International Biodeterioration & Biodegradation 81 (2013) 82–87

Contents lists available at SciVerse ScienceDirect

International Biodeterioration & Biodegradation

journal homepage: www.elsevier.com/locate/ibiod

Microbial community structure and microbial activities related to CO_2 storage capacities of a salt cavern

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ARTICLE INFO

Article history:
Received 29 August 2011
Received in revised form
15 July 2012
Accepted 7 August 2012
Available online 31 August 2012

Keywords:
CO_2 storage
Salt cavern
Acetohalobium
Homoacetogenic bacteria
Methanogens

ABSTRACT

Salt caverns are cavities that form in salt deposits and can be used for hydrocarbon or gas storage as well as waste disposal. Despite high salt concentrations in those environments, microbial life still occurs and affects the storage or disposal capacities of the cavern. In order to predict what may occur over time it is important to understand the types of microorganisms present in salt caverns and the environmental impact of their activities. For example if salt caverns were to be used for CO_2 storage, the fate of CO_2 will be impacted by microbial activities. In the presence of H_2 microorganisms can convert CO_2 to acetic acid and water or to methane and water. Samples from a salt cavern plant used to store oily sand were obtained and used to determine microbial community composition by pyrosequencing. Community analyses showed a halophilic, thermophilic community including the genera *Methanohalophilus*, *Methanolobus* and others capable of producing methane. Oil-degrading genera (*Halanaerobium* and *Marinobacter*), sulfate-reducing genera (*Desulfovermiculus* and *Desulfovibrio*) as well as the genus *Acetohalobium* capable of producing acetic acid in high salt environments were also prevalent. Determination of microbial activities related to CO_2 fixation or transformation (homoacetogenic activity and hydrogenotrophic methanogenic activity) indicated that the salt caverns have homoacetogenic activity at both high and low salt concentration. Methanogenic activity was observed with low salt concentration only. Thus if salt caverns were to be used for CO_2 storage production of acetic acid, but not of methane, is expected if H_2 is present.

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1. Introduction

Salt caverns are formed in underground salt deposits. The Western Canada Sedimentary Basin contains several extensive salt deposits contained primarily within strata of the Devonian Elk Point Group (Grobe, 2000). Large cavities of up to 5.10^5 m^3 in volume can be created by solution mining, whereby water is injected into a salt bed or dome and the brine solution is pumped out (Bachu, 2000; Voormeij and Simandl, 2004). Since salt is highly impermeable, salt caverns provide a long-term solution for underground storage of petroleum, natural gas and disposal of wastes (Crossley, 1998; Bachu, 2000; Lux, 2009).

Salt caverns are also considered for CO_2 sequestration. The accumulation of greenhouse gases (GHG) such as CO_2, CH_4 and N_2O in the atmosphere leading to climate change is a major concern of the modern world. Because of its relative abundance compared with the other greenhouse gases, CO_2 is by far the most important, being responsible for about 64% of the enhanced "greenhouse effect" (Bryant, 1997). CO_2 sequestration in the subsurface is a novel and promising technology to mitigate further increases in atmospheric GHG (Dooley et al., 1999; Dooley and Wise, 2003). CO_2 sequestration in salt caverns is an expensive form of geological sequestration of CO_2, and may not be implemented on a large scale (Bachu, 2000; Voormeij and Simandl, 2004). However, situations may arise when CO_2 sequestration in salt caverns may be one of few available options. For example oil sands deposits in northeastern Alberta (Canada) are located in a region where the options for CO_2 geological storage by other means, such as oil and gas reservoirs, coal beds and deep saline aquifers, are limited (Bachu and Stewart, 2002). Several extensive thick salt beds are present in the area and CO_2 sequestration in salt caverns may become an attractive option for reducing CO_2 emissions of the oil sands industry.

If we are to use salt caverns successfully and safely for CO_2 sequestration we need to understand the long-term fate of the stored CO_2. The influence of microorganisms on CO_2 storage is still unknown. Preliminary information collected on a variety of hydrocarbon resource environments has indicated that many of the subsurface community members are able to use CO_2 for energy

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http://dx.doi.org/10.1016/j.ibiod.2012.08.001

metabolism and/or as a carbon source (Watanabe et al., 2002; Gieg et al., 2010; Shartau et al., 2010; Mayumi et al., 2011; Park et al., 2011). Homoacetogenic bacteria for example couple the oxidation of hydrogen with the reduction of CO_2 to produce acetic acid and water. H_2^- and CO_2^-utilizing methanogens (hydrogenotrophic methanogens) can also participate in the bioconversion of CO_2 to methane using H_2 as a reductant.

The present study was conducted to determine the microbial community structure and microbial activities affecting CO_2 sequestration in salt caverns. CO_2 being considered as an important energy metabolism and/or as a carbon source in subsurface ecosystems (Pedersen, 2000) microbes able to reduce CO_2 (i.e. homoacetogenic bacteria or hydrogenotrophic methanogens) should be detected. We also expected many halophilic anaerobic microbes to be present in salt caverns. In this context, the microbial community structures of two salt caverns have been determined by pyrosequencing. Microbial activities related to CO_2 fixation and storage were also investigated focusing on the conversion of CO_2 to acetic acid or methane by homoacetogenic bacteria and methanogens.

2. Materials and methods

2.1. Sample collection from a salt cavern storage plant

Samples were received from Baker Hughes representing two salt caverns (Table 1) on November 9, 2010. The salt cavern plant is located near Lindbergh (Alberta, Canada) and is used to store oily sand, produced as part of a nearby heavy oil production operation. The location of this storage plant makes it also suitable for sequestration of CO_2 produced from the oil sands industry in northeastern Alberta. Sample SC01 was obtained from the facility produced water tank. Sample SC02 and sample SC03 were brine samples obtained from two salt caverns return lines. SC02 came from a salt cavern that has been used for oily sand disposal since 1991 while SC03 came from a salt cavern that will soon be used for oily sand disposal. Both caverns are anaerobic. Sample SC04 was obtained downstream of a brine tank receiving the brines of the two salt caverns. The reported temperatures for SC01, SC02, SC03 and SC04 at the time of sampling were 80 °C, 40 °C, 50 °C and 50 °C, respectively. Samples were shipped at ambient temperature (20 °C) in 1 L Nalgene bottles filled up to the top (no head space), were received the day after collection and were immediately stored at room temperature in an anaerobic hood containing an atmosphere of 90% N_2 and 10% CO_2. Cell pellets obtained by centrifugation were stored at −70 °C and serum bottles for microbial activity measurements were inoculated within 48 h after receiving the samples.

2.2. Microbial community analysis

Microbial community analyses were performed with 454 pyrosequencing as previously described (Park et al., 2011). Briefly, genomic DNA was isolated based on a procedure modified from Marmur (1961) where cell pellets stored at −70 °C were submitted to bead-beating and lysozyme in order to lyse the cells before DNA purification. DNA samples were amplified through 2-step PCR amplification using 16S rRNA gene primers 926Fw (AAACTYAAAKGAATTGRCGG) and 1392R (ACGGGCGGTGTGTRC) in the first PCR (25 cycles) followed by a second PCR (10 cycles) with FLX titanium amplicon primers 454T_RA_X and 156 454T_FwB, which have the sequences for 16S rRNA gene primers 926Fw and 1392R as their 3′-ends. Primer 454T_RA_X has a 25 nucleotide A-adaptor (CGTATCGCCTCCCTCGCGCCATCAG) and a 10 nucleotide multiplex identifier barcode sequence, whereas primer 454T_FwB has a 25 nucleotide B-adaptor sequence (CTATGCGCCTTGCCAGCCCGCTCAG). Concentrations of purified PCR products were determined by a Qubit Fluorometer (Invitrogen), using a Quant-iT™ dsDNA HS Assay Kit (Invitrogen). Additional quality controls were done at the McGill University Innovation Centre (Montreal, Quebec), where pyrosequencing was performed using a Genome Sequencer FLX Instrument and a GS FLX Titanium Series Kit XLR70 (Roche Diagnostics Corporation).

Pyrosequence data analysis was conducted with Phoenix 2, a 16S rRNA gene data analysis pipeline (Park et al., 2011). Sequences which passed quality control and chimeric sequence removal were clustered into Operational Taxonomic Units (OTUs) at 3% distance by using the average linkage algorithm (Schloss and Westcott, 2011). A taxonomic consensus of representative sequences from each OTU was derived from the recurring species within 5% of the best bitscore from a BLAST search against the SILVA database. Amplicon libraries were clustered into a Newick-formatted tree using the UPGMA algorithm with the distance between communities calculated with the thetaYC coefficient as a measurement of similarity between the structures of two communities (Park et al., 2011). Fast Unifrac and Dendroscope were used to generate and visualize the dendrogram. The entire set of the raw reads is available from the Sequence Read Archive (SRA) at NCBI under accession number: SRA048644.

2.3. Determination of homoacetogenic and methanogenic activities in salt cavern brine samples

Homoacetogenic and methanogenic activities were determined for the two salt cavern brine return samples SC02 and SC03 in low salt medium containing (g L^{-1}) NH_4CL (1), KH_2PO_4 (0.33), K_2HPO_4 (0.45), $MgSO_4 \cdot 7H_2O$ (0.10) yeast extract (2) and 0.15 mL L^{-1} of 1% (w/v) resazurin. A high salt variety of this medium contained 160 g L^{-1} of NaCl. Following autoclaving and gassing with 10% CO_2, 90% N_2, 0.5 g L^{-1} of L-cysteine, 1 mL L^{-1} of trace elements solution, 1 mL L^{-1} of tungsten-selenite solution, 30 mL L^{-1} of 1 M $NaHCO_3$ and 1 mL L^{-1} of 1 M Na_2S, all formulated according to Widdel and Bak (1992), were added and the pH was adjusted to 7 with 2 M HCl. Medium (45 ml) was dispensed into 120 ml serum bottles containing a gas phase of 67% H_2 and 33% CO_2 and closed with butyl rubber stoppers. A volume of 5 ml of either SC02 or SC03 was added to inoculate duplicate bottles within 48 h after receiving the samples and these were incubated at 30 °C or 60 °C. Anaerobic water (5 mL) was added to the control bottles.

Homoacetogenic and methanogenic activities were monitored by measuring the formation of acetic acid and methane,

Table 1
Properties of salt cavern plant samples received in November 2010; data provided by Baker Hughes. Cl^-: chloride (ppm); HCO_3^-: bicarbonate (ppm); SO_4^{2-}: sulfate (ppm); Na^+: sodium (ppm); Mg^{2+}: magnesium (ppm); Ca^{2+}: calcium (ppm); TDS: Total Dissolved Solids (ppm); T: temperature (°C).

Sample	Description	Cl^-	HCO_3^-	SO_4^{2-}	Na^+	Mg^{2+}	Ca^{2+}	TDS	pH	T
SC01	Facility produced water	33,900	140	23	18,500	733	1570	55,145	6.5	80
SC02	14–28 cavern brine return	159,000	169	113	109,000	756	1670	270,967	7.1	40
SC03	15–28 cavern brine return	174,000	144	2020	114,000	709	2220	293,358	6.6	50
SC04	Disposal pump	164,000	149	291	97,900	655	1630	264,909	7.0	50

respectively, at 30 and at 60 °C. Acetic acid concentrations in the liquid phase were determined by high-performance liquid chromatography as described elsewhere (Lambo et al., 2008). Methane concentrations in the head space were determined by gas chromatography as described elsewhere (Ramos-Padron et al., 2011).

3. Results

3.1. Microbial community structure in SC01–SC04 samples

Following determination of 18,080 16S rRNA gene sequences from the four different samples, the Mothur software package was used to generate a dendrogram showing the relatedness of the microbial community compositions of the samples obtained (Fig. 1). Two major clusters were observed composed of (i) SC01, SC02 and SC04 and (ii) SC03, indicating the latter to be distinct.

At the phylum level, the microbial community was dominated by *Euryarchaeota* (62.7–74.2%) in SC01, SC02 and SC04 (Fig. 2A). In contrast, the phyla *Firmicutes* (49.8%) and *Proteobacteria* (30.1%) were dominant in sample SC03. Pyrosequencing data showed that at the class level (Fig. 2B), samples SC01, SC02 and SC04, were dominated by *Methanomicrobia* (61.7–71.3%) followed by *Gammaproteobacteria* (18.7%) in SC01 and *Deltaproteobacteria* (18.5% and 15%) and *Clostridia* (15.2% and 20.8%) in SC02 and SC04, respectively. In contrast, for the salt cavern brine return sample SC03, *Clostridia* (49.8%) and *Deltaproteobacteria* (29.7%) were detected as the dominant microbial class followed by *Methanomicrobia* (18.5%). *Methanococci* (0–2.0%), *Thermococci* (0.2–0.6%) and *Halobacteria* (0–0.4%) were detected at lower fractions in the different samples.

The percentage of reads at the genus level shows interesting differences between the samples (Table 2). Although *Methanohalophilus* is the dominant genus in samples SC01, SC02 and SC04, the pattern for the abundance of the other *Methanomicrobia* is different for these three samples. *Methermicoccus* is more abundant in SC01 (5.7%) compared to SC02 and SC04 (0.1%), while *Methanolobus* is more prevalent in SC02 and SC04 (18.3% and 15.6%, respectively) than in sample SC01 (0.3%). *Methanohalobium* (16.2%) were detected in salt cavern brine return sample SC03 and at a lower fractions in sample SC04 and SC02 (3.3% and 0.1%, respectively). In SC03 sample the most abundant genus was *Acetohalobium* (45.4%). *Acetohalobium* was also detected at a lower fraction in samples SC02 (0.5%) and SC04 (9.8%) but not in sample SC01. In addition, other microorganisms detected in the samples included various thermophiles, halophiles, sulfate-reducers and fermentative bacteria (*Methanothermococcus*, *Thermococcus*, *Thermosipho, Pelobacter*, *Halanaerobium, Halocella, Halomonas, Desulfovibrio, Desulfovermiculus*, *Dethiosulfatibacter*, *Desulfohalobium*, *Kosmotoga*).

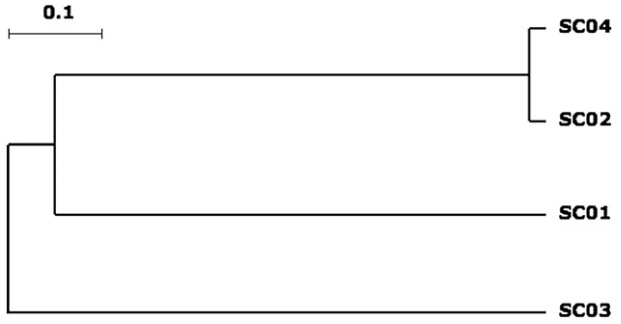


Fig. 1. Dendrogram of microbial community compositions in the salt cavern water samples analyzed in this study (SC01, SC02, SC03 and SC04). The dendrogram was generated as described in the text.

3.2. Microbial activities related to CO_2 storage

At low salinity (no added NaCl) the cavern brine return sample SC02 formed acetic acid or methane more rapidly than sample SC03, as shown in Fig. 3. Acetic acid production was detected within the first 7 days of incubation with SC02 reaching a maximum of 8 mM at the end of the experiment (64 days). With SC03 the acetic acid production started between 17 and 26 days and reached a maximum of 7 mM after 64 days. Methanogenic activity also started earlier with SC02 compared to SC03 (10–20 days and 30–45 days, respectively) with a higher amount of methane produced with SC02 compared to SC03 (22.5 mM and 4.3 mM, respectively). For both samples homoacetogenic activity was detected earlier than methanogenic activity.

At high salinity the cavern brine return sample SC03 was more active in formation of acetic acid than the cavern brine return sample SC02 (Fig. 4A). Acetic acid was detected with samples SC02 and SC03 after 7 days of incubation (3 mM and 5 mM, respectively). The acetic acid remained between 0 and 2 mM with SC02 until the end of the experiment, while it reached a maximum of 12 mM after 64 days with SC03. Methanogenic activity was not detected in either of the samples during the 64 days of incubation (Fig. 4B).

Microbial activity was not detected in any salinity condition at 60 °C.

4. Discussion

Our initial focus was to determine which types of microbes are present in salt caverns and if these can impact CO_2 sequestration. Due to the high salinity and increased temperature of this environment we expected many of these microbes to be obligately halophilic, i.e. capable of growth only under high salt conditions, and thermophilic. Halophilic microbes have been described in other high salt environments like the Dead Sea (Bodaker et al., 2010). Due to the difficulties to cultivate these microbes we resorted to DNA-based technologies to determine the microbial community composition in samples SC01 to SC04. SC02 and SC03, both taken from the brine return lines from the two salt caverns of the plant, were distantly correlated (Fig. 1). Phylogenetic distance between these two samples was also reflected by phylum and class differences in the microbial community profiles (Fig. 2). This observation correlates with the overall water chemistry (Table 1) which indicates higher sulfate concentration (2020 ppm) in the brine return sample from the unused salt cavern (SC03) compared to the brine return sample from the salt cavern used for oily sand disposal (SC02, 113 ppm). SC03 better represents the original state of the local subsurface compared to SC02. Storage of oily sand must have had an important impact on the microbial community of the salt cavern that yielded sample SC02. Anaerobic water-mediated hydrocarbon degradation is typically a three-step process in which complex organic materials are converted to the end products of methane and carbon dioxide (Zengler et al., 1999; Pallasser, 2000; Jones et al., 2008). Numerous microbial groups are involved in this process and can be activated by the addition of hydrocarbons. The final step, conversion of acetate to methane and carbon dioxide, is performed by methanogens which are members of the phylum *Euryarchaeota*. These microbes are less represented in the SC03 sample compared to the other samples (Fig. 2 and Table 2).

Phylogenic data at the genus level correlated with salinity (concentration of chlorides between 33,900–174,000 ppm) and temperature conditions (samples temperature between 40 and 80 °C) of the salt caverns. Species of the main methanogens genera *Methanohalophilus* (SC01, SC02 and SC04) and *Methanohalobium* (SC03) have been documented to be halophilic, methylotrophic

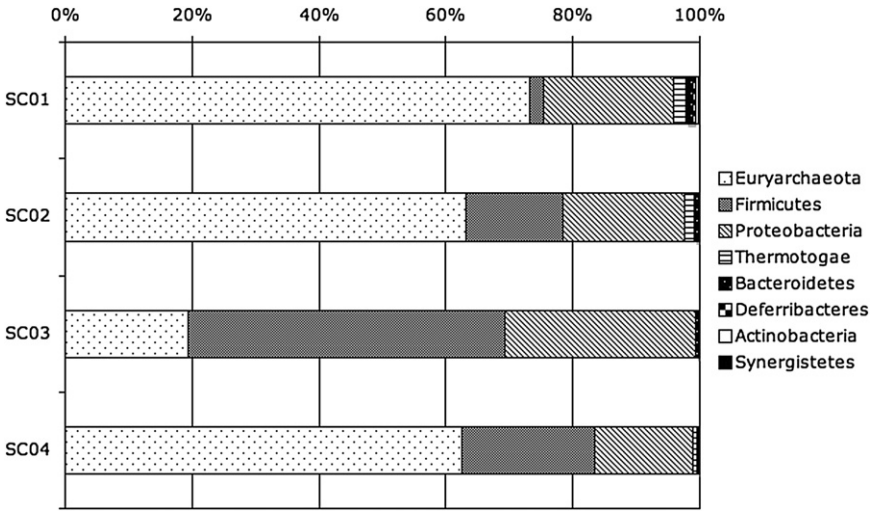


Fig. 2. Stacked-bar representation of microbial community composition at the phylum level of salt cavern water samples. The percentage fraction (%) of reads for each group is indicated.

methanogens. *Methanohalophilus* and *Methanohalobium* species reduce methyl compounds to produce methane but no growth occurs with H_2–CO_2. They have been isolated from a variety of saline environments (Zhilina and Zavarzin, 1987; Mathrani et al., 1988; Paterek and Smith, 1988; Liu et al., 1990).

Among the genus *Methermicoccus* (5.7% in SC01), *Methermicoccus shengliensis* has been documented as a thermophilic, methylotrophic methanogen isolated from oil-production water (*in situ* temperature 75–80 °C) in the Shengli oilfield, China (Cheng et al., 2007). The sampling temperature of water sample SC01 was 80 °C (Table 1). In contrast, *Methanolobus* is more prevalent in samples SC02 and SC04 where the temperature was lower (40 °C and 50 °C, respectively). *Methanolobus* is a mesophilic methylotrophic methanogen. It is found in various hydrocarbon resource environments such coal seams (Doerfert et al., 2009) and natural gas reservoirs (Ehinger et al., 2009; Mochimaru et al., 2009). *Methanothermococcus*, detected in all samples, are thermophilic H_2–CO_2 utilizing methanogens (Huber et al., 1982; Takai et al., 2002).

The most abundant genus in the salt cavern brine return sample SC03 is *Acetohalobium* (45.4%). This genus consists of halophilic, homoacetogenic bacteria. *Acetohalobium arabaticum* participates

Table 2
Community composition of the salt cavern water samples at the genus level. The total number of pyrosequencing reads (*N*) is indicated, as well as the fraction of pyrosequencing reads (%) belonging to a given genus. Genera representing less than 0.1% of the total were not included.

Samples	SC01	SC02	SC03	SC04	Average
Methanohalophilus	61.5	43.3	1.7	42.8	37.3
Acetohalobium	0.0	0.5	45.4	9.8	13.9
Methanolobus	0.3	18.3	0.4	15.6	8.7
Desulfovermiculus	0.0	5.3	20.4	6.4	8.0
Halanaerobium	0.0	13.3	4.3	9.3	6.7
Methanohalobium	0.0	0.1	16.2	3.3	4.9
Desulfovibrio	0.0	3.2	9.1	3.6	4.0
Pelobacter	0.2	9.6	0.1	4.8	3.7
Marinobacter	12.1	0.4	0.1	0.1	3.2
Methermicoccus	5.7	0.1	0.1	0.0	1.5
Marinobacterium	5.5	0.0	0.0	0.0	1.4
Methanothermococcus	2.0	0.8	0.1	0.4	0.8
Dethiosulfatibacter	0.0	0.7	0.0	1.3	0.5
Caminicella	1.0	0.5	0.1	0.3	0.5
Thermococcus	0.6	0.5	0.2	0.3	0.4
Kosmotoga	0.5	0.6	0.0	0.1	0.3
Flavobacterium	0.7	0.0	0.0	0.1	0.2
Anaerophaga	0.0	0.7	0.0	0.1	0.2
Halocella	0.5	0.1	0.0	0.1	0.2
Halomonas	0.5	0.1	0.0	0.0	0.1
Carboxydocella	0.5	0.0	0.0	0.0	0.1
Thermosipho	0.4	0.1	0.0	0.0	0.1
Limnohabitans	0.5	0.0	0.0	0.0	0.1
Thiomicrospira	0.5	0.0	0.0	0.0	0.1
Desulfohalobium	0.0	0.3	0.1	0.1	0.1
Total %	93.0	98.6	98.3	98.6	97.1
Total reads (*N*)	2686	4773	5737	4884	4520

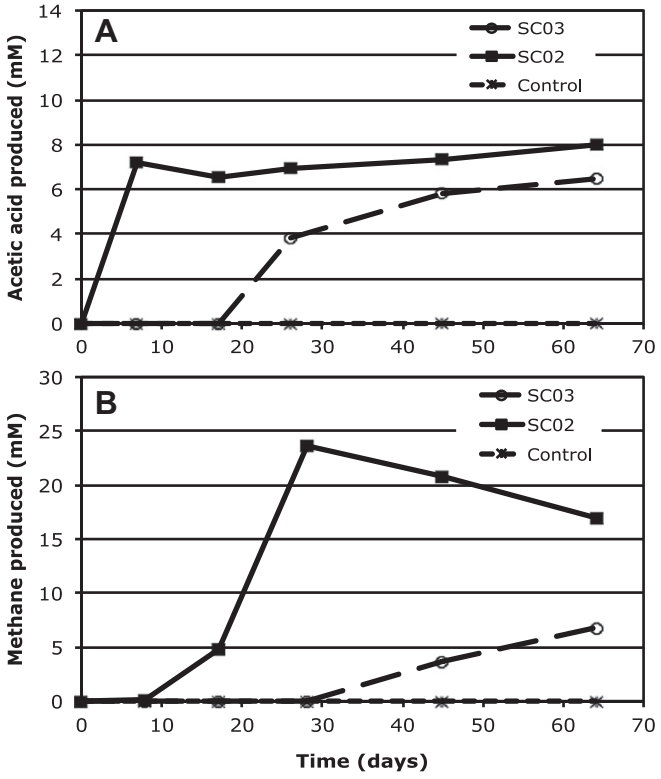


Fig. 3. Production of (A) acetic acid and (B) methane by cavern brine return samples SC02 and SC03. Activities were determined in low salt medium (no NaCl added). Results shown are averages of duplicate serum bottles.

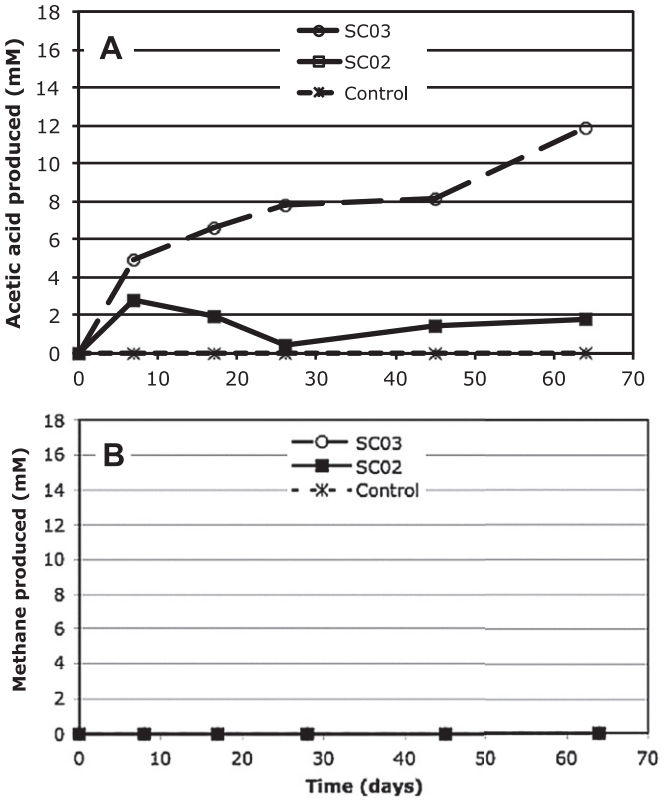


Fig. 4. Production of (A) acetic acid and (B) methane by cavern brine return samples SC02 and SC03. Activities were determined in high salt medium (160 g L^{-1} of NaCl added). Results shown are averages of duplicate serum bottles.

together with other halophilic bacteria (and methanogens) in the C1-trophic chain in hypersaline environments (Zhilina and Zavarzin, 1990) thus contributing to the production of acetic acid.

The next most abundant genus in sample SC03 is *Desulfovermiculus* (20.4%), belonging to the class *Deltaproteobacteria*. *Desulfovermiculus halophilus* is a facultatively chemolithoautotrophic, moderately halophilic, sulfate-reducing bacterium and has been isolated from a highly mineralized formation water of an oil field (Belyakova et al., 2006).

The thermophilic and halophilic nature of the salt cavern environment is also indicated by the presence of the lower abundant class *Thermococci* (0.2%–0.6%), *Thermosipho* (0%–0.4%), *Halocella* (0–0.5%) and *Halomonas* (0%–0.5%).

In view of the presence of acetogens and methanogens in the salt cavern brine return samples as determined by DNA sequence analysis, homoacetogenic and methanogenic activities were expected. The higher homoacetogenic and methanogenic activities detected in SC02 sample at low salinity indicates that oily sand disposal in the SC02 salt cavern led to a more active microbial community. Methanogenic activity from H_2–CO_2 with sample SC02 also correlated with the detection of *Methanothermococcus* in this sample. Homoacetogenic activity at high salinity was expected in the salt cavern brine sample SC03 where *Acetohalobium* represents the main bacterial species.

With the significant growth of heavy oil production in northeastern Alberta, the Lindbergh caverns are ideally situated to handle the waste generated from the primary production facilities. They could also potentially be used for future CO_2 sequestration. The microbial community structure and the homoacetogenic and methanogenic activities detected in the salt caverns indicate that even at high salinity, microbes inhabiting the Lindberg caverns can convert CO_2 to acetate provided H_2 is present. This would limit the risk of leaking CO_2 from the storage site. Methanogens, which were prominent in the microbial community of the salt caverns could convert CO_2 or acetate to methane. However, we have not been able to demonstrate this activity under the high salinity conditions in the cavern.

Acknowledgments

This work was supported by a grant from ISEEE and from the NSERC CMC-NCE in Carbon Capture and Storage as well as by an NSERC Industrial Research Chair Award to GV. The latter was also supported by Baker Hughes Incorporated, British Petroleum, Intertek/CML, Commercial Microbiology Limited (Intertek), the Computer Modelling Group Limited, ConocoPhillips Company, YPF SA, Aramco Services, Shell Canada Limited, Suncor Energy Developments Inc., and Yara International ASA, as well as by the Alberta Innovates Energy and Environment Solutions. This work was also supported by Genome Canada, Genome Alberta and Genome BC. We thank Kirk Miner from Baker Hughes for providing the samples. Xiaoli Dong and Christoph Sensen are thanked for the bioinformatics support, Sean Caffrey and Rhonda Clark for administrative support.

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