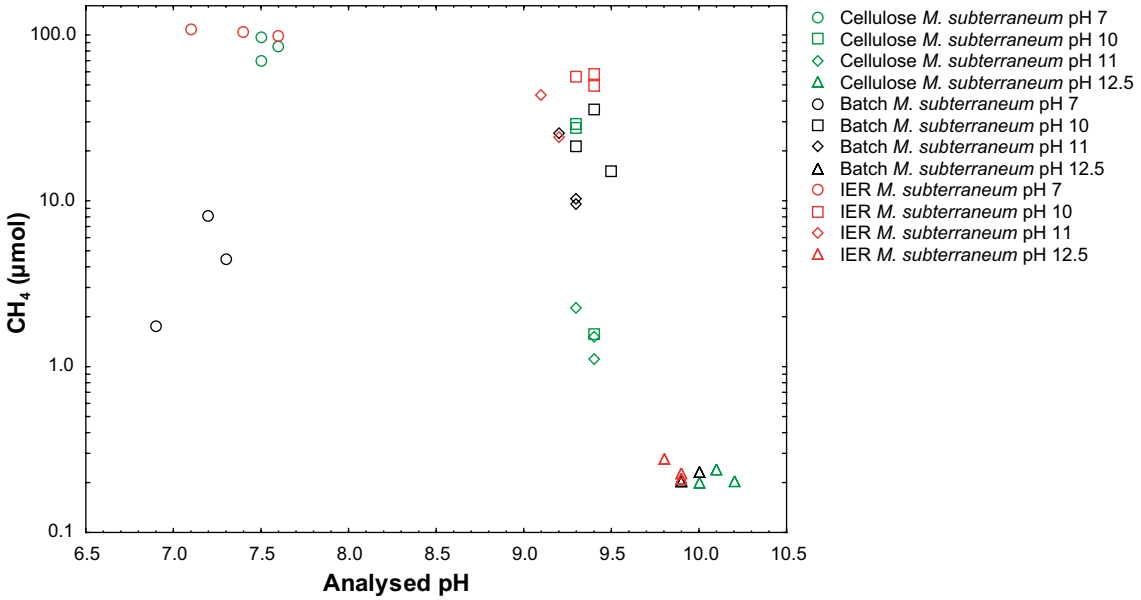

Figure 3-2. *Analysed methane plotted against analysed pH from serum bottles after 8 weeks of incubation. The serum bottles contained 50 mL enrichment medium and were inoculated with M. subterraneum. Additionally, the serum bottles contained material (cellulose or IER). Bottles labelled "Batch" contained only medium (2.2.2). The pH in the name description indicates the expected pH. The detection limit was 0.04 µmol.*

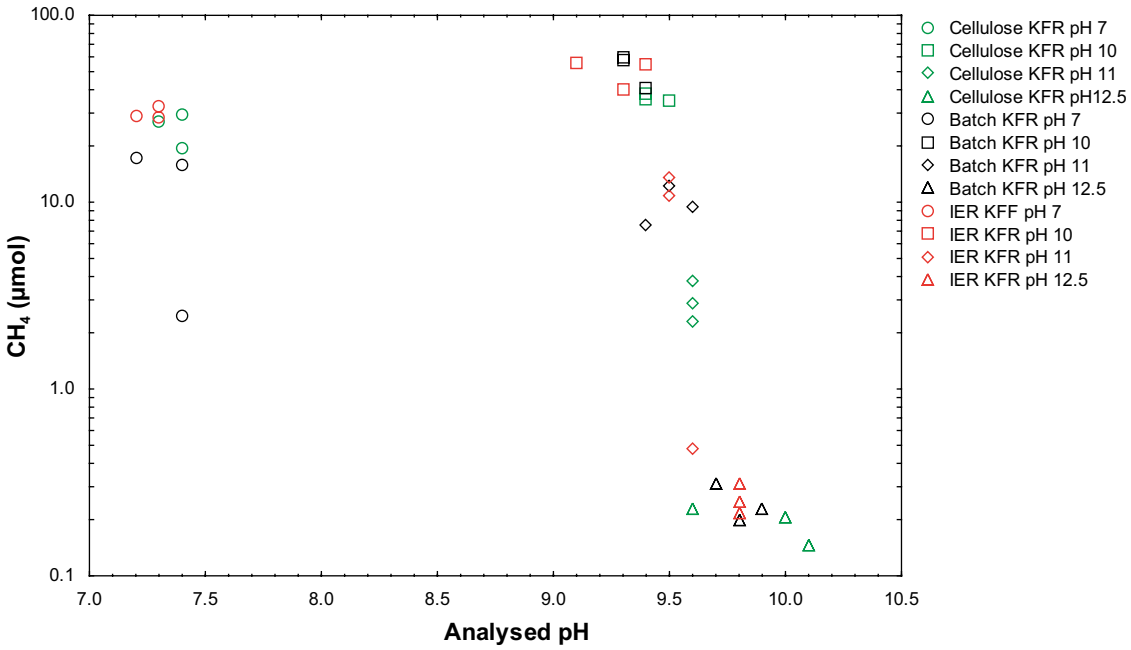

Figure 3-3. *Analysed methane plotted against analysed pH from serum bottles after 8 weeks of incubation. The serum bottles contained 50 mL enrichment medium and were inoculated with KFR. Additionally, the serum bottles contained material (cellulose or IER). Bottles labelled "Batch" contained only medium (2.2.2). The pH in the name description indicates the expected pH. The detection limit was 0.04 µmol.*

3.3 Microbial methane production in LLW and ILW

The steel container 1 continuously lost pressure after the first sampling. The leakage could not be stopped and therefore the last two analysis of steel container 1 were excluded from Figure 3-4. Figure 3-4 shows that methane was detected in all steel containers and the concentrations increased over time. As expected, the concentration of methane was higher at neutral pH in steel container 4 in comparison to steel container 1 and 2 with alkaline pH. The analysed pH of steel container 2 and 4 was as expected and the bitumen in steel container 3 increased the pH to 8 (Table 3-3). However, the pH of steel container 1 was lower than expected (Table 3-3).

Steel container 3 showed, in comparison to the other steel containers, a high concentration of CO_2 (Table 3-3). Steel container 1 and 3 had higher concentration of H_2 than steel container 2 and steel container 4 had the lowest concentration of H_2. The isotope fractionation values (δC^{13}_{CH4}) ranged from −13 ‰ to −18 ‰ (Table 3-3).

Table 3-3 Analysed pH, CO_2 and H_2 for each steel container after 6 months incubation and CH_4 after 6, 7 and 9 months incubation. Measurement uncertainty is 15 % for the gas analyses.

Sample name	6 months					7 months	9 months
	pH*	CH_4 (µL L^{-1})	CO_2 (µL L^{-1})	H_2 (µL L^{-1})	δC^{13}_{CH4} (‰)	CH_4 (µL L^{-1})	CH_4 (µL L^{-1})
Steel container 1 $Ca(OH)_2$ and IER-mixture	9.9	278	95	4 025	−14	89	27
Steel container 2 Cement and IER-mixture	12.7	24	43	616	−13	27	37
Steel container 3 Bitumen and IER-mixture	8.0	195	2 109	5 202	−14	267	471
Steel container 4 IER-mixture	7.7	3 846	225	< 4	−18	4 436	6 279

* *pH after 7 months were 9.9, 12.6, 8.1 and 7.6 respectively.*

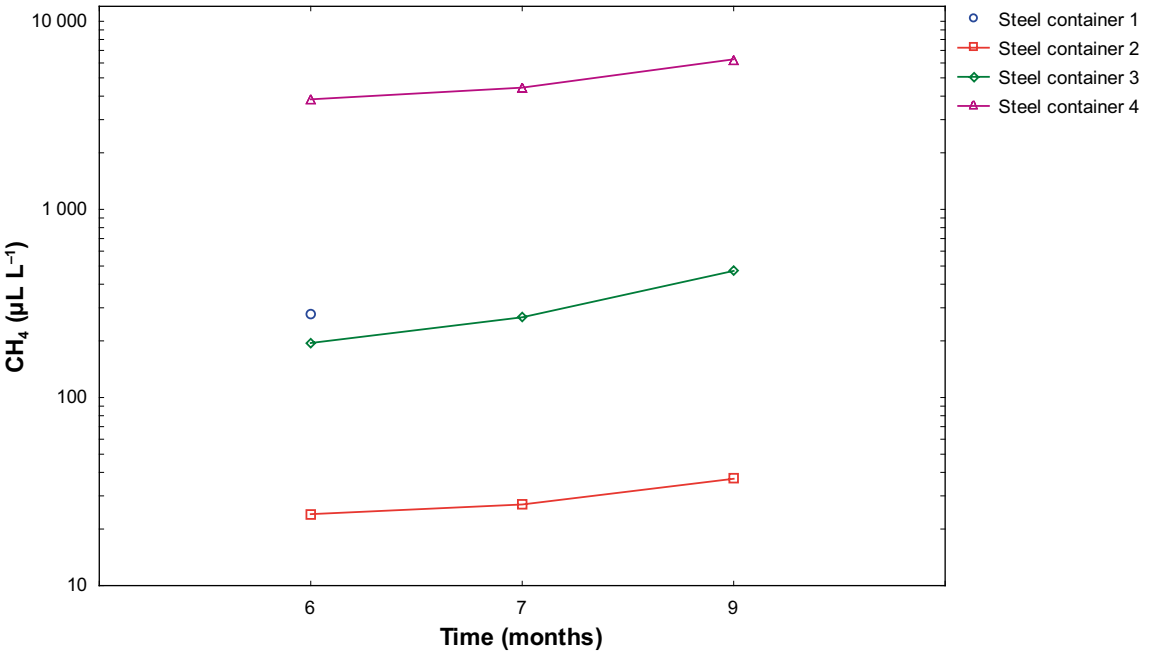

Figure 3-4. *Methane development over time for all steel containers. For information about steel containers see Table 2-2.*

4 Discussion

4.1 Confirmation of methanogens in SFR-groundwater

Microorganisms of the domain Archaea exist in a broad spectrum of habitats and have also been identified in deep granitic rock aquifers (Kotelnikova and Pedersen 1997). Archaea are adapted to extreme conditions and have members in the physiological groups of halophiles, thermophiles, alkaliphiles, and acidophiles (DeLong and Pace 2001). Hence, the conditions at the SFR-site are not preventative to microbial activity. The presence of methanogens in SFR-groundwater was confirmed by cultivating them in different growth media designed for methanogens (Table 3-1). The Archaea group of methanogens will be of importance for the repository because of their capability to generate methane which could lead to increasing gas pressure. Methanogens use either acetate (acetoclastic methanogenesis) or hydrogen plus carbon dioxide (hydrogenotrophic methanogenesis) as substrates. Methylated amines and short chained alcohols can also serve to a minor extent as substrate (King 1988, Widdel 1986). The cultivation investigation showed that methane production was highest with C_1 and C_2 organic acids. The growth was expected to be fastest with short chained organic acids because methanogens are not able to degrade more complex carbons. They are otherwise reliant on the growth of fermenter and their degradation products such as acetate or formate (McInerney et al. 1979). This was shown in media containing the longer chained organic acids valeric acid, propionic acid and butyric acid where the methane production was lower than in bottles with C_1 and C_2 organic acids. It is known that both short carboxylic acids like formic acid and acetic acids and longer mono-carboxylic acids such as propionic acid and butanoic acid leach from Eurobitumen and potentially will leach into the water at the SFR (Walczak 2000, Kagawa et al. 2000). Eurobitumen used in Belgium is heat-treated and possibly oxidised and has different physical properties to other bituminous materials such as used in France, which are softer. These organic acids can fuel methanogenesis in the repository as shown in the cultivations experiment with SFR-groundwater.

Culture-independent molecular analyses help to identify microbes that are lost through cultivation methods. Therefore, the gene mcrA was targeted for PCR amplification in extracted DNA. This gene is exclusive to methanogens (Steinberg and Regan 2009). It was not possible to quantify or determine methanogens through the molecular approach. The PCR approach probably failed due to too low amounts of DNA from methanogens for the analysis to be successful.

4.2 Methanogenesis at alkaline pH

The enriched methanogens from 1.5 were used to perform the follow-up experiment to analyse the pH dependence of methanogenesis. Methanogens are reported to have their pH optima mainly near neutral pH (Williams and Crawford 1984). There are however examples of methanogenesis at pH 4 or lower from peat bogs (Jones et al. 1987) and examples of growth at pH 9 like alkaliphilic methanogens such as *Methanobacterium thermoalcaliphilum* (Blotevogel et al. 1985). *M. subterraneum* was observed to produce methane up to pH 9.2 but not above (Kotelnikova et al. 1998).

Therefore, it was expected that methanogenesis would be inhibited at pH $\geq$ 10. It was observed that the pH at the end of the incubation time deviated from the expected pH. The difference might be due to the buffer capacity of bicarbonate which during the incubation time lowered the pH. In the future, buffer capacity over incubation time must be under better control to obtain targeted pH. Despite this, important information could be obtained from the results.

In these experiments methanogenesis was observed up to pH 10.2 for *M. subterraneum* and up to pH 10.1 for cultivations from SFR-groundwater. Therefore, the pH threshold was higher than previously described for *M. subterraneum.* Microcosms are heterogeneous therefore it is possible that the micro-organisms grew at a lower pH that the analysed pH. In case the pH decreases below 10 in regions of the repository, methanogenesis will be possible. The optimum for methane production was at neutral pH as described for *M. subterraneum*.

The optimum for KFR cultures seemed to be at pH 10 because methanogenesis was increased in comparison to pH 7 in the series Batch and IER. A metagenomic analysis of the KFR cultures would elucidate if these results are due to alkaliphilic methanogens or syntrophic consortia.

It was also observed that *M. subterraneum* cultivations with IER showed on average greater methane production in comparison to the series Cellulose and Batch (Figure 3-2, Table 6-4). It is known that microbes can adhere to ion exchangers and microbial growth is in general enhanced when they can adhere to solid surfaces (Flemming 1987). IERs filtering property enriches nutrients on the resin, thus providing a carbon source for microbial growth (Schubert and Esanu 1972, Saunders 1954). There is no evidence that microbes can degrade IER under anaerobic conditions (Warthmann et al. 2013). However, the functional groups of IER can be utilised. Methylamines released from basic resins like Amberlite IRA-400 can be used as electron donator for methanogenesis (Van Loon and Hummel 1995, Purwantini et al. 2014). Under ILW and LLW storage conditions, IER will be degraded mainly by radiolysis and release the sulfonyl or amine functional groups. Irradiation of basic IER such as Amberlite lead to generation of H_2 which is as well an electron donor for methanogenesis (Dhiman and LaVerne 2013). Chemical degradation will not occur during storage because IER lack partial positive charged carbon atoms for a nucleophilic attack of OH-ions on the polymer chain (Van Loon and Hummel 1995).

The added material cellulose showed lower amounts of methane at alkaline pH in comparison to the other two cultivation series, independent of the inoculum. Cellulose hydrolysis under hyperalkaline pH to water-soluble organics, mainly isosaccharinic acid (ISA) (Glaus et al. 1999, Greenfield et al. 1994, Van Loon et al. 1999). ISA can be used by fermentative and methanogenic microorganism up to a pH of 10.0 (Rout et al. 2015). Bassil et al. observed that microbial communities from lime-kiln waste degraded cellulose at hyperalkaline pH, producing acetate and thereby decreasing the pH (Bassil et al. 2015). However, in these experiments the pH was probably not sufficiently alkaline to generate ISA.

Furthermore, the cellulose might have not been degraded by microorganisms in this experiment because of the short incubation time of 8 weeks in comparison to 30 months reported by Bassil et al. (2015).

4.3 Microbial methane production under LLW and ILW conditions

In the steel container experiment microbial methane production under LLW and ILW conditions was investigated. The enriched Archaea from the SFR-groundwater were incubated in steel containers which contained different combinations of material. It was demonstrated that methane developed in all steel containers and the concentrations increased over time (Figure 3-4). The hydrogen in the steel containers likely originated from anaerobic corrosion of the steel. The lower amount of H_2 in steel container 2 is consistent with that hyperalkaline pH passivates steel corrosion and it is possible that some H_2 was consumed by methanogenesis. Parts of the repository with large amounts of concrete will for a long period be under hyperalkaline conditions and therefore H_2 from steel corrosion will be limited. Bitumen filled containers will be near neutral as demonstrated by steel container 3. The concentration of CO_2 varied in the containers. The low CO_2 concentrations in steel container 1 and 2 were due to the high pH. At high pH most, CO_2 is found as carbonates and will not show on a gas chromatograph.

The final concentrations of methane were dependent on pH as in the previous experiment and the optimum for methane generation was as before observed at neutral pH. It was observed that methane production was 19-times lower if IER was imbedded in bitumen compared to IER in contact with water.

A reason for the higher CO_2 concentration might be biodegradation of the bitumen. Biodegradation of bitumen is well studied but is expected to be slow under anoxic conditions (Wolfram et al. 1997). Albeit, anaerobic microorganisms can degrade hydrocarbons directly from the matrix and water-soluble carboxylic acids that leach from the bitumen. N_2 and CO_2 are generated through the degradation (Jacquot et al. 1997). It might be that these microorganisms outcompeted methanogens and therefore the rate of methanogenesis was lower. Yet it raises the prospect that with increased incubation time the rate of methane generation could increase, and the degradation products can be used as substrates for methanogenesis.

2-C^{13} labelled acetate was used to distinguish between heterotrophic and autotrophic produced methane in the steel containers. $\delta C^{13}CH_4$ originating from the acetoclastic methanogenesis should have values lower than −100 ‰ because all methane would have a ^{13}C signature. However, microbially generated methane with hydrogen plus carbon dioxide would have a $\delta C^{13}CH_4$ signature that would be somewhat lower than the signature of the CO_2 that was added to the steel containers and this signature should correspond negatively to the amount of methane produced. Therefore, judged from $\delta C^{13}CH_4$ values and the low concentration of molecular hydrogen gas in steel container 4, the observed methane formation was mainly of autotrophic origin. Autotrophic methane production appears to have competed successfully with acetoclastic production. This process reduces the molar amount of gas from 5 to 1 which reduces the risk for detrimental high gas pressure in the repository if the conditions are favourable for methanogenesis.

The possibility of methanogenesis is as discussed dependant on a lot of factors and their interplay. Hyperalkaline pH (> 12.5) will prevent methanogenesis in the repository. Although low pH niches may be present within cementitious regions of the repository which could allow methanogenesis. An alkaline pH increases the dissolved organic carbon concentrations from bitumen (Walczak 2000). Organic acids can be degraded by anaerobic microbes and provide substrates like acetate, H_2, CO_2 for methanogens. Furthermore, the pH is affected by CO_2 buffering and acidity from organic acids, both generated through radiolytic, chemical and microbial degradation (Kagawa et al. 2000, Wolf and Bachofen 1991, Wolfram et al. 1997). Finally, the micro environments present in the SFR and groundwater determines which microbial processes will take place and at which rate.

4.4 Possibility of $^{14}CH_4$ generation

Neretnieks and Moreno calculated that in case of microbial activity more than 10 % of the radioactive carbon in the waste could escape to the biosphere over a time span from a 100 to 1 000 years (Neretnieks and Moreno 2015). Hydrogen from scrap metal corrosion (iron and aluminium) and inorganic and organic ^{14}C and ^{12}C compounds are ideal for methanogenesis to take place. The generated methane and hydrogen will form bubbles in SFR1 and be carried out. However, the amount of methane gas is proportional to the amount of hydrogen from metal corrosion (Neretnieks and Moreno 2015).

The conducted experiments reported here showed that methanogens are present at the SFR and are able to perform methanogenesis under alkaline pH. The rate of reaction under repository conditions is difficult to predict. The investigation of Microbial methane production in LLW and ILW showed that at pH > 12.5 only 37 μL L^{-1} methane was generated in 9 months. In case the pH in large parts of the repository stays hyperalkaline the generation of large amounts of $^{14}CH_4$ is unlikely. Yet if the pH decreases the rate of methane generation could increase as the steel container 1 showed after 6 months. Therefore, the generation of $^{14}CH_4$ through methanogenesis is possible but dependant on substrates, pH and the micro environments present in the SFR-groundwater.

4.5 Uncertainties

The understanding of the present micro environments at the SFR is of importance including variation of pH. The identification of methanogens should be pursued because it would shed light on the possible microbial activities at the SFR and how it could affect the storage safety. DNA could be extracted from the cultivations and be analysed using molecular DNA methods. However, this only will identify methanogens that are cultivable from the SFR-groundwater.

Furthermore, the analysis of ISA and acetate from cellulose should be included to investigate if cellulose is degraded by microorganism in the SFR-groundwater to eventually produce methane.

Moreover, steel container 3, with bitumen, can be analysed for carboxylic acids and N_2. It would show if the bitumen was biodegraded and hydrocarbons leached into the water phase. In addition, the combination of IER embedded in bitumen and surrounded by concrete could be analysed for possible microbial methane generation. Future experiments should reconsider the addition of CO_2 and calculate the amount of alkalinity is required to maintain pH.

4.6 Conclusions

- Methanogens are present in SFR-groundwater.
- The present methanogens can use C_1 and C_2 organic acids and H_2 for methanogenesis.
- The methanogens cultivated from the SFR-groundwater are active up to pH 10 but their activity rapidly decreases after pH 9.5.
- Waste simulant experiments with IER have generated methane including an experiment buffered at pH 12.5 by cement.
- Autotrophic methane production appears to have competed successfully with acetoclastic production. This process reduces the molar amount of gas from 5 to 1 which reduces the risk for detrimental high gas pressure in the repository.

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Appendix

Table A-1. Media combinations for Methanogens in SFR-groundwater.

Sample name	Organic carbon sources (mM)	Tissue paper (mg)	Filter paper (mg)
KFR02 Medium 1:1	Na-acetate (20) + Formate (30) + Methanol (37)	–	–
KFR02 Medium 1:2	Na-acetate (20) + Formate (30) + Methanol (37)	–	–
KFR02 Medium 2:1	Valeric acid (10) + Propionic acid (10) + Butyric acid (10) + tissue paper	110	–
KFR02 Medium 2:2	Valeric acid (10) + Propionic acid (10) + Butyric acid (10) + tissue paper	107	–
KFR02 Medium 3:1	Glucose (20) + filter paper	–	321
KFR02 Medium 3:2	Glucose (20) + filter paper	–	309
KFR02 Medium 4:1	Valeric acid (1) + Propionic acid (1) + Butyric acid (1) + cellulose (10)	–	–
KFR02 Medium 4:2	Valeric acid (1) + Propionic acid (1) + Butyric acid (1) + cellulose (10)	–	–
Negative control Medium 1:1	Na-acetate (20) + Formate (30) + Methanol (37)	–	–
Negative control Medium 1:2	Na-acetate (20) + Formate (30) + Methanol (37)	–	–
Positive control Medium 1:1	Na-acetate (20) + Formate (30) + Methanol (37)	–	–
Positive control Medium 1:2	Na-acetate (20) + Formate (30) + Methanol (37)	–	–
KFR03 Medium 1:1	Na-acetate (20) + Formate (30) + Methanol (37)	–	–
KFR03 Medium 1:2	Na-acetate (20) + Formate (30) + Methanol (37)	–	–
KFR03 Medium 2:1	Valeric acid (10) + Propionic acid (10) + Butyric acid (10) + tissue paper	122	–
KFR03 Medium 2:2	Valeric acid (10) + Propionic acid (10) + Butyric acid (10) + tissue paper	114	–
KFR03 Medium 3:1	Glucose (20) + filter paper	–	327
KFR03 Medium 3:2	Glucose (20) + filter paper	–	320
KFR03 Medium 4:1	Valeric acid (1) + Propionic acid (1) + Butyric acid (1) + cellulose (10)	–	–
KFR03 Medium 4:2	Valeric acid (1) + Propionic acid (1) + Butyric acid (1) + cellulose (10)	–	–
Negative control Medium 2:1	Valeric acid (10) + Propionic acid (10) + Butyric acid (10) + tissue paper	112	–
Negative control Medium 2:2	Valeric acid (10) + Propionic acid (10) + Butyric acid (10) + tissue paper	110	–
Positive control Medium 2:1	Valeric acid (10) + Propionic acid (10) + Butyric acid (10) + tissue paper	115	–
Positive control Medium 2:2	Valeric acid (10) + Propionic acid (10) + Butyric acid (10) + tissue paper	120	–
KFR105 Medium 1:1	Na-acetate (20) + Formate (30) + Methanol (37)	–	–
KFR105 Medium 1:2	Na-acetate (20) + Formate (30) + Methanol (37)	–	–
KFR105 Medium 2:1	Valeric acid (10) + Propionic acid (10) + Butyric acid (10) + tissue paper	114	–
KFR105 Medium 2:2	Valeric acid (10) + Propionic acid (10) + Butyric acid (10) + tissue paper	116	–
KFR105 Medium 3:1	Glucose (20) + filter paper	–	310
KFR105 Medium 3:2	Glucose (20) + filter paper	–	325
KFR105 Medium 4:1	Valeric acid (1) + Propionic acid (1) + Butyric acid (1) + cellulose (10)	–	–
KFR105 Medium 4:2	Valeric acid (1) + Propionic acid (1) + Butyric acid (1) + cellulose (10)	–	–
Negative control Medium 3:1	Glucose (20) + filter paper	–	325
Negative control Medium 3:2	Glucose (20) + filter paper	–	320
Positive control Medium 3:1	Glucose (20) + filter paper	–	313
Positive control Medium 3:2	Glucose (20) + filter paper	–	309
KFR04 Medium 1:1	Na-acetate (20) + Formate (30) + Methanol (37)	–	–
KFR04 Medium 1:2	Na-acetate (20) + Formate (30) + Methanol (37)	–	–
KFR04 Medium 2:1	Valeric acid (10) + Propionic acid (10) + Butyric acid (10) + tissue paper	116	–

Sample name	Organic carbon sources (mM)	Tissue paper (mg)	Filter paper (mg)
KFR04 Medium 2:2	Valeric acid (10) + Propionic acid (10) + Butyric acid (10) + tissue paper	123	–
KFR04 Medium 3:1	Glucose (20) + filter paper	–	313
KFR04 Medium 3:2	Glucose (20) + filter paper	–	310
KFR04 Medium 4:1	Valeric acid (1) + Propionic acid (1) + Butyric acid (1) + cellulose (10)	–	–
KFR04 Medium 4:2	Valeric acid (1) + Propionic acid (1) + Butyric acid (1) + cellulose (10)	–	–
Negative control Medium 4:1	Valeric acid (1) + Propionic acid (1) + Butyric acid (1) + cellulose (10)	–	–
Negative control Medium 4:2	Valeric acid (1) + Propionic acid (1) + Butyric acid (1) + cellulose (10)	–	–
Positive control Medium 4:1	Valeric acid (1) + Propionic acid (1) + Butyric acid (1) + cellulose (10)	–	–
Positive control Medium 4:2	Valeric acid (1) + Propionic acid (1) + Butyric acid (1) + cellulose (10)	–	–
KFR08 Medium 1:1	Na-acetate (20) + Formate (30) + Methanol (37)	–	–
KFR08 Medium 1:2	Na-acetate (20) + Formate (30) + Methanol (37)	–	–
KFR08 Medium 2:1	Valeric acid (10) + Propionic acid (10) + Butyric acid (10) + tissue paper	118	–
KFR08 Medium 2:2	Valeric acid (10) + Propionic acid (10) + Butyric acid (10) + tissue paper	120	–
KFR08 Medium 3:1	Glucose (20) + filter paper	–	315
KFR08 Medium 3:2	Glucose (20) + filter paper	–	316
KFR08 Medium 4:1	Valeric acid (1) + Propionic acid (1) + Butyric acid (1) + cellulose (10)	–	–
KFR08 Medium 4:2	Valeric acid (1) + Propionic acid (1) + Butyric acid (1) + cellulose (10)	–	–
KFR7A Medium 1:1	Na-acetate (20) + Formate (30) + Methanol (37)	–	–
KFR7A Medium 1:2	Na-acetate (20) + Formate (30) + Methanol (37)	–	–
KFR7A Medium 2:1	Valeric acid (10) + Propionic acid (10) + Butyric acid (10) + tissue paper	118	–
KFR7A Medium 2:2	Valeric acid (10) + Propionic acid (10) + Butyric acid (10) + tissue paper	117	–
KFR7A Medium 3:1	Glucose (20) + filter paper	–	313
KFR7A Medium 3:2	Glucose (20) + filter paper	–	302
KFR7A Medium 4:1	Valeric acid (1) + Propionic acid (1) + Butyric acid (1) + cellulose (10)	–	–
KFR7A Medium 4:2	Valeric acid (1) + Propionic acid (1) + Butyric acid (1) + cellulose (10)	–	–

Table A-2. Sources of microorganisms for Methanogens in SFR-groundwater.

Sample name	Inoculum
KFR02 Medium 1:1	KFR02:2
KFR02 Medium 1:2	KFR02:2
KFR02 Medium 2:1	KFR02:2
KFR02 Medium 2:2	KFR02:2
KFR02 Medium 3:1	KFR02:2
KFR02 Medium 3:2	KFR02:2
KFR02 Medium 4:1	KFR02:2
KFR02 Medium 4:2	KFR02:2
Negative control Medium 1:1	–
Negative control Medium 1:2	–
Positive control Medium 1:1	Methanobacterium subteraneum
Positive control Medium 1:2	Methanobacterium subteraneum

Sample name	Inoculum
KFR03 Medium 1:1	KFR03:4
KFR03 Medium 1:2	KFR03:4
KFR03 Medium 2:1	KFR03:4
KFR03 Medium 2:2	KFR03:4
KFR03 Medium 3:1	KFR03:4
KFR03 Medium 3:2	KFR03:4
KFR03 Medium 4:1	KFR03:4
KFR03 Medium 4:2	KFR03:4
Negative control Medium 2:1	–
Negative control Medium 2:2	–
Positive control Medium 2:1	Methanobacterium subteraneum
Positive control Medium 2:2	Methanobacterium subteraneum
KFR105 Medium 1:1	KFR105:1
KFR105 Medium 1:2	KFR105:1
KFR105 Medium 2:1	KFR105:1
KFR105 Medium 2:2	KFR105:1
KFR105 Medium 3:1	KFR105:1
KFR105 Medium 3:2	KFR105:1
KFR105 Medium 4:1	KFR105:1
KFR105 Medium 4:2	KFR105:1
Negative control Medium 3:1	–
Negative control Medium 3:2	–
Positive control Medium 3:1	Methanobacterium subteraneum
Positive control Medium 3:2	Methanobacterium subteraneum
KFR04 Medium 1:1	KFR04:2
KFR04 Medium 1:2	KFR04:2
KFR04 Medium 2:1	KFR04:2
KFR04 Medium 2:2	KFR04:2
KFR04 Medium 3:1	KFR04:2
KFR04 Medium 3:2	KFR04:2
KFR04 Medium 4:1	KFR04:2
KFR04 Medium 4:2	KFR04:2
Negative control Medium 4:1	–
Negative control Medium 4:2	–
Positive control Medium 4:1	Methanobacterium subteraneum
Positive control Medium 4:2	Methanobacterium subteraneum
KFR08 Medium 1:1	KFR08:1
KFR08 Medium 1:2	KFR08:1
KFR08 Medium 2:1	KFR08:1
KFR08 Medium 2:2	KFR08:1
KFR08 Medium 3:1	KFR08:1
KFR08 Medium 3:2	KFR08:1
KFR08 Medium 4:1	KFR08:1
KFR08 Medium 4:2	KFR08:1
KFR7A Medium 1:1	KFR7A:1
KFR7A Medium 1:2	KFR7A:1
KFR7A Medium 2:1	KFR7A:1
KFR7A Medium 2:2	KFR7A:1
KFR7A Medium 3:1	KFR7A:1
KFR7A Medium 3:2	KFR7A:1
KFR7A Medium 4:1	KFR7A:1
KFR7A Medium 4:2	KFR7A:1

Table A-3 Analysed pressure and methane in cultivation bottles containing different SFR-groundwater and with different media compositions.

Sample name	Analysed pressure (bar)	Injected Volume (µL)	Analysed CH_4 (µL L^{-1})	Volume of gas phase (L)	CH_4 in gas phase (µL)	CH_4 (µmol)
KFR02 Medium 1:1	1.28	100	553	0.045	31.8	1.30
KFR02 Medium 1:2	1.74	100	21	0.038	1.37	0.06
KFR02 Medium 2:1	1.99	100	253	0.041	20.7	0.85
KFR02 Medium 2:2	1.67	100	8	0.041	0.54	0.02
KFR02 Medium 3:1	0.25	100	34	0.045	0.39	0.02
KFR02 Medium 3:2	0.63	100	275	0.038	6.56	0.27
KFR02 Medium 4:1	1.22	100	52	0.041	2.60	0.11
KFR02 Medium 4:2	0.46	100	418	0.041	8.02	0.33
KFR03 Medium 1:1	1.55	100	66600	0.045	4666	190
KFR03 Medium 1:2	1.66	100	54	0.038	3.36	0.14
KFR03 Medium 2:1	2.37	100	63	0.041	6.12	0.25
KFR03 Medium 2:2	1.71	100	130	0.041	9.16	0.37
KFR03 Medium 3:1	0.44	100	9237	0.045	182	7.46
KFR03 Medium 3:2	0.79	100	19	0.038	0.57	0.02
KFR03 Medium 4:1	1.11	100	19	0.041	0.88	0.04
KFR03 Medium 4:2	0.72	100	152	0.041	4.52	0.18
KFR105 Medium 1:1	0.49	100	14	0.045	0.32	0.01
KFR105 Medium 1:2	1.52	100	12	0.038	0.71	0.03
KFR105 Medium 2:1	1.77	100	14	0.041	1.08	0.04
KFR105 Medium 2:2	1.66	100	14	0.041	0.97	0.04
KFR105 Medium 3:1	1.68	100	22	0.045	1.66	0.07
KFR105 Medium 3:2	1.63	100	16	0.038	1.08	0.04
KFR105 Medium 4:1	2.45	100	7	0.041	0.74	0.03
KFR105 Medium 4:2	1.89	100	8	0.041	0.59	0.02
KFR04 Medium 1:1	1.28	100	553	0.045	31.8	1.30
KFR04 Medium 1:2	1.74	100	21	0.038	1.37	0.06
KFR04 Medium 2:1	1.99	100	253	0.041	20.7	0.85
KFR04 Medium 2:2	1.67	100	8	0.041	0.54	0.02
KFR04 Medium 3:1	0.25	100	34	0.045	0.39	0.02
KFR04 Medium 3:2	0.63	100	275	0.038	6.56	0.27
KFR04 Medium 4:1	1.22	100	52	0.044	2.60	0.11
KFR04 Medium 4:2	0.46	100	418	0.041	8.02	0.33
KFR08 Medium 1:1	1.55	100	66600	0.045	4666	190
KFR08 Medium 1:2	1.66	100	54	0.041	3.36	0.14
KFR08 Medium 2:1	2.37	100	63	0.041	6.12	0.25
KFR08 Medium 2:2	1.71	100	130	0.041	9.16	0.37
KFR08 Medium 3:1	0.44	100	9237	0.045	182	7.46
KFR08 Medium 3:2	0.79	100	19	0.038	0.57	0.02
KFR08 Medium 4:1	1.11	100	19	0.041	0.88	0.04
KFR08 Medium 4:2	0.72	100	152	0.041	4.52	0.18
KFR7A Medium 1:1	0.49	100	14	0.045	0.32	0.01
KFR7A Medium 1:2	1.52	100	12	0.038	0.71	0.03
KFR7A Medium 2:1	1.77	100	14	0.044	1.08	0.04
KFR7A Medium 2:2	1.66	100	14	0.041	0.97	0.04
KFR7A Medium 3:1	1.68	100	22	0.045	1.66	0.07
KFR7A Medium 3:2	1.63	100	16	0.041	1.08	0.04
KFR7A Medium 4:1	2.45	100	7	0.041	0.74	0.03
KFR7A Medium 4:2	1.89	100	8	0.041	0.59	0.02
Negative control Medium 1:1	1.14	100	0	0.050	0	0
Positive control Medium 1:1	1.18	100	46207	0.050	2717	110
Negative control Medium 2:1	1.24	100	0	0.048	0	0
Positive control Medium 2:1	1.20	100	296	0.045	16	0.7
Negative control Medium 3:1	1.13	100	0	0.049	0	0
Positive control Medium 3:1	1.16	100	134	0.046	7.10	0.3
Negative control Medium 4:1	1.24	100	0	0.049	0	0
Positive control Medium 4:1	1.26	100	175	0.046	10.1	0.4

Table A-4. Analysed pH, pressure and methane of M. subterraneum cultivations.

Sample name	Analysed pH	Analysed pressure (bar)	Injected Volume (µL)	Analysed CH_4 (µL L^{-1})	CH_4 in gas phase (µL)	CH_4 (µmol)	Average (µmol)	Standard deviation (µmol)
Cellulose M. subterraneum pH 7_1	7.6	1.13	100	37400	2130	85.2	84.2	±11.2
Cellulose M. subterraneum pH 7_2	7.5	1.45	100	33500	2438	97.5		
Cellulose M. subterraneum pH 7_3	7.5	1.87	100	18600	1748	69.9		
Cellulose M. subterraneum pH 10_1	9.3	1.24	100	11000	690	27.6	19.4	±12.6
Cellulose M. subterraneum pH 10_2	9.4	1.37	100	575	39	1.58		
Cellulose M. subterraneum pH 10_3	9.3	1.13	100	12800	729	29.1		
Cellulose M. subterraneum pH 11_1	9.4	2.00	100	279	28	1.12	1.63	±0.48
Cellulose M. subterraneum pH 11_2	9.3	2.02	100	558	57	2.26		
Cellulose M. subterraneum pH 11_3	9.4	1.97	100	382	38	1.51		
Cellulose M. subterraneum pH 12.5_1	10.2	1.84	100	55	5	0.20	0.21	±0.02
Cellulose M. subterraneum pH 12.5_2	10	1.99	100	50	5	0.20		
Cellulose M. subterraneum pH 12.5_3	10.1	2.00	100	60	6	0.24		
Batch M. subterraneum pH 7_1	7.3	0.78	100	2820	110	4.41	4.75	±2.60
Batch M. subterraneum pH 7_2	7.2	1.99	100	2020	202	8.08		
Batch M. subterraneum pH 7_3	6.9	2.04	100	427	44	1.75		
Batch M. subterraneum pH 10_1	9.3	1.08	100	9850	537	21.4	23.9	±8.60
Batch M. subterraneum pH 10_2	9.5	1.05	100	7110	374	14.9		
Batch M. subterraneum pH 10_3	9.4	1.08	100	16400	889	35.5		
Batch M. subterraneum pH 11_1	9.3	1.96	100	2640	259	10.3	15.1	±7.35
Batch M. subterraneum pH 11_2	9.3	1.97	100	2430	240	9.58		
Batch M. subterraneum pH 11_3	9.2	1.95	100	6520	639	25.5		
Batch M. subterraneum pH 12.5_1	10	1.95	100	59	6	0.23	0.21	±0.01
Batch M. subterraneum pH 12.5_2	9.9	1.96	100	52	5	0.20		
Batch M. subterraneum pH 12.5_3	9.9	1.89	100	54	5	0.20		
IER M. subterraneum pH 7_1	7.1	1.24	100	43200	2697	107	103	±4.14
IER M. subterraneum pH 7_2	7.6	1.06	100	45800	2450	97.9		
IER M. subterraneum pH 7_3	7.4	1.23	100	42400	2622	104		
IER M. subterraneum pH 10_1	9.4	1.20	100	24100	1457	58.2	54.6	±3.73
IER M. subterraneum pH 10_2	9.3	1.24	100	22500	1406	56.2		
IER M. subterraneum pH 10_3	9.4	1.10	100	22300	1238	49.5		
IER M. subterraneum pH 11_1	9.1	1.85	100	11700	1092	43.6	33.8	±9.83
IER M. subterraneum pH 11_3	9.2	1.95	100	6140	601	24.0		
IER M. subterraneum pH 12.5_1	9.9	2.04	100	56	6	0.23	0.24	±0.03
IER M. subterraneum pH 12.5_2	9.9	1.94	100	54	5	0.21		
IER M. subterraneum pH 12.5_3	9.8	1.97	100	70	7	0.28		

Table A-5. Analysed pH, pressure and methane of KFR cultivations.

Sample name	Analysed pH	Analysed pressure (bar)	Injected Volume (µL)	Analysed CH_4 (µL L^{-1})	CH_4 in gas phase (µL)	CH_4 (µmol)	Average (µmol)	Standard deviation (µmol)
Cellulose KFR pH 7_1	7.4	0.85	100	17700	757	29.6	25.3	±4.25
Cellulose KFR pH 7_2	7.3	0.95	100	14300	686	26.8		
Cellulose KFR pH 7_3	7.4	0.91	100	10900	500	19.5		
Cellulose KFR pH 10_1	9.4	1.42	100	13500	967	37.9	36.2	±1.22
Cellulose KFR pH 10_2	9.4	1.51	100	12000	914	35.8		
Cellulose KFR pH 10_3	9.5	1.36	100	13100	893	35.0		
Cellulose KFR pH 11_1	9.6	1.66	100	1150	96	3.7	2.99	±0.60
Cellulose KFR pH 11_2	9.6	1.69	100	870	74	2.89		
Cellulose KFR pH 11_3	9.6	1.65	100	711	59	2.30		
Cellulose KFR pH 12.5_1	9.6	1.90	100	61	6	0.23	0.19	±0.03
Cellulose KFR pH 12.5_2	10.1	1.59	100	47	4	0.15		
Cellulose KFR pH 12.5_3	10	2.00	100	53	5	0.21		
Batch KFR pH 7_1	7.4	0.81	100	1540	63	2.48	11.9	±6.70
Batch KFR pH 7_2	7.2	0.96	100	9180	444	17.3		
Batch KFR pH 7_3	7.4	0.80	100	10000	405	15.8		
Batch KFR pH 10_1	9.9	1.22	100	17000	1041	40.7	52.7	±8.55
Batch KFR pH 10_2	9.3	1.27	100	24000	1531	60.0		
Batch KFR pH 10_3	9.3	1.10	100	26400	1469	57.5		
Batch KFR pH 11_1	9.4	1.68	100	2270	192	7.51	9.77	±1.95
Batch KFR pH 11_2	9.5	1.58	100	3940	313	12.2		
Batch KFR pH 11_3	9.6	1.38	100	3520	243	9.53		
Batch KFR pH 12.5_1	9.9	2.02	100	58	6	0.23	0.25	±0.05
Batch KFR pH 12.5_2	9.8	1.99	100	51	5	0.20		
Batch KFR pH 12.5_3	9.7	1.94	100	82	8	0.31		
IER KFR pH 7_1	7.2	0.86	100	17000	735	28.8	29.8	±1.87
IER KFR pH 7_2	7.3	0.87	100	19000	828	32.4		
IER KFR pH 7_3	7.3	0.95	100	15100	721	28.2		
IER KFR pH 10_1	9.1	1.04	100	27200	1426	55.8	50.1	±7.15
IER KFR pH 10_2	9.3	1.22	100	16600	1022	40.0		
IER KFR pH 10_3	9.4	1.16	100	23900	1389	54.4		
IER KFR pH 11_1	9.5	1.58	100	4350	346	13.5	8.30	±5.64
IER KFR pH 11_2	9.5	1.61	100	3430	278	10.8		
IER KFR pH 11_3	9.6	1.57	100	156	12	0.48		
IER KFR pH 12.5_1	9.8	2.00	100	80	8	0.31	0.26	±0.04
IER KFR pH 12.5_2	9.8	1.98	100	56	6	0.22		
IER KFR pH 12.5_3	9.8	1.94	100	65	6	0.25		

Table A-6. Analysed pH, pressure and methane of medium controls.

Sample name	Analysed pH	Analysed pressure (bar)	Injected Volume (µL)	Analysed CH_4 (µL L^{-1})	CH_4 in gas phase (µL)
Medium control Cellulose pH 7	7.1	2.00	100	0	0
Medium control IER pH 7	9.1	1.92	100	0	0
Medium control Cellulose pH 10	9.2	1.86	100	0	0
Medium control IER pH 10	10.2	1.79	100	0	0
Medium control Cellulose pH 11	7.1	1.95	100	0	0
Medium control IER pH 11	9.2	1.94	100	0	0
Medium control Cellulose pH 12.5	9.5	2.00	100	0	0
Medium control IER pH 12.5	9.9	1.52	100	0	0

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