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The biogeochemistry of gas generation from low-level nuclear waste: Microbiological characterization during 18 years study under *in situ* conditions

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ABSTRACT

In Finland low level radioactive waste (LLW) contains considerable amounts of cellulose and hemicellulose-based material. Metals are also present in LLW and steel containers are used to store and dispose waste. The microbial degradation of cellulose and hemicellulose, together with the utilization of hydrogen generated by metal corrosion, will result in gas generation under final repository conditions. Microbially mediated LLW degradation and gas generation processes can influence the performance of multi-barrier systems, such as by accelerating corrosion and can affect the mobility of radionuclides from the repository. 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. A significant observation from the GGE was 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 became less significant. The results demonstrate that LLW is converted to methane and carbon dioxide by a succession of anaerobic processes within a complex microbial consortium. Several genes related to cellulose and hemicellulose hydrolysis were detected using bacterial 16S rRNA gene sequencing and PICRUSt bioinformatics software. In addition, microbial groups with potential to metabolise formed saccharides to acetate, hydrogen and volatile fatty acids were detected. Hydrogenotrophic methanogens dominated after one year of operation, which was related to the utilization of hydrogen generated by anaerobic corrosion of steel and metallic waste. Acetoclastic methanogens were detected for the first time in 2005, coinciding with an increase in gas generation rate. Sulphate reducers were the most significant microbial group competing with methanogens for electron donors and their relative ratio compared to methanogens decreased considerably during the operation of the GGE. From the microbiological point of view, the results are consistent with the sequence of microbial processes simulated by previous biogeochemical modelling studies of the experiment.

1. Introduction

In addition to energy, nuclear power plants produce high-level radioactive (HLW), intermediate- (ILW) and low-level nuclear waste (LLW). Because of the differences in classification systems, the composition of LLW varies in different countries. In Finland LLW (activity < 1 MBq/kg) is mainly composed of scrap metals and operational maintenance waste containing materials like paper sheets, cardboard, cotton gloves and different kind of plastics (Posiva, 2016). At Olkiluoto, Finland, compressible miscellaneous maintenance waste is compacted in steel drums and non-compressible waste packed into steel boxes and both of them further into concrete containers (boxes or

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drums). The concrete boxes are emplaced in the bedrock silo of the LILW disposal facility, called VLJ Repository, at a depth of 60–110 m. Approximately 30% of the operational maintenance waste of the nuclear power units OL1 and OL2 in Olkiluoto, has been found to be composed of materials containing cellulose and hemicellulose which are biodegradable, and hence a source of carbon for microbes (Kumar et al., 2008; Perez et al., 2002). A Gas Generation Experiment (GGE) has been in operating at the repository since 1997 to examine in detail how organic-containing LLW degrades under repository conditions and in particular to quantify the rate of microbial gas generation.

Microbiological effects are important to the geological disposal of LLW, mediating processes of gas generation and enhancing corrosion of steel containers and metallic waste (Rodwell, 2000; Rizoulis et al., 2012). Microbial and chemical processes have the potential to influence the performance of the multibarrier system and have to be considered in the repository safety assessments. Methane and carbon dioxide are generated as a result of microbiological degradation of organic materials and this could lead to overpressure in the repository and migration of water-borne radionuclides in the fractures of crystalline bedrock and may drive transport of radionuclide contaminated groundwater to the biosphere (Metcalfe et al., 2008). In addition, irradiated metals and waste materials can contain radionuclides (e.g., ^{14}C and ^{79}Se), which can be volatilized to the biosphere in the form of gas (Peitzsch et al., 2010). The formation of H_2 by corrosion of metals in anaerobic conditions may further contribute to gas generation where H_2 is used as an electron donor in microbial processes (Libert et al., 2011). Soluble organic degradation products also have the potential to enhance corrosion and form aqueous complexes with radionuclides, which may affect their mobility and release from the repository (Stockdale and Bryan, 2013; Glaus et al., 2004).

Gas generation from radioactive waste containing organics has been discussed in the context of repository conditions including Opalinus clay (Leupin et al., 2016), high salinity environments (Wang et al., 1997; Molecke, 1979; Gillow and Francis, 2011; Wang and Francis, 2004), crystalline bedrock (Neretnieks and Moreno, 2014) and near surface repositories (Humphreys et al., 1997; Dumitru et al., 2007; Francis et al., 1980; Molnár et al., 2010). Modelling approaches to represent gas generation have also been developed (Humphreys et al., 1997; Small et al., 2008; Suckling et al., 2015; Leupin et al., 2016). Despite the significance of gas generation from organic containing radioactive waste there are few large-scale and long-term experimental studies that simulate the *in situ* repository conditions apart from the GGE (Small et al., 2008), and similar large-scale experiments more recently commissioned in South Korea (Choung et al., 2014; Ahn et al., 2019), which have included detailed microbiological characterization (Ahn et al., 2019).

Under anaerobic conditions cellulose and hemicellulose (generally comprising more easily hydrolysable amorphous material compared to cellulose) can be converted to methane and carbon dioxide by successive action of microbial species during degradation process (Nelson et al., 2011). Methanogens compete for electron donors with other energetically more favoured microbial processes such as sulphate and nitrate reduction (Madigan et al., 2015). Microbial processes and activity is controlled by geochemistry, including the availability of electron acceptors and donors, pH, and oxidation-reduction potential as well as physical effects such as temperature of the final repository (Pedersen et al., 2012; Rizoulis et al., 2012; Duro et al., 2013). Extreme alkaline pH (pH > 12.5) is expected to limit microbial processes (Rizoulis et al., 2012), which is one of the reasons concrete materials are used in the engineered barrier system of repositories. Other advantages of concrete include low-cost, physical and chemical stability and low solubility of many radionuclides in alkaline conditions (Ojovan and Lee, 2013). Alkaline conditions may however cause abiotic hydrolysis of cellulose and formation of isosaccharinic acid which can form soluble complexes with radionuclides increasing their mobility (Askarieh et al., 2000). However, recent work (Bassil et al., 2015; Rout et al., 2014) provides evidence that isosaccharinic acid can be degraded by microorganisms under alkaline (pH < 11) conditions.

The Gas Generation Experiment (GGE) is a large-scale *in situ* simulation experiment that has been established in 1997 to examine gas generation from drums of LLW under initial conditions representative of the geological disposal for operating waste at the VLJ Repository, Olkiluoto, Finland (Small et al., 2008). 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 some preliminary microbiological studies was first published by Small et al. (2008). Small et al. (2017) provided an update of the gas generation and aqueous chemical data after 18 years of operation of the GGE, over which time it has been observed that the initial alkaline pH buffered by concrete present in the water-dominated regions of the experiment has been neutralised. Small et al. (2008, 2017) also present results of a biogeochemical model that simulates the key anaerobic microbial processes that are likely to occur and their effect on the rate of gas generation and evolution of pH and other chemical variables. The biogeochemical model was developed using the Generalised Repository Model (GRM) developed to simulate gas generation processes and the chemical (pH, Eh) evolution of a UK LLW repository (Small et al., 2008) and was initially undertaken as a blind-test.

Most of the studies concentrating on gas generation in final repository conditions and involving microbial aspects have been based on cultivation-based methods. However, it has been estimated that only 1% of all microbial species can been grown in laboratory conditions, and therefore molecular techniques providing better insight on microbial communities have gradually taken over such conventional cultivation methods. The aim of this study was to use DNA sequencing techniques to examine microbial function and community structure of samples recovered during biodegradation of cellulose- and hemicellulose-based materials in the GGE between 1998 and 2013 to provide insight to the gas generation processes occurring over this extended period. In addition, the results are used to validate the previous GRM biogeochemical modelling and interpretation of the geochemical data.

2. Materials and methods

2.1. Experimental design of GGE

The GGE is located underground at the VLJ Repository, a geological disposal facility for low- and intermediate -level operating waste, at Olkiluoto, Finland and is operated by TVO. Sixteen carbon-steel waste drums (200 dm^3, exterior of the drums painted) were emplaced in a concrete box, as used in the repository, which was enclosed in an acid proof steel tank. The volume of the tank was 20 m^3. Drums were filled with representative maintenance waste from nuclear power units including cellulose-and hemicellulose-based material (paper, cardboard, cotton cloth and gloves) and other materials (polyethylene (PE), polyvinylchloride (PVC) glass fibre latex gloves, natural rubber, polycarbonate cloth, metal) (Fig. 1). The amount of cellulosic materials inside the drums varied from 5 to 95 w-% and are listed by Small et al. (2008). After closure, the repository is planned to be filled with locally sourced river water, with which the GGE was filled. The water was not treated in order to introduce its microbe biota into the experiment. This initial filling water had pH of 7.6 and contained 5.5 mg L^{-1} organic carbon (Table 2). The GGE was maintained at 8–11 °C. Electrical conductivity, pH, Eh and dissolved oxygen in the tank water were continuously monitored by on-line equipment and sample loop located at the drum lid level (Fig. 1). The volume and composition of the released gas was measured (e.g. CH_4, CO_2, H_2, O_2, N_2) by gas chromatography periodically. Fig. 1 shows the location of various sample lines installed in the GGE. Water samples were taken at certain time intervals and analysed for alkalinity, major anions and cations (e.g. SO_4^{2-}, Fe^{2+}, NH_4^+) in TVO's own laboratory and for quality assurance in external

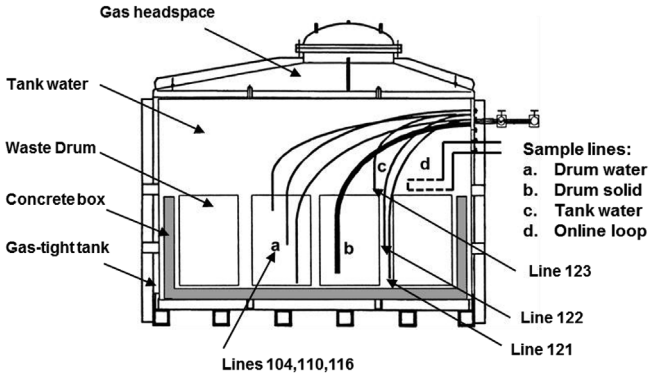

Fig. 1. Schematic of the GGE showing different types of sampling lines (a, b, c) and online analyses (d) and location of Lines 104, 110, 116, 121, 122 and 123 that were used to take water samples for microbiological analysis. The dead volumes of the tubing are approximately 0.1–0.4 dm^3. Also capsules containing a piece of drum steel and LLW were loaded to the experiment (b, drum solid) (after Small et al., 2008).

laboratories. The experimental design and of sampling information are described in more detail by Small et al. (2008, 2017).

2.2. Sampling

Water samples for microbiological analysis were collected from the sampling lines, which sample different levels of the GGE tank (bottom/middle/drum-lid level), and from the drums (Fig. 1). Some of the sampling lines have clogged during operation of the GGE and have been used at different times. Between 1998 and 2005 microbiological water samples were taken from the bottom (Line 121) and the drum-lid level (Line 123) of the GGE tank, transported to the laboratory at 4 °C, stored at −20 °C before analysis and analysed at the same time as samples taken in 2013 (Table 1). In 2013, samples were also taken from the middle level of the tank (Line 122) and from the drums containing maintenance waste (Lines 104, 110, 116) with different proportions of cellulose (Table 1). Samplings were performed anaerobically and aseptically from the line outlets with sterile silicon tubes into sterile, anaerobic headspace vials (vol. 120 mL) sealed with the butyl rubber septum and the aluminium cap (Bellco Glass, NJ, USA). Groundwater from the fractured bedrock of the repository was added in the tank in order to include microbes from the geosphere. Also capsules containing a piece of drum steel and LLW were loaded to in the GGE, analyses are ongoing and results will be presented separately.

Chemical parameters were analysed by TVO's laboratory and further details are presented in Small et al. (2008). Volatile fatty acid analyses were carried out by capillary electrophoresis at VTT. Separations of the carboxylic acid standards and filtrated samples were performed with a P/ACE MDQ capillary electrophoresis instrument (Beckman-Coulter, Fullerton, CA, USA) equipped with a UV detector.

Table 1
Water samples taken from the GGE for microbiological analyses in 2013. Between the time period of 1998 and 2005, and 2015, samples were taken only from Lines 121 and 123. Sampling dates are marked in Fig. 2.

	Sampling line	Description
Tank water	121	Water from the bottom of the tank
	122	Water from the middle level of the drums
	123	Water from the lid level of the drums
Waste drums*	104	Drum 1596024** containing 5 w-% cellulosic material
	110	Drum 1596026**containing 39 w-% cellulosic material
	116	Drum 1596022** containing 95 w-% cellulosic material
Groundwater	The groundwater from the fractured bedrock of the VLJ Repository	

* For clarity in this paper each drum is identified by the sampling line.
** Drum number referred to by Small et al. (2008).

2.3. Total number of microbes

The number of microorganisms in water samples was determined with fluorescent staining with 4′-6′-diamidino-2-phelylindole (DAPI, Sigma) as described by Bomberg et al. (2010). A 1–5 ml water sample was stained with 50 µl of 2.5 mg/ml DAPI suspended in 200 µl of 2.5% glutaraldehyde (Merck, NJ). DAPI is a fluorescent stain, which binds preferentially to certain regions of double-stranded DNA found in bacterial, archaeal and fungal cells. The stained samples were collected on 0.2 µm polycarbonate Isopore Membrane filters (GTPB, Millipore, Billercia, MA, USA). The number of cells was counted from 20 random microscopy fields using an epifluorescence microscopy (Olympus BX60, Olympus Optical Ltd, Tokyo, Japan).

2.4. Nucleic acid extraction

Microbial biomass for nucleic acid (DNA and RNA) extractions was concentrated by filtration of 50–100 mL water on cellulose acetate filters (0.2 µm pore size, Corning) by vacuum suction. DNA is considered to describe the total microbial community and RNA the active fraction of the community. DNA was extracted from the filters with PowerWater DNA Isolation kit (MoBio Laboratories, USA) and RNA with the PowerWater RNA isolation kit (MoBio Laboratories, USA). Extractions were performed according to the manufacturer's instructions and DNA/RNA was eluated with 100 µL of molecular grade water. A negative DNA isolation control was also included.

2.5. Reverse transcription of RNA

RNA samples were translated to complementary DNA (cDNA) with the SuperScript III (Invitrogen, Life technologies, Ltd, UK) and Random hexamers (Promega) as described by Purkamo et al. (2013). A negative reagent control was included.

2.6. Quantitative PCR analysis

In order to quantify the numbers of bacteria, archaea, sulphate reducers and methanogens in the water samples real-time quantitative polymerase chain reaction (qPCR) was applied. The DNA was extracted from the biomass samples (collected during 1998–2015) and were studied by targeting qPCR on 16S rRNA for bacteria and archaea, *dsr*B gene for sulphate reducers and *mcr*A gene for methanogens. In addition, RNA extracted from the 2013 sampled biomass was used to quantitate transcripts of *dsr*B and *mcr*A describing the active fraction of microbial community. PCR reactions were performed in 10 µL reactions, containing 2.5 pmol of each primer, KAPA™ SYBR® Fast 2 × Master mix LightCycler 480 (Kapa Biosystems, Woburn, MA, USA), and 1 µL template DNA or cDNA using LightCycler 480 (Roche, Basel, Switzerland). No-template controls were included in every run and triple reactions were performed for each sample. qPCR was performed on a Roche LightCycler 480 (Roche Applied Science, Germany) on the white 96-well plates (Roche Applied Science, Germany) sealed with transparent adhesive seals (4titude, UK). Gene copy numbers were calculated by comparing the amplification result to that of a dilution series of plasmids containing bacterial 16S rRNA, *mcr*A and *dsr*B gene inserts, or a dilution series of genomic DNA of *Halobacterium salinarum* for archaea. Inhibition of the qPCR reactions was tested by adding sample (1 µL) to *dsr*B gene dilution serie and comparing the result to a dilution series without sample addition.

Bacterial quantification was performed with primers P1 and P2 (Muyzer et al., 1993), which specifically target the sequences flanking the V3 region of the bacterial 16S rRNA gene. The amplification

conditions were an initial denaturation step of 15 min at 95 °C to activate the polymerase, followed by 45 amplification cycles consisting of 10 s denaturation at 95 °C, 35 s annealing at 57 °C and 30 s elongation at 72 °C, and a final extension for 3 min at 72 °C. Sample fluorescence was measured at the end of each elongation phase. The specificity of the qPCR was tested with a melting curve analysis consisting of a denaturation step of 10 s at 95 °C followed by re-annealing at 65 °C for 1 min and a gradual temperature rise to 95 °C, while fluorescence was continually measured.

Archaea were quantified using 400 base pairs (bp) fragment of the 16S rRNA gene copies which were amplified with primers ARC344f (Bano et al., 2004) and Ar774r (modified from Barns et al., 1994). The qPCR conditions consisted of an initial denaturation at 95 °C for 10 min followed by 45 amplification cycles of 15 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C with a quantification measurement at the end of each elongation, and a final extension step of 3 min at 72 °C. The melting curve analysis was as described for the bacteria.

Quantification of sulphate reducers was done by qPCR targeting the 370 bp fragment of the *dsr*B gene copies and transcripts with primers DSRp2060F and DRS4R (Geets et al., 2006; Wagner et al., 1998). Methanogens were quantified by qPCR of 330 bp fragment gene copies and transcripts targeting on *mcr*A which were amplified with primers ME1 and reverse complemented ME3 (5- TGTGTGAAWCCKACDCCACC-3) (Modified from Hales et al., 1996). The qPCR conditions for *dsr*B and *mcr*A was as described for the bacteria, except that the annealing temperature was 54 °C.

2.7. Amplification library preparation

The amplification libraries of bacterial and archaeal 16S rRNA gene fragments for high throughput sequencing were prepared by PCR from the DNA samples as described by Bomberg and Itävaara (2013). The library preparation, emulsion PCR and pyrosequencing were conducted with a Genome Sequencer FLX 454 System according to manufacturer's protocol (454 Life Sciences/Roche Applied Biosystems, Branford, CT, USA).

2.8. Sequence processing and analysis

The sequence reads obtained from the 454 high-throughput sequencing were partly processed with in-house pipeline. The sequence reads were first subjected to quality control using the Mothur software v. 1.31.2 (Schloss et al., 2009). During this step, adapters, barcodes and primers were removed from the sequence reads, and the quality of basecalls was assessed in order to remove erroneous reads from the data set. Subsequently, chimeric sequence reads, which are a type of sequencing artefact arising from sequences from separate sources fusing into one, were removed from the data set with the USEARCH algorithm version 5.2.236 (Edgar, 2010) by de novo detection and through similarity searches against the Greengenes reference database 13_8 (DeSantis et al., 2006).

Operational Taxonomic Units (OTUs), were detected from the chimera-filtered sequence data set following the open-reference OTU-picking protocol of QIIME v. 1.7.0 (Caporaso et al., 2010). First, all reads that failed to hit the Greengenes reference database (DeSantis et al., 2006) with a minimum of 60% identity threshold were discarded as sequencing error. Subsequently, closed-reference OTUs were picked at 97% clustering identity against the Greengenes database, and de novo OTUs were picked from a randomly subsampled sequence subset that failed the closed-reference OTU-picking stage. Next, singleton OTUs, i.e. OTUs that were represented by a single sequence, were filtered from the data set. Finally, taxonomy from the domain-level up to species-level was assigned to OTUs via representative OTU sequences with the RDP classifier algorithm at minimum of 80% confidence (Wang et al., 2007).

2.9. Metabolic predictions

De novo 16S OTUs were filtered from the bacterial biom file (otu_table_mc2_w_tax.biom) that resulted from open-reference OTU-picking against Greengenes 13_8. The new OTU table was used as input for the PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) software (Langille et al., 2013) on the web-based Galaxy application. PICRUSt software links taxonomic assignments of marker genes, such as 16S, to phylogenetically nearest sequenced reference genomes, from which total metagenomes are extrapolated. Because 16S rRNA copy number varies greatly among bacteria, the OTU table was normalized by dividing the abundance of each organism by its predicted 16S copy number (normalize_by_copy_number.py). PICRUSt also calculated the nearest sequenced taxon index (NSTI) which quantify the availability on nearby genome representatives for each sample. Annotated genes related to lignocellulose degradation in each sample were analysed.

3. Results

3.1. Gas generation, gas composition and changes in water chemistry

The data related to gas generation and other chemical parameters measured from the GGE were recently presented by Small et al. (2017). Consequently, only selected chemical data are presented here. Gas generation started approximately after one year of operation and has varied from 0.5 m^3/year to 1.3 m^3/year, with an increase apparent after around 2005 (Fig. 2). The dominant component in the gas released from the tank has been methane (80–95 vol %) with minor amounts of CO_2, O_2, N_2 and H_2. The concentration of CO_2 has stayed below three volume-% but has unfortunately not been measured since 2009. Small et al. (2017) discussed with the aid of geochemical speciation modelling that, considering increasing levels of dissolved inorganic carbon (10–14 mM) and neutral pH the CO_2 content of the gas phase is likely to have increased to around 10 volume-% after 2009.

Data also shows that chemical conditions in various compartments

Table 2
Selected values for water chemistry in the filling water, GGE in tank water (lid level of the tank, Line 123) and inside the waste drum with the sampling Line 104. DOC = dissolved organic carbon; DIC = dissolved inorganic carbon. nd = not determined.

Time	mg L^{-1}	pH	DOC	DIC	SO_4^{2-}	S (-II)	N_{tot}	NH_4	NO_3^-	Fe_{tot}	Fe^{2+}	Ca
1997 (2nd October)	Initial filling water	7.6	5.5	nd	26	nd	1.5	< 0.05	4.1	0.30	nd	11
1998 (2nd September)	Tank Line 123	11.2	18	nd	49	0.16	0.9	1.8	0.03	0.73	0.4	19
	Drum Line 104	7.3	990	nd	12	0.32	32	23	0.2	310	nd	300
2000 (13th September)	Tank Line 123	9.1	69	34	0.6	6.8	2.8	1.6	0.05	1.7	1.9	9
	Drum Line 104	6.8	1330	36	4	0.2	29	24	0.13	350	370	340
2005 (21st September)	Tank Line 123	7.8	98	24	< 1	< 0.01	2	4	< 1	2.1	0.8	18
	Drum Line 104	6.6	1200	88	< 0.2	0.07	25	19	0.28	410	380	270
2013 (26th February/11th March)	Tank Line 123	7.0	25	173	0.5	0.03	3	6	< 0.4	53	54	48
	Drum Line 104	6.5	600	240	0.2	0.02	11	6.4	0.12	435	420	140

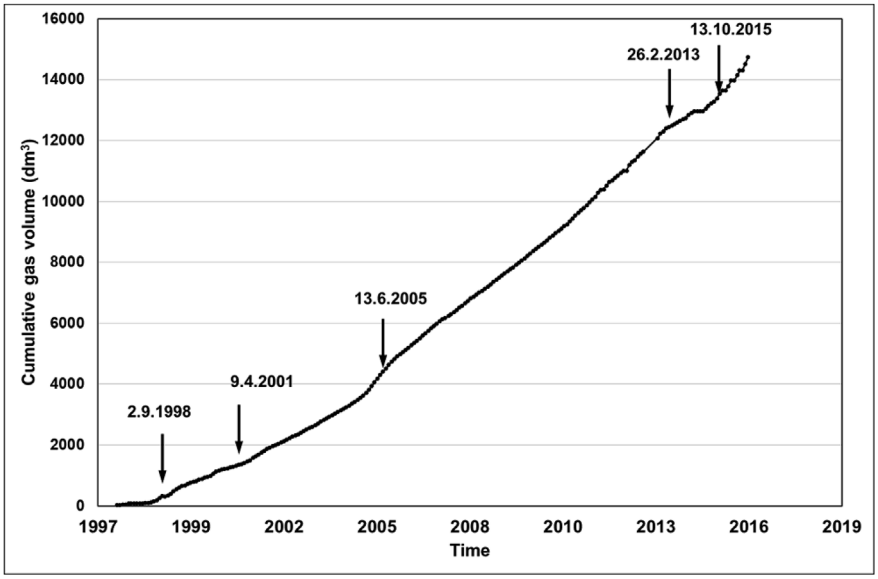

Fig. 2. Cumulative gas generation in the GGE. Arrows represent microbiological samplings from the experiment.

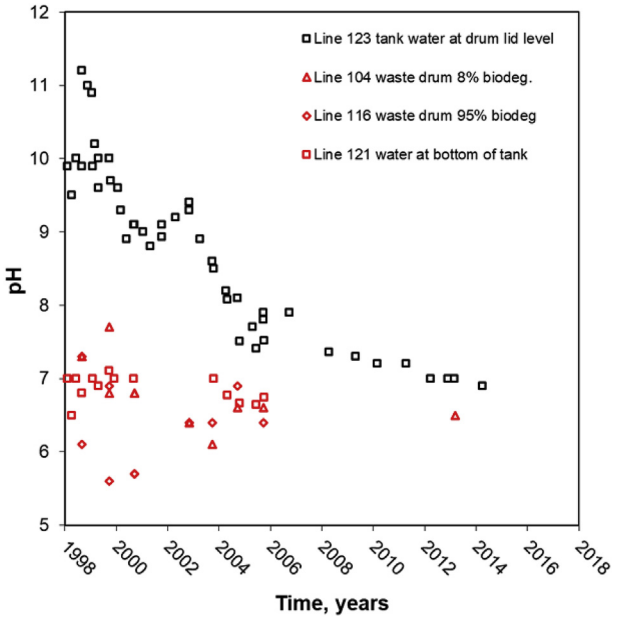

Fig. 3. Evolution of pH in various compartments of the GGE (adapted from Small et al., 2017).

of GGE tank have been very heterogeneous (Table 2). During the first years of operation of the GGE, water at the drum-lid level of the tank was alkaline (pH 10–11) but pH remained close to neutral inside the drums and in the tank water at the bottom of the tank, where there is an accumulation of organic matter originating from river water (Fig. 3). The alkalinity of the tank water has gradually declined, presumably as a result of CO_2 adsorbed into the tank water and microbial metabolites such as volatile fatty acids generated during biodegradation of LLW. The surface of the concrete is likely to have been carbonated as a result of the high concentrations of dissolved inorganic carbon and this may have further reduced the alkaline buffering effect of the concrete. The amount of dissolved organic carbon (DOC) was considerably higher inside the drums compared to the tank water. Dissolved inorganic carbon (DIC) in the tank water increased significantly during operation of the GGE (Table 2) and was higher inside the drums. Volatile fatty acids (VFAs) which are intermediate products of anaerobic degradation of organic matter were detected in the tank water in the beginning of the GGE but were below detection limit in 2013 (Table 3). In 2013, VFAs were found only inside the waste drums in the samples from Lines 104 and 110.

Sulphate reduction was evident in the decline in aqueous sulphate in water sampled from waste drums and from the tank water (Line 123) after 2 years (Table 2). Aqueous sulphide was also recorded at high concentrations in the tank water (2×10^{-4} M, Table 2) and has since declined to below 3×10^{-6} M, most likely due to equilibration of FeS corrosion products as indicated by geochemical modelling (Small et al., 2017).

3.2. Quantity of bacteria and archaea in GGE tank water 1998–2015

The abundance of bacteria and archaea in the tank water during operation of GGE were estimated by analysing the number of 16S rRNA

Table 3
Volatile fatty acids in the GGE measured from tank water and from water inside drums. Samples from the tank bottom (sampling Line 121), the drum-lid level of the tank (Line 123), from water of the three drums (Lines 104, 110 and 116), and groundwater from the fractured bedrock of the VLJ Repository.

VFA g L^{-1}	Tank water, bottom (Line 121)				Tank water drum-lid level (123)	Drum 5% cellulose (104)	Drum 39% cellulose (110)	Drum 95% cellulose (116)	Groundwater
	1998	2001	2005	2013	2013				
Acetic	1.338	0.796	< 0.005[a]	< 0.005	< 0.005	0.952	1.160	< 0.005	< 0.005
Propionic	0.497	0.179	0.258	< 0.005	< 0.005	0.126	0.393	< 0.005	< 0.005
Butyric	0.110	0.019	0.025	< 0.005	< 0.005	0.017	0.165	0.013	< 0.005
Valeric	0.040	0.014	0.000	< 0.005	< 0.005	0.021	0.094	< 0.005	< 0.005

[a] Detection limit 0.005 g L^{-1}

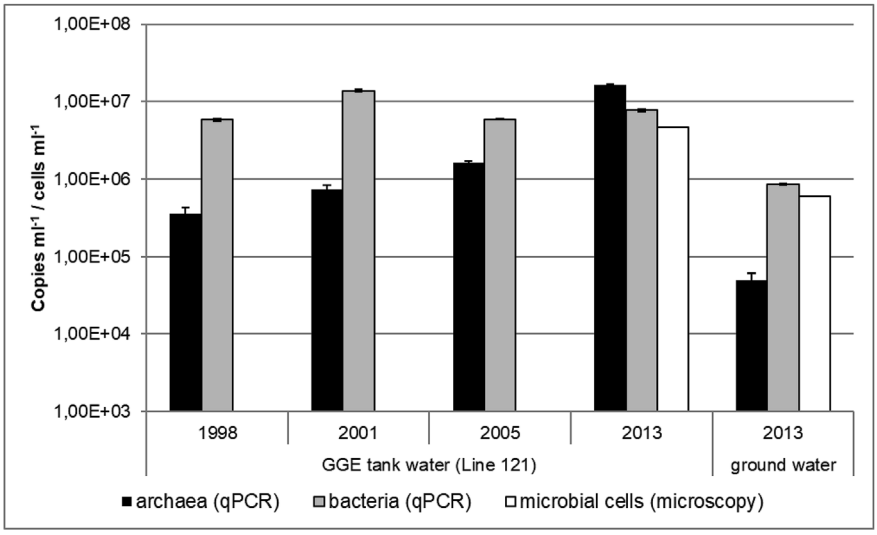

Fig. 4. The amount of bacterial and archaeal 16S rRNA gene copies analysed by qPCR and the number of microbial cells in the GGE tank water determined by microscopy (Line 121, bottom of the tank) in 1998, 2001, 2005 and 2013. The groundwater taken from the fractured bedrock of the VLJ Repository served as a reference.

gene copies in the isolated DNA samples using qPCR. The amount of bacteria and archaea in GGE tank water collected from the bottom of the tank were general higher than in the groundwater (Fig. 4).

The quantity of archaea has increased in the tank water during the operation of GGE. In the sample collected in 1998, the number of archaeal 16S rRNA genes was 3.6×10^5 copies mL^{-1} whereas in 2013 the number of genes was 1.6×10^7 copies mL^{-1}. The amount of bacteria has remained at the same level as indicated by the number of 16S rRNA gene copies that range between 2.1×10^6 and 1.4×10^7 copies mL^{-1} (Figs. 4 and 5a, Fig. 6a).

In 2013, the total number of microbial cells in the GGE tank water were stained and counted by epifluorescence microscopy. The number of cells at the bottom of the tank was 4.6×10^6 mL^{-1}, which was slightly lower compared to the values obtained by qPCR. Several factors in the qPCR analysis have the potential to over- or underestimate the 'real' microbial values. Microbial cells can contain several 16S rRNA gene copies per cell, which can lead to overestimation of cell numbers by qPCR. Bacterial cells can contain 1 to 15 gene copies per cell and copy number seem to be taxon-specific at least by some extent (Klappenbach et al., 2001). It has been reported that the majority of archaeal cells contain only single gene copy per cell but especially species belonging to Euryarchaeota can have one or more gene copies per cell (Lee et al., 2009).

3.3. Methanogens and sulphate reducing bacteria in the GGE tank water in 1998–2015

The abundance of sulphate reducing bacteria (SRB) and methanogens in the tank water samples were analysed based on quantification of the marker genes *dsr*B and *mcr*A, respectively.

The amount of methanogens increased in the tank water, both at the bottom and lid level during operation of the GGE (Fig. 5b). For example, the number of *mcr*A gene copies in the samples from the bottom of the tank (Line 121) was 5.1×10^3 copies mL^{-1} in 1998 and 2.3×10^5 copies mL^{-1} in 2015 (Fig. 5b). This is in accordance to the general increase in the amount of archaea in the tank water as methanogens belong to the domain of archaea (Fig. 4). The amount of methanogens was higher at the bottom of the tank compared to the lid level until 2005, but afterwards the methanogens were equally abundant at both levels.

The amount of SRBs at the bottom of the tank has been relatively steady throughout operation of the GGE (Fig. 5c), which is in accordance with the overall bacterial abundancies quantified with 16S rRNA genes (Fig. 5a). Similar to the methanogens, the SRBs were more abundant at the bottom of the tank in 1998 and 1999 compared to the drum-lid level (Fig. 5c). After that, SRBs have been equally abundant at both levels and the maximum amount of *dsr*B gene copies (1.3×10^5 gene copies mL^{-1}) was found in a sample collected in 2013 from the lid level. The ratio of sulphate reducers to methanogens decreased from 2.1 to 0.1 in the drum-lid level and 7.4 to 0.1 at the bottom of the tank between 1998 and 2015 (Table 4).

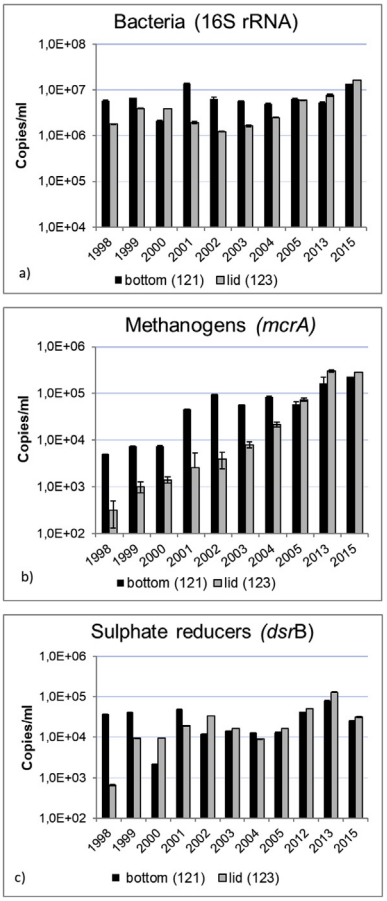

Fig. 5. Bacterial 16S rRNA (a), mcrA (b) and dsrB (c) gene copies in 1 mL of tank water between 1998 and 2015. Samples taken from tank water at the bottom (Line 121) and at the lid level (Line 123) of the GGE tank.

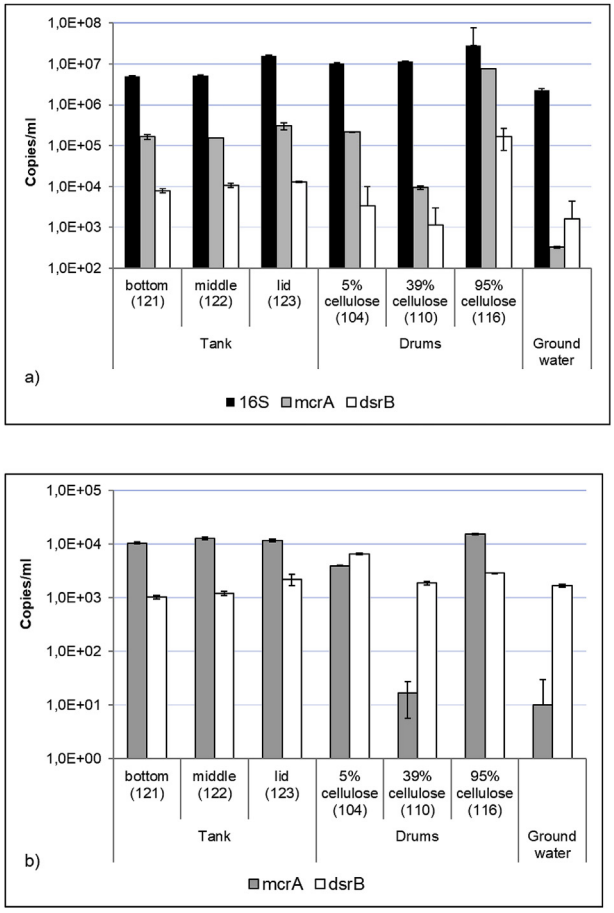

Fig. 6. The bacterial 16S rRNA, mcrA and dsrB gene copies (a) and mcrA and dsrB transcripts (b) in 1 mL in 2013 in various compartments in the GGE. Transcripts describe active fraction of microbial community and gene copies the total microbial community.

3.4. The quantity and activity of sulphate reducers and methanogens in 2013

The abundancies and activities of SRBs and methanogens in the GGE tank water (bottom, middle and lid level of the drums) and in three waste drums (sampled from Lines 104, 110 and 116) were compared using the qPCR based quantification of *dsr*B and *mcr*A gene copies (DNA-based) and transcripts (RNA-based), respectively. DNA represents the total microbial population (including both active and dormant microbes) and RNA active part of the microbial population. Because RNA does not remain stable during long storage, RNA was only extracted from samples taken in 2013 and were used for RNA-based analyses (transcripts).

The SRBs and methanogens were most abundant in the samples taken from the drum, which contained the highest amount of cellulose- and hemicellulose-based waste (Line 116)(Fig. 6a). The amounts of SRBs and methanogens in the other drums and in the tank water were one to two log units lower. No significant differences in the abundance of *mcr*A and *dsr*B gene copies were found between the samples collected from the tank water at the bottom, middle and lid level of the drums. Both SRB and methanogen abundances were in general higher in the GGE samples than in the groundwater reference.

The number of *mcr*A transcripts, reflecting active fraction of methanogens, in the tank water samples and in the waste with the lowest and highest cellulose contents (5% and 95%) was around three log units higher than in the groundwater sample and in the sample from drum 110 with an intermediate cellulose content of 39% (Fig. 6b). The activity level of SRBs (*dsr*B transcripts) in all sampling points (bottom, middle, lid level of the drums, in drum) in the GGE tank and in the groundwater sample was within one log unit.

3.5. Bacterial communities in the GGE

Bacterial communities in the GGE were studied using 454 pyrosequencing of 16S rRNA genes. The analysis included samples from the bottom of the tank collected in 1998–2013 (Line 121). Samples from the GGE tank at the lid- (Line 123) and middle level of the drums (Line 122) of the GGE tank and from the waste drums (Lines 104, 110, 116) were collected in 2013. In addition, a groundwater sample taken from the repository was also analysed as a reference in 2013.

DNA sequencing of microbiomes resulted in 995 (in groundwater) to 13406 (in the sample from the Line 116) sequence reads with mean count of 7522. In the beginning of the GGE experiment in 1998, Bacteroidetes (34% of the sequence reads) and Firmicutes (34% of the sequence reads) were the most abundant phyla in the water samples taken from the bottom of the tank. Their relative proportion in the community decreased until 2005 with increasing amounts of unidentified bacteria OTU, bacterial phyla OD1 (also known as Parcubacteria). In 2013, diversities of this sample and other tank water samples were very alike at the phyla-level (Fig. 7). The most dominant bacterial phyla in these tank waters in 2013 were Bacteroidetes (19–23%), Firmicutes (10–12%), Spirochaetes (10–13%), Proteobacteria (6–7%), Cloroflexi (14–17%) and phyla WWE1 (Waste Water of Evry 1, Candidate phylum Cloacimonetes) (8–10%). In contrast, in 1998, 2001 and 2005 phyla of Chroloflexi, Spirochaetes and WWE1 were almost absent in tank water.

The drum water diversities and relative portions of phyla diverged from each other and from tank water. Bacteroidetes were abundant in all the waste drums in 2013 representing 31–58% of the bacterial community (Fig. 7). Firmicutes were also abundant in the drums, and especially in the drum containing 5% of cellulose (Line 104) where it formed 34% of the bacterial community. Acidobacteria (20% of the community) were found especially in the waste drum with intermediate cellulose content (Line 110). Although Bacteroidetes was the most abundant phylum in the drum containing the highest amount of cellulose (Line 116), many other phyla such as Firmicutes, Spirochaetes, Elusimicrobia and OP11 (Cancidate phylum Microgenomates) were found in relatively high relative abundancy. The phylum WWE1, which was relatively abundant in the tank water samples in 2013 was detected in waste drums in relatively small quantities (0.1–0.8%). SRBs belonging to the phyla Proteobacteria and family of Desulfovibrionaceae were detected in all tank water samples and inside the drums containing 5% and 39% of cellulose (Lines 104 and 110) in 2013.

Table 4
The ratio of sulphate reducers to methanogens in the tank water between 1998 and 2015 estimated based on mcrA (methanogens) and dsrB (sulphate reducers) gene copies in qPCR analysis.

	1998	1999	2000	2001	2002	2003	2004	2005	2013	2015
Tank, bottom (Line 121)	7.4	5.6	0.3	1.1	0.1	0.3	0.2	0.2	0.5	0.1
Tank, drum-lid level (Line 123)	2.1	9.3	6.8	7.5	8.4	2.0	0.4	0.2	0.4	0.1

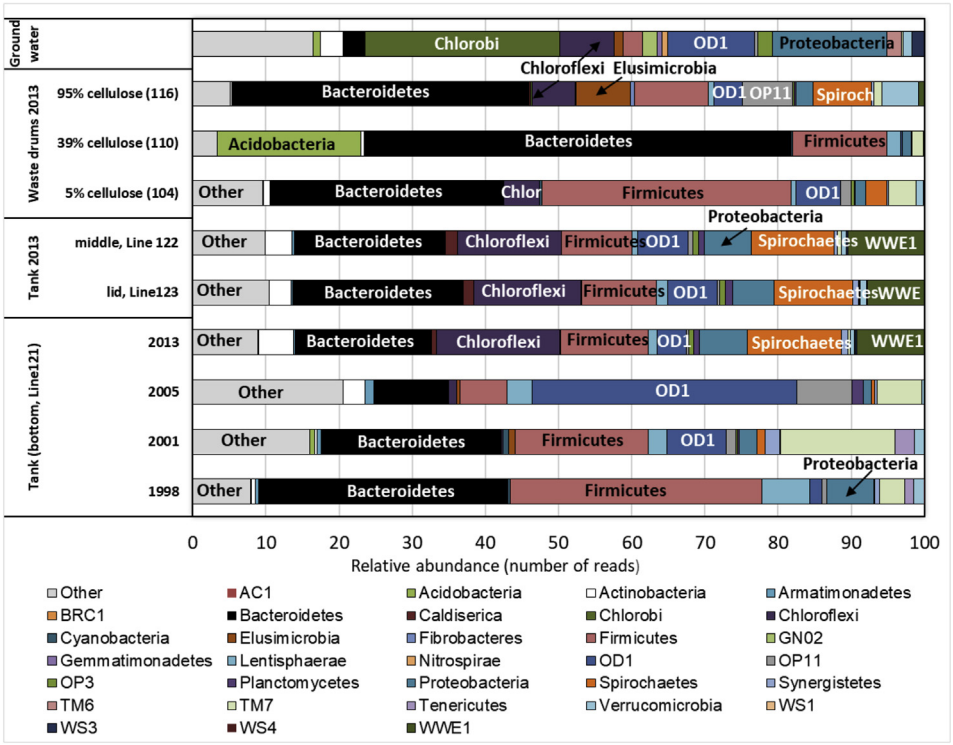

Fig. 7. Relative abundance of bacterial community composition in GGE water samples grouped by phyla-level.

3.6. Archaeal communities

The diversity of archaeal community in the bottom of the GGE tank (Line121) in 1998–2013 and the waste drums (Lines 104,110, 116) in 2013 were also analysed with pyrosequencing in order to further examine the methanogens and other archaea.

The mean sequence read count was 18468 with a range from 10747 sequence reads in the tank water (Line 121, in 2013) to 30175 sequence reads in the drum 104 in 2013. Euryarchaeota was the predominant phylum in all the samples constituting 94–100% of total archaeal community. Within Euryarchaeota three classes were identified including Methanobacteria, Methanomicrobia and Thermoplasmata. Orders Methanobacteriales and Methanomicrobiales represented approximately 62–93% of archaeal community. Hydrogenotrophic methanogenic families Methanoregulaceae, Methanobacteriaceae and Methanospirillaceae dominated the tank water samples in 1998, 2001 and 2005 (Fig. 8). Methanoregulaceae was the most dominant family in the earliest sample collected in 1998, while in 2001 and 2005 the Methanobacteriaceae and Methanospirillaceae became more abundant in the tank water. In 2013, Methanoregulaceae represented again 45% of the tank water community. Acetate utilizing family Methanosaetae belonging to family Methanosaetaceae was not detected from the tank water in 1998 and 2001 but formed 1.9% of the sequence reads in 2005 and 31.4% of sequence reads in 2013.

The archaeal communities in the waste drums were rather similar to each other. Methanoregulaceae and Methanobacteriaceae were the most dominant families in the drums forming 47–59% and 13–25% of the total community, respectively. Genera Methanosarcina (belonging to family Methanosarcinaceae) and Methanosaeta formed 5–32% of the sequence reads in 2013.

3.7. Predicted cellulose degradation

Metabolic functions related to hydrolysis of cellulosic waste in the GGE were predicted using the open-source PICRUSt software, which compares the identified 16S rRNA gene sequences to those of known genome sequenced species thus giving an estimate of the possible gene contents of the uncultured microbial communities.

The accuracy of predicted metabolic functions was evaluated by nearest sequenced taxon index NSTI by PICRUSt. An NSTI value of 1 indicates no similarity to the closest sequenced taxon, while NSTI value 0 indicates high similarity. The NSTI of the bacterial communities varied between 0.102 in water sample taken from the drum 110 in 2013 and 0.189 in tank water sample taken from Line 121 in 2001.

The PICRUSt analysis revealed several predicted genes that could be linked to the degradation of cellulosic wastes including endo-1.4-beta-xylanase (EC:3.2.1.8), beta-mannosidase (EC:3.2.1.25), mannan endo-1,4-beta-mannosidase (EC:3.2.1.78), endoglucanase (EC:3.2.1.4), beta-glucosidase (EC:3.2.1.21) and cellulose 1,4-beta-cellobiosidase (cellobiohydrolase, EC:3.2.1.91). (Table 5). According to the average number of predicted genes, no significant differences could be seen over time in the water samples taken from the bottom of the tank (Line 121). The highest number of predicted genes linked to hemicellulose and cellulose degradation was found from the drum with intermediate content of cellulose, 39% (Line 110).

4. Discussion

Significant gas generation in the GGE started after one year of operation and methane was detected after 6 months indicating microbial degradation of cellulose and hemicellulose in LLW. Gas generation has continued at a generally constant rate, with a notable increase occurred around 8 years, which coincided with neutralization of the tank water (Figs. 2 and 3). The microbiological characterization studies reported here including pyrosequencing analysis and interpretation using PICRUSt of extracted DNA in recently collected samples sheds further light on the complex consortium of microbial groups present and active in the experiment. Fig. 9 provides a summary of the main microbial groups present in this consortium and the chemical substrates and metabolites present. The most significant microbial groups influencing the gas generation in GGE were cellulose and hemicellulose hydrolysing microbes, fermentative bacteria, methanogens and sulphate reducers.

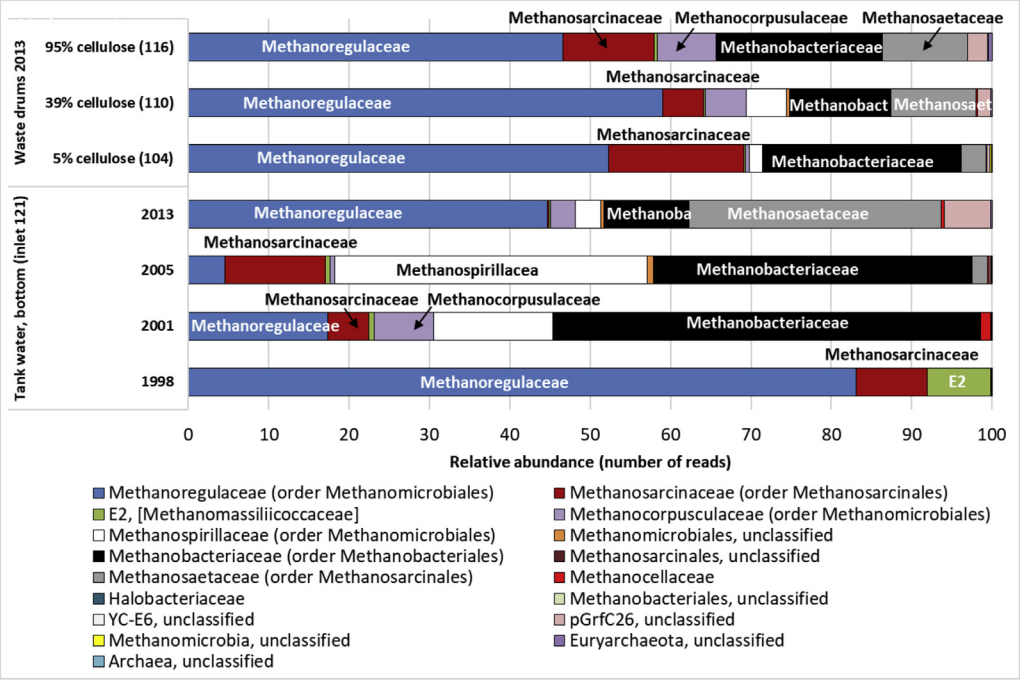

Fig. 8. Relative abundance of archaeal communities in GGE presented as taxonomic family-level.

In the following subsections the evidence supporting the interpretation of the sequence of the microbial processes illustrated in Fig. 9 is presented, followed by discussions of the effects of heterogeneity in the initial condition of the GGE.

4.1. Hydrolysis of cellulose and hemicellulose

In this study, several microbial groups having potential to hydrolyse cellulose and hemicellulose were detected on the basis of 454 pyrosequencing (Fig. 7). Firmicutes were one of the most dominant bacterial phylum in the GGE, comprising 7–34% of the sequences of bacterial community in GGE tank water. In anaerobic digestion the majority of cellulose degraders have been shown to belong to the Firmicutes phylum and especially lineages of the clostridia (Burrell et al., 2004; O'Sullivan et al., 2005). Also bacterial phylum WWE1, with relative abundance of 8–10% of the bacterial community in 2013, has been detected from the mesophilic anaerobic digester at a wastewater treatment plant (Chouari et al., 2005) and it has been suggested that phyla WWE1 has a role either in extracellular cellulose hydrolysis process and/or in the utilization of fermentation products (Limam et al., 2014).

Metabolic functions related to degradation of cellulose and hemicellulose were also predicted using PICRUSt software. Degradation of cellulose- and hemicellulose-based waste requires a concerted action of several extracellular enzymes including endo-enzymes cleaving internally the main chain, exo-enzymes releasing monomeric or dimeric sugars, and enzymes cleaving the side chains of the polymers or associated oligosaccharides (De Souza, 2013). PICRUSt analysis gave indication of the degradation potential of cellulose- and hemicellulose-based material by the microbial consortium in the GGE, but this finding should be treated with cautions since the quality of these results is dependent on the availability of reference genomes in databases used in the analysis. No significant differences in the average number of predicted genes linked to cellulose and hemicellulose degradation could be seen over time in the water sample taken from the bottom of the tank. This indicates that the activity of cellulose degraders has been at the same level at the bottom of the tank during the GGE. In 2013, the highest number of predicted genes linked to hemicellulose and cellulose

Table 5
Average number of predicted genes related to degradation of cellulosic wastes by PICRUSt. Samples taken from the tank water (bottom, drum-lid level, drum-middle level) and from the drums.

Year	1998	2001	2013	2013	2013	2013	2013	2013
KEGG gene description	Tank bottom (Line 121)	Tank bottom (Line 121)	Tank bottom (Line 121)	Tank, drum-lid (Line 123)	Tank, drum-middle (Line 122)	Drum (Line 104) 5% cellulose-based	Drum (Line 110) 39% cellulose-based	Drum (Line 116) 95% cellulose-based
Hemicellulose degradation								
mannan endo-1,4-beta-mannosidase [EC:3.2.1.78]	0	9	10	28	24	8	98	24
beta-mannosidase [EC:3.2.1.25]	307	215	434	423	426	266	890	362
endo-1.4-beta-xylanase [EC:3.2.1.8]	349	241	235	244	260	186	1962	225
Cellulose degradation								
endoglucanase [EC:3.2.1.4]	505	356	688	727	826	934	1008	713
beta-glucosidase [EC:3.2.1.21]	349	241	235	244	260	186	1962	225
cellulose 1,4-beta-cellobiosidase cellobiohydrolase, [EC:3.2.1.91]	60	32	3	9	5	137	4	7

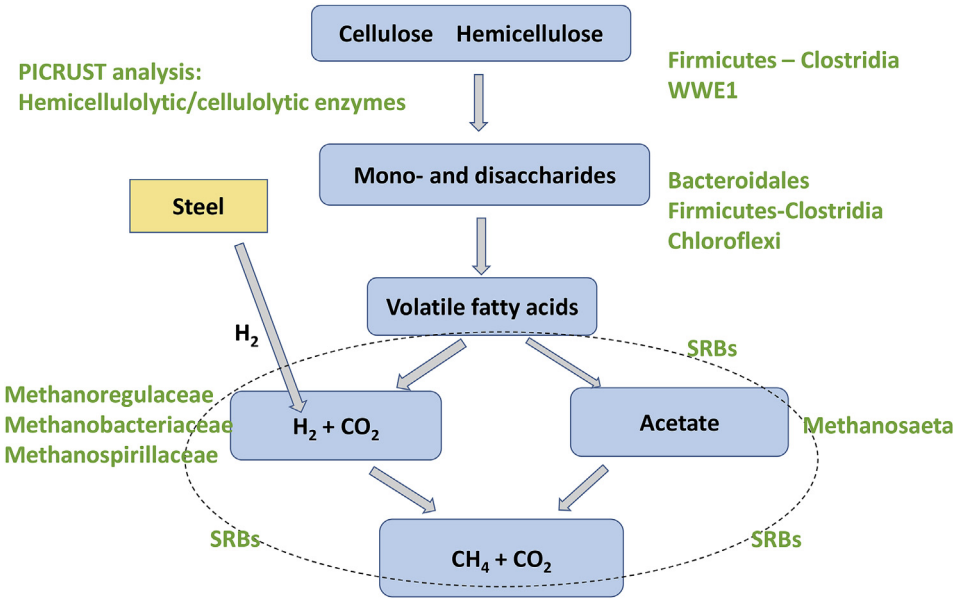

Fig. 9. Sequence of microbial processes during biodegradation of LLW in GGE, microbial groups identified by pyrosequencing and PICRUSt analysis are labelled in green. Figure modified from Small et al. (2008). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

degradation was found in the waste drum 110 containing 39 w-% cellulose-based waste. The drum 110 also contained the highest concentration of volatile fatty acids, which can result from intensive degradation of cellulose and hemicellulose when these metabolites are not consumed fast enough by other microbes.

4.2. Fermentative bacteria

After cellulose and hemicellulose are hydrolysed to mono- and disaccharides they can be further utilized as a substrate by fermentative bacteria, more specifically acidogenic and acetogenic bacteria (Demirel and Scherer, 2008). As a result, hydrogen, volatile fatty acids (VFAs), acetate and carbon dioxide are formed. VFAs including especially acetic and propionic acid were detected from the tank water in 1998, 2001 and 2005 but their concentrations were below detection limit in 2013 (Table 3). This indicates that VFAs were consumed by other bacteria like *Syntrophomonas* and *Syntrophobacter* that were found in tank water in 2005 and 2013. It has been shown, that the accumulation of VFAs can result in the inhibition of activity of methanogens (Franke-Whittle et al., 2014). In 2013, considerable concentrations of VFAs were detected inside the drums sampled by Line 104 and especially by Line 110 containing 5% and 39% of cellulosic maintenance waste, respectively. This could be related to the fact that the drum 110 contained significantly less active methanogens than other drums and tank water (Fig. 6).

The majority of the bacterial community in the GGE was composed of Bacteroidetes with most of the sequences assigned to the order Bacteroidales (Fig. 6) containing saccharolytic species, which can ferment sugars to acetate and succinate (Madigan et al., 2015). In addition, the class Clostridia include saccharolytic species capable of fermenting sugars to produce butyric acid, acetone or butanol as end products (Madigan et al., 2015). Some Clostridia were identified at genus level to *Syntrophomonas* belonging to syntrophic acetogens capable of butyrate and propionate degradation (Nelson et al., 2011). The second largest phylum in tank water in 2013 was Chloroflexi with most OTUs belonging to orders of Anaerolineales and Dehalococcoidetes. Chloroflexi has been shown to form a major part of the bacterial sequences in anaerobic digestion (Nelson et al., 2011) and have been detected in digested sludge (Rivière et al., 2009) and in sediments (Hug et al., 2013). Chloroflexi is a highly diverse group of microbes including species which are able to ferment sugar to acetate and has also been commonly found with anaerobic ammonium oxidation (Pereira et al., 2017). Another abundant bacterial phyla OD1 in the GGE, has been detected in anoxic environments and have been associated with fermentation of simple sugars to organic acids such as acetate and butyrate (Nelson and Stegen, 2015; Wrighton et al., 2014).

4.3. Methane formation

The archaeal community structure of the GGE is quite typical of that observed in anaerobic digestion (Nelson et al., 2011). In the final stage of the anaerobic biodegradation process CH_4 is formed from acetate (acetoclastic methanogens) or from hydrogen and CO_2 (hydrogenotrophic methanogens). On the basis of qPCR results the amount of methanogens increased during the operation of the GGE and was typically higher in the compartments with lower pH and higher amount of organic substrate (Fig. 6). Because the *mcr*A gene can be found in all methanogens, qPCR analysis does not reveal anything about the relative amounts of acetoclastic and hydrogenotrophic methanogens. According to the sequencing results, however, the relative abundance of Methanobacteriales and Methanomicrobiales represented approximately 62–93% of archaeal population in the GGE between 1998 and 2013 and these orders typically utilize H_2/CO_2 or formate as carbon source (Madigan et al., 2015) (Fig. 8). Families Methanosarcinaceae and Methanosaeta belonging to the order Methanosarcinales are known to be able to convert acetate to CH_4 and their relative abundance ranged from 5 to 32% of archaeal population in the GGE. As Methanosarcina can use also other substrates like H_2/CO_2, methanol and methylamines, whereas Methanosaeta can utilize only acetate as a carbon source (Kendal and Boone, 2006; Madigan et al., 2015). Acetate utilizing Methanosaeta were detected only in 2005 and 2013 when it formed 15–32% of archaeal population. The increasing amount of Methanosaeta also coincides with the decreasing amount of acetate and other fatty acids. These results are consistent with the GRM model (Small et al., 2017) which predicted domination of hydrogen utilizing methanogens in the beginning of GGE and acetate utilizing methanogens after approximately 2003 (Small et al., 2017). The increasing amount of acetoclastic methanogens in the GGE is also linked to increased gas generation and decreased amount of DOC.

4.4. Sulphate reduction

Methanogens compete with SRBs for electron donors such as H_2 (Libert et al., 2011) which can influence the gas generation rate. SRBs

are a diverse group of anaerobic bacteria and archaea that can use sulphate as a terminal electron acceptor and release hydrogen sulphide as a metabolic by-product (Muyzer and Stams, 2008). The DNA-based qPCR analysis with *dsr*B marker gene indicates that the number of SRBs has remained approximately at the same level at the bottom of the tank and has increased at the lid level of the tank during the operation of the GGE (Fig. 5). Although the amount of *dsr*B marker genes has remained high during the operation of GGE, the relative ratio of sulphate reducers compared to methanogens has decreased considerably both in the lid and the bottom level of the tank between 1998 and 2013 (Table 4). In addition, the amount of *dsr*B transcripts reflecting the active part of the population was quite small in 2013 (Fig. 6). Because the amount of *dsr*B transcripts was not analysed before the year 2013 there is unfortunately no information about their amount before that. The amount of sulphate has been close to the detection limit since 2000, which could indicate that sulphate, leached out from the waste material inside the drums, is rapidly consumed by SRBs. It has been shown that SRBs can use other electron acceptors besides sulphate (Muyzer and Stams, 2008) which could also explain the fact that SRBs are detected in the GGE. According to the modelling, the amount of SRBs is gradually decreased when they are replaced by hydrogenotrophic methanogens, however the waste as a source of sulphate is not represented in the model (Small et al., 2017).

SRBs are also known to affect various corrosion mechanisms and cause corrosion of steel in anoxic conditions (Bryant et al., 1991; Kakooei et al., 2012; Lee et al., 1995). SRBs produce the corrosive chemical agent hydrogen sulphide and by consuming excess hydrogen, they are believed to stimulate corrosion process. In this study, *dsr*B sequences belonging to the orders of Desulfovibrionales were detected in all samples taken from GGE tank water and inside the waste drums 104 and 110 in 2013. Desulfovibrionales include family of Desulfovibrionaceae, which is demonstrated to contain isolates participating in the corrosion processes (Enning and Garrelfs, 2014).

In addition to SRBs, also denitrifying bacteria can compete with methanogens for electron donors, although in the GGE the inventory of nitrate is low. Nitrogen is an essential element for microbial metabolism and the necessary nitrogen was probably leached out from solid waste materials inside the drums. In future studies, the role of denitrifying bacteria should also be assessed. The denitrifying capability has been detected in numerous groups of bacteria and archaea (Philippot, 2002) which makes difficult to evaluate denitrifying bacteria based on molecular sequencing results.

4.5. Heterogeneity in the GGE

In the beginning of the GGE the chemical conditions were very heterogeneous and especially variation in pH was significant ranging from pH 11 to pH 6 (Small et al., 2008). Concrete materials created alkaline environment at the drum-lid level of the tank, but pH was close to neutral at the bottom of the tank and inside the waste drums. In addition, more organic carbon (DOC) was available at the bottom of the tank due to settled organic matter from the river water and inside the drums containing LLW rich in cellulose and hemicellulose. These heterogeneous conditions created optimal niches for microbial activity, which could also be seen as differences in the microbial abundances in different compartments of the GGE e.g. the amounts of methanogens and SRBs were higher at the bottom of the tank compared to the lid level during the first years of the experiment (Fig. 5). It should also be noted that the samples taken from the sampling lines do not necessarily represent average composition inside the drums because there could be heterogeneous conditions inside one specific drum.

In 2013, chemical conditions in the various compartments in the GGE were stabilized and pH of the tank water was close to neutral. As a result, of more homogeneous conditions no significant differences were detected in the microbial abundances and community structure between the tank bottom and the drum-lid level (Figs. 6–8). However, there were still differences in microbial community structure and activity when samples inside the drums and tank water were compared. For example, the activity of methanogens seemed to be smaller inside waste drum 110 containing 39% of cellulosic biodegradable waste compared to the tank water and the other drums with higher and lower cellulose content (Fig. 6). In addition, bacterial phyla WWE1 was detected inside the waste drums in minor quantities although it was more abundant in the tank water.

5. Conclusions

The application of DNA-based high-throughput sequencing technology and qPCR allowed the characterisation of archaeal and bacterial communities in the GGE. Heterogeneous chemical (pH) conditions in the GGE created optimal niches for microbial action and gas generation starting approximately after one year of operation although alkaline conditions limited microbial activity in certain compartments in the GGE tank for longer. This can be seen e.g. in the abundance of methanogens and SRBs, which was higher at the bottom of the tank compared to the drum-lid level during the first years of GGE. By the year 2013, quite similar microbial community structures and activities were observed inside the waste drums and in tank water indicating a development to more homogeneous chemical conditions.

The results demonstrate that cellulosic and hemicellulosic components in LLW were converted to methane and carbon dioxide as a successive action of a complex microbial consortium. The dominant component in the gas phase has been methane as CO_2 has probably reacted with alkaline tank water resulting in the precipitation of $CaCO_3$. The most significant microbial groups influencing the gas generation in GGE were cellulose and hemicellulose hydrolysing microbes, fermentative bacteria, methanogens and SRBs. Several genes related to cellulose and hemicellulose degradation (hydrolysis) were detected using bacterial 16S rRNA gene sequences and PICRUSt. In addition, several microbial groups with potential to hydrolyse cellulose and hemicellulose and metabolise sugars to acetate and hydrogen or volatile fatty acids were detected by the 454 high-throughput sequencing. Both acetoclastic and hydrogenotrophic methanogens were found in the GGE but the formation of CH_4 from H_2 and CO_2 seemed to be more favourable metabolic route compared to the one utilizing acetate, especially in the beginning of GGE. Methanogens compete with sulphate reducing bacteria for electron donors, which can influence the gas generation rate. The relative ratio of SRBs compared to methanogens decreased considerably both in the drum-lid and bottom level of the tank between 1998 and 2013 although sulphate reduction appears to function simultaneously with methane formation. Rapid degradation of cellulose and hemicellulose and the extensive formation of volatile fatty acids seemed to reduce the activity of methanogens and gas formation, at least temporarily.

Biogeochemical processes in the GGE have previously been modelled using the Generalised Repository Model (GRM) (Small et al., 2008, 2017). Our findings, concerning the sequential development of anaerobic processes, elucidated using DNA pyrosequencing, are consistent with this biogeochemical model. This builds confidence in our interpretation of the biogeochemical processes occurring in the GGE, and the wider application of biogeochemical models to LLW repository studies. The study highlights the value of combining molecular-based microbiological characterization with geochemical and modelling studies to interpret the complex coupled biogeochemical processes of this unique long-term gas generation experiment.

Declaration of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apgeochem.2019.04.002.

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