Structural analysis of microbiomes from salt caverns used for underground gas storage

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Abstract

Salt caverns are a safe and well-proven reservoir for large-scale natural gas storage and hence, a potential hydrogen storage. Contrary to natural gas, hydrogen is a favorable energy source for many microorganisms. Microorganisms are ubiquitously abundant in the upper lithosphere and therefore expected to be present also in subsurface geological formations potentially selected for H_2 storage, such as salt caverns. Thus, future salt cavern hydrogen storage requires monitoring of the cavern microbiome, to evaluate and prevent unwanted microbial activities. In this study, we analyzed the microbiomes of brines sampled from the bottom of five German natural gas storage caverns. All brines were colonized by microorganisms in considerable cell numbers ranging from $2x10^6$ cells ml^{-1} to $7x10^6$ cells ml^{-1}. The structures of the microbiomes were characterized by 16S rRNA gene amplicon sequencing. A core community detected in all five studied caverns consists of members affiliated to the *Halobacteria*, *Halanaerobiales* and *Balneolales*. Further, a phylotype belonging to the extremely halophilic, lithoautotrophic and sulfate-reducing genus *Desulfovermiculus* was found. Examination of microbial activity also included measuring hydrochemical parameters in order to assess the salt concentrations and the availability of nutrients and potential microbial carbon sources or metabolites. NaCl (4.7 M) was the main salt and sulfate (at average 40.8 mM) the main electron acceptor; methanol (up to 37.5 mM) and ethanol (up to 6.9 mM) were of anthropogenic origin and found in higher concentrations. Some putative microbial metabolites were found in lower concentrations (butyrate, ≤0.7 mM; formate, ≤0.08 mM; acetate, ≤0.5 mM; lactate, ≤0.06 mM); their potential relation to microbial activity is discussed. We propose a guideline for sampling and subsequent chemical and molecular biological analysis for future characterization of microbial communities of salt cavern brines.

Keywords: Halophiles; salt cavern; hydrogen; underground gas storage; sulfate reduction; microbial diversity

Introduction

Power to gas, the electrochemical conversion of excess energy from solar and wind plants into hydrogen (H_2), is a promising concept that requires large-scale underground gas storage (UGS). Due to the chemical properties of halite and long-term operating experience with natural gas, artificial caverns in salt deposits have been proposed to be excellent storage cavities for H_2 [1-5]. Respective caverns are formed through solution mining in underground salt bodies at depths between 500 and 2000 m below surface [1]. This process leaves a sump at the bottom of each cavern. The nature of the salt body shapes the chemical composition of the brine in the sump. In case of north-western Europe an underlying salt body was formed in the late Permian age, due to water evaporation from the Zechstein Sea. This lead to a precipitation series forming thalassohaline evaporate deposits consisting mainly of halite (NaCl), anhydrite/gypsum ($Ca[SO_4]$/$Ca[SO_4]\cdot H_2O$), carbonate ($CaCO_3$), claystone and others, such as carnallite ($KCl\cdot MgCl_2\cdot 6\ H_2O$;), sylvite (KCl), poly-halite ($K_2SO_4\cdot Mg\ SO_4\cdot 2Ca\ SO_4\cdot 2\ H_2O$) and kieserite ($Mg\ SO_4\cdot H_2O$) [6, 7]. Also microorganisms can be included in evaporates [8, 9] and subsequently freed during the process of solution mining. The sulfate minerals in Permian salts are potential electron acceptors for microbial energy generation, while the presence of carbonate rock may provide an inorganic carbon source for anabolism and cell growth. This allows the assumption that autotrophic microbial life is feasible under certain circumstances, given the presence of a suitable electron donor. Introduction of H_2, a potent electron donor, into salt caverns that have so far been used as a natural gas reservoir might stimulate microbial growth. In the presence of sulfate reducing microorganisms, sulfate reduction with H_2 is accompanied by the formation of hydrogen sulfide (H_2S), which has severe negative impacts on gas quality, cavern infrastructure and health as it is highly corrosive and toxic. As many hydrogenotrophic sulfate-reducing microorganisms are not fully autotrophic [10], the availability of consumable organic substances could be the key for their long-term activity. Thus the presence of homo-acetogenic microorganisms in the salt caverns producing acetate by reduction of carbon dioxide with H_2, might be a key process for H_2 oxidation by halophilic sulfate reducers. Hydrogenotrophic methanogens may also compete for H_2, but were not yet detected in salt saturated environments. To date, most studies on extremely halophilic microbial communities have been done with samples from solar salterns, soda and salt lakes [11, 12]. It was proven that reduction of sulfur species is possible at a salinity close to saturation and that anaerobic microorganisms, for example methanogens and sulfate-reducers, can be enriched from such environments [13-15]. Environmental studies showed sulfate-reduction rates of 12.6 $\mu mol*dm^{-3}day^{-1}$ at salinities above 400 g l^{-1} Na^+ [16, 17].

So far, only a few H_2 storage facilities are in operation in the United States (Clemens Dome, Moss Bluff) and the United Kingdom (Teeside)[18], and further information about microbial life in UGS in deep subsurface salt formations is scarce (Table 1). More research on the structure and putative

functions of indigenous microbiomes of underground reservoirs is important to assess the prospects and challenges of future H_2 UGS [19-21]. With this study we provide a first insight into the microbiomes of five natural gas storage salt caverns located in the North European Zechstein formation. Brines were sampled at operating conditions and subsequently analyzed for their microbial community composition (16S amplicon sequencing) and content of ions, fatty acids and organic compounds. This allows for a discussion of putative microbial metabolic processes in the cavern brine under a natural gas atmosphere and an outlook regarding potential effects during H_2 storage. Furthermore, a field sampling and monitoring guideline to support investigation of microbial activity is provided for storage facility operators.

Table 1 Overview of studies investigating microbial diversity in underground salt formations

	Storage site	Storage object	Object of study	NaCl	Microbial studies	Reference
1	North Germany, salt cavern	Oil	Oil, brine	0.4 M	CFU count, enrichment cultures	[22]
2	United States, salt mine	Transuranic waste	Brine, rock salt	3.9 M	CFU count, enrichment cultures	[8]
3	Canada, salt cavern	Oily sand	Salt cavern brine	1.87 M	16S rRNA amplicon sequencing	[23]
4	Canada, salt cavern	Empty (in preparation for future oily sand storage)	Salt cavern brine	2.0 M	16S rRNA amplicon sequencing	[23]
5	Mid Germany, Salt cavern	Natural gas, Town gas (1970s)	Brine	4.7 M	16S rRNA amplicon sequencing, hydrochemical analysis	This study
6	Mid Germany, Salt cavern	Natural gas, Town gas (1970s)	Brine	4.7 M	16S rRNA amplicon sequencing, hydrochemical analysis	This study
7	Mid Germany, Salt cavern	Natural gas	Brine	4.7 M	16S rRNA amplicon sequencing, hydrochemical analysis	This study
8	Mid Germany, Salt cavern	Natural gas	Brine	4.7 M	16S rRNA amplicon sequencing, hydrochemical analysis	This study
9	Mid Germany, Salt cavern	Natural gas	Brine	4.7 M	16S rRNA amplicon sequencing, hydrochemical analysis	This study

Materials and methods

Field sampling and site description

Five caverns (Cav-A, Cav-B, Cav-C, Cav-D, Cav-E), located in the Zechstein formation of Central Germany, were sampled. They have been used as UGS for 25-30 years and have been charged with natural gas. Two caverns, Cav-A and Cav-B, were charged with town gas (up to 50 % H_2) before transitioning to natural gas in mid 1990. The cavities lie at a depth of 0.5 to 1 km below surface, with

diameters ranging from 50 to 100 m. According to their location, the brine was expected to consist mainly of halite, sulfate and carbonate (88>11>0.5%) (Figure 1D). At the time point of sampling, all caverns were operating and charged with natural gas, which required cooperation with SOCON sonar control (Kavernenvermessung GmbH; Giessen, Germany) for sampling. In brief, a high-pressure air lock (Figure 1A) was installed for measurements and sampling. Subsequently, a probe was lowered down for a sonar cavern survey. Echo sounding and conductivity measurements were used to determine cavity dimensions, interface and brine depth, in order to estimate if and to what extent sampling of brine was possible. Having determined the geological dimensions, the sensor was changed for the modular sampling probe. It was equipped with chucks for one to four sterilized stainless steel flasks, each with a capacity of 2 l (Figure 1BC) and having a valve motor to control closure. The sampling device was inserted with the valves closed. After immersion into the brine the bottles were opened, left there for roughly 60 minutes to be flooded with brine and slowly recovered, the valves still being open. Decompression was performed with 4 bar min^{-1} once the sampling device reached the high-pressure air lock. Having completed decompression, the valves were closed and the flasks were removed from the sampling device. The liquid was transferred to N_2 flushed bottles and sealed. The pH value was measured using a pH electrode (LE438) equipped with an integrated temperature sensor for field use connected to a FiveGoTM pH-Meter (Mettler Toledo,Columbus, Ohio, USA). Within 8 hours after sampling, 2x500 ml of brine were filtered through polyethersulfone filters (PES, 0.1 µm; Sartorius, Göttingen, Germany), which were stored at -80 ◦C until field sampling was completed (2 months).

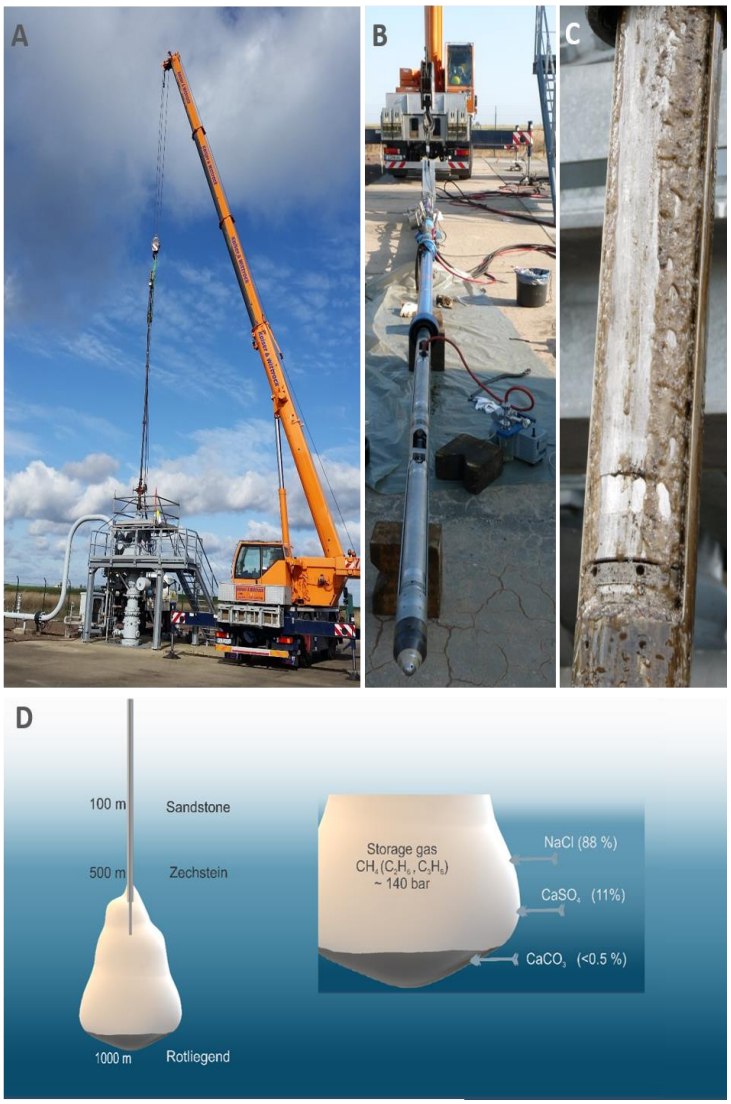

Figure 1: Documentation of the field sampling procedure and cavern dimensions
A: Installation of high-pressure air lock; B: Sampling probe with two slots for 2 l stainless-steel flask; C: Stainless-steel flask after recovery from salt cavern; D: schematic overview of cavern dimensions and brine composition

Determination of total cell counts

Microbial cell numbers were determined immediately after sampling by staining with 4'6-diamidino-2-phenylindole (DAPI). In brief, cells in 10 ml sample volume were fixed with 2 ml 12 % paraformaldehyde in phosphate-buffered saline for 16h at 4 °C. Subsequently, they were filtered onto polycarbonate filters (0.1 µm; Merck, Darmstadt, Germany) and washed with PBS, while increasing ethanol concentration (0%, 30%, 50%, 70%, 80%; 2 ml each). Filter pieces were incubated in a 50 µl DAPI solution (1 µg ml^{-1}, Thermo Fisher Scientific, Waltham, MA, USA) at RT for 10 minutes, shielded from light. After washing with sterilized MilliQ and 80 % ethanol, filter pieces were incubated with Citifluor vectashield (CitiFluorTM AF1, Mounting Medium [Science Services GmbH, München, Germany] mixed 4:1 with Vectashield™ Mounting Medium [LINARIS Biologische Produkte

GmbH, Dossenheim, Germany]) for 1.5 h at -20 °C und finally analyzed using Imager.Z2 (Carl Zeiss Microscopy GmbH, Jena, Germany).

Hydrochemical analysis

The hydrochemical composition of brine samples was determined according to standard methods provided by Deutsches Institut fuer Normung e.V. (DIN). The hydrocarbon-index was determined using solvent extraction and subsequent gas chromatography according to DIN EN ISO 9377-2; dissolved organic carbon (DOC) and total inorganic carbon (TIC) were analyzed by nondispersive infrared CO_2 monitoring according to DIN EN 1484; methanol, ethanol and isopropanol concentrations were determined by gas chromatography according to DIN EN ISO 22155; carboxylic acids including acetate, formate and butyrate as well as chloride and sulfate were analyzed by ion chromatography according to DIN EN ISO 10304-1; nitrate, nitrite, ammonia and phosphate concentrations were determined by spectral photometry according to DIN ISO 15923-1; and iron, potassium and sodium were analyzed by inductively coupled plasma mass spectrometry (ICP-MS) according to DIN EN ISO 17294-2.

DNA extraction

Prior to DNA extraction from the cavern samples, a set of extremely halophilic enrichment cultures from other origins was used to determine the most efficient (high gDNA yield and alpha diversity) DNA extraction method. Different kits (DNeasy PowerWater Kit, DNeasy Tissue Kit Qiagen, according to manufacturer's instruction) and a modified phenol-chloroform extraction method according to Maher et al. [24] were tested (Table SI-1, Figures SI-1, SI-2). Subsequently, 16S rRNA gene fragments were amplified and digested using restriction enzymes *Hae*III, *Rsa*I and *Bst*-UI to be analyzed via T-RFLP according to Ziganshin et al. [25]. On the basis of DNA yield, purity and T-RFLP results (Table SI-1, Figures SI-1, SI-2), the phenol-chloroform extraction method was selected to extract microbial gDNA from cavern sumps. First, microbial cells were lysed during incubation of PES filters in 4 ml lysis solution (150 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.5 % SDS, 0.4 mg ml^{-1} Proteinase K (Machery-Nagel™, Schwerte, Germany) at 55 °C in a hybridization oven (rotation stage 6; Mini MKII Hybaid, MWG Biotech) for 1h. Next, the filter was removed and DNA was separated from cell debris during centrifugation (3,000 x g, RT, 20 min) with 1:1 phenol-chloroform-isoamylalcohol (ROTI Aqua-P/C/I, 25:24:1, CarlRoth, Karlsruhe, Germany). Phenol residues were removed through centrifugation of the aqueous phase with an equal volume of chloroform-isoamylalcohol (ROTI C/I, 24:1, CarlRoth, Karlsruhe, Germany). The resulting aqueous phase was used to precipitate DNA at -20 °C for 16 h with sodium acetate (pH 5.5, 3 M) at a final concentration of 0.3 M and 6 µl glycogen (Serva, Heidelberg, Germany) and subsequently mixed with 2.5 volumes of icecold ethanol abs. Finally, gDNA was pelleted (40 min, 11400 x g, 4 ◦C), washed twice with freshly prepared 70% ethanol (10 min, 11400 x g, 4 ◦C), dried (desiccator, 15 min, RT) and resuspended in 50 µl PCR-grade water.

Library preparation

For amplicon sequencing, the variable V4 region of the 16S rRNA gene was amplified using universal primers 515F and 806R (F: GTGCCAGCMGCCGCGGTAA; R: GGACTACHVGGGTWTCTAAT) [26]. Briefly, 25 µl PCR reaction mixture was prepared containing 1-5 µl undiluted and 10-fold dilutions of gDNA template, 12.5 µl MyTaq^{TM} Mix (Bioline, Heidelerg, Germany), 1 µl bovine serum albumin (2 mg ml^{-1}) and 1 µl (0.2 µM) of each primer with the Illumina overhang adapter sequence. For amplification, initial denaturation was carried out for 1 min at 96 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 50 °C and 30 s at 72 °C with a final elongation for 5 min. Resulting amplicons were purified and indexed according to manufacturer's instructions ('16S Metagenomic Sequencing Library Preparation', Illumina, California, USA). DNA concentration was determined based on the interaction with fluorescent dye (Qubit^{TM} 4 Fluorometer, Thermo Fisher Scientific, Waltham, Massachusetts, USA) and the amplicons were diluted to a final concentration of 4 nM.

Amplicon sequencing and data analysis

The sequencing library was pooled of equimolar concentrations (4 nM) of each sample and 2x300 bp paired-end reads were generated from a final library concentration of 14 pM using Illumina's MiSeq platform. The raw, demultiplexed reads were trimmed and analyzed using qiime2 Version 2021.2 [27]. In doing so, the primer sequences of the reads obtained were removed using Cutadapt Version 1.15 [28]. Dada2 Version 1.18.0 [29] was used create amplicon sequence variants (ASVs) from the forward and reverse reads with an allowed error rate of 2 bases in the forward read and 4 in the reverse read, finally read quality was assessed using fastqc and mulitqc (Versions 0.11.5 and 1.10.1). Obtained ASVs were taxonomically assigned to the recent ribosomal RNA archive (release 138.1) of the Silva Database [30, 31] using a fitted classifier [32]. Results were stored in a biom file and further processed in R (Version 4.1.0) using phyloseq and microbiome [33, 34]. The core community was extracted by filtering for most prevalent species using a detection limit of 0.01 and prevalence of 50%. Sequences were deposited at the European Nucleotide Archive (ENA) under the primary accession number PRJEB49822.

Results

Field sampling

Five gas storage caverns, Cav-A, Cav-B, Cav-C, Cav-D and Cav-E, served as sampling location. They are located in the Stassfurth salt of the German Zechstein sequence [35]. The caverns were built at depths between 765 and 1025 m below surface and each has a volume of approximately 500.000 m^3 and operated with a maximum cavern head pressure of 126 bar. Due to gas charging and discharging in the past decades, water could have been removed from the cavern, so it was therefore not clear

how much brine was left for sampling. Indeed, the brine depth varied from 2.5 to 8 m in the different caverns and more than one sampling was necessary to recover sufficient fluid for caverns Cav-A, Cav-B and Cav-D. Information on physico-chemical and microbiological cavern data are summarized in Table 2. The brine pH value of all samples was around 6.2 (±0.2), the temperature ranged from 24.5 to 27.9 °C and the cavern head pressure from 76 to 126 bar. Microbial cell number ranged from 2.0 to 7.0 x 10^6 cells ml^{-1}.

Table 2 Field sampling and cavern operation details on the cavern geometry obtained through sonar cavern survey (brine level, depth and ground), the number of inlets necessary to recover enough sampling material, brine pH value and temperature (directly measured on the field site), as well as cavern head pressure during operation and the microbial cell number based on DAPI staining.

Cavern	**Cav-A**	**Cav-B**	**Cav-C**	**Cav-D**	**Cav-E**
Brine level / m	876.8	863	919.0	866.2	908.6
Ground / m	879.3	871	926.2	868.9	916.3
Brine depth / m	2.5	8	7.2	2.7	7.7
Inlets	2	3	1	2	1
Brine pH	6.2	6.2	6.0	6.4	6.5
Brine temperature / °C	27.9	24.5	27.8	25.8	25.9
Pressure / bar	119	76	107	126	120
Cell number / N ml^{-1}	2.5E+6	7.0E+6	2.0E+6	4.0E+6	3.9E+6

Hydrochemistry

NaCl concentrations were uniformly 4.7 M, corresponding to 27.5 % w/v (Table 3). In line with the geological profile, sulfate was found in concentrations ranging from 34.7 to 52.0 mM. Potassium (0.8 -1.6 mM) and ammonium (0.2-0.7 mM) were also found in all caverns albeit in lower concentrations. Bioavailable nitrogen sources are a key element for microbial growth and ammonium the energetically most favored form for assimilation. Nitrate, nitrite, phosphate and iron (total iron) were found in trace concentrations in some caverns. The carbon pool for potential production of microbial cell material was found to be diverse. Concentrations of dissolved organic carbon (DOC) ranged from 190 to 460 mg l^{-1} and total inorganic carbon (TIC; comprising CO_2, H_2CO_3, HCO_3^-, CO_3^{2-}) ranged from 55 to 120 mg l^{-1}. The hydrocarbon-index (HCI), resembling the presence of mineral oil compounds in aqueous solutions, ranged from 0.2 to 7 mg l^{-1} brine. It was highest in Cav-A (7 mg l^{-1}) and below the detection limit in Cav-D. Notably, methanol (11.8-37.6 mM), ethanol (0.4-6.9 mM), isopropanol (0.005-0.9 mM) and butyrate (0.5-0.7 mM) were found in all caverns. Low concentrations of formate were detected in all caverns (0.01-0.08 mM), except in cavern Cav-C. In contrast, this cavern was the only one where lactate (0.06 mM) was detected. Acetate was only present in Cav-A and Cav-B (0.5 mM), the caverns that formerly stored gas from coal gasification (coal gas, with H_2 concentration up

to 50%). Propionate, iso-butyrate, n-valerate and caproate were below the detection limit.

Table 3 Chemical composition of five brine samples retrieved from caverns located in the Zechstein formation. The concentrations are in mM, except the hydrocarbon-index, dissolved organic carbon (DOC) and total inorganic carbon (TIC), which are shown in mg l^{-1}.

Compound	**Cav-A**	**Cav-B**	**Cav-C**	**Cav-D**	**Cav-E**
Hydrocarbon-Index	7.0	0.25	0.51	*<0.3*	0.58
DOC	460	220	270	250	190
TIC	100	120	73	62	55
Methanol	37.45	19.35	23.72	31.21	11.86
Ethanol	6.95	0.41	0.91	2.61	4.78
Isopropanol	0.865	0.07	0.008	0.005	0.073
Formate	0.009	0.084	*<0.002*	0.009	0.011
Acetate	0.508	0.454	*<0.002*	*<0.002*	*<0.002*
Lactate	*<0.001*	*<0.001*	0.062	*<0.001*	*<0.001*
Propionate	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*
n-Butyrate	0.499	0.717	0.494	0.54	0.517
iso-Butyrate	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*
n-Valerate	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*
Caproate	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*
Chloride	5259.6	5366.3	5666.0	5359.6	5263.1
Nitrite	0.001	*<0.00043*	0.00043	*<0.00043*	*<0.00043*
Nitrate	0.013	*<0.008*	0.011	*<0.008*	0.034
Sulfate	36.45	34.65	39.34	41.76	51.96
Ammonium	0.70	0.62	0.56	0.44	0.21
Phosphate	0.03	*<0.001*	0.01	0.04	0.01
Fe(II)/Fe(III)	*<0.005*	0.013	*<0.005*	0.076	*<0.005*
Potassium	1.56	1.51	1.46	1.13	0.80
Sodium	4723.4	4794.7	4759.9	4726.0	4653.8

Microbial community composition

A total of 518,088 reads were obtained after sequencing. Read count per sample ranged from 34,682 to 71,260 reads, except sample Cav-B.1, which had 14,408 reads. In total, 881 ASVs were extracted from the dataset and 118 archaeal, one eukaryotic and 762 bacterial ASVs were found and assigned at least to the phylum level. Most prevalent phyla were *Proteobacteria*, *Halobacterota*, *Bacteroidota*, *Firmicutes*, *Actinobacteriota*, *Halanaerobiaeota* and *Desulfobacterota*, comprising 91 % of the microbial community (Figure 2). The remaining 9% are distributed among 21 further phyla. The dominating phyla were usually represented by 2-4 families, except for phylum *Firmicutes*, which was represented by 9 families and phylum *Desulfobacterota*, dominated only by the family *Desulfohalobiaceae* (Figure 2).

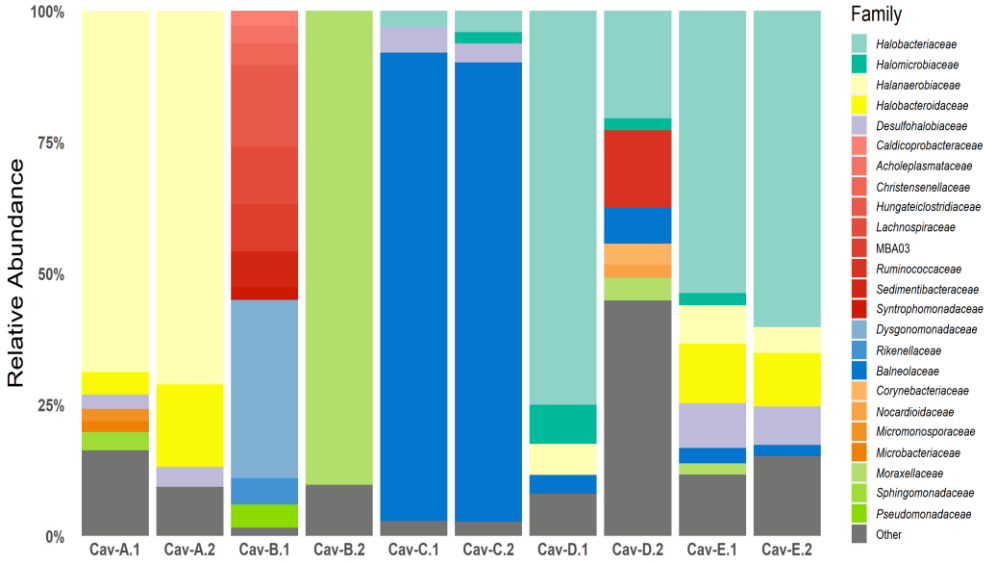

Figure 2: Microbial community composition in five salt caverns based on amplicon sequencing of the 16S-V4 region: Shown are microbial families with a relative abundance >3%. The DNA extraction, amplification and sequencing was performed in duplicates. Read count for Cav-B.1 was significantly lower than in all other samples. Shades of turquoise: phylum *Halobacterota*; yellow: phylum *Halanaerobiaeota*; lilac: *Desulfobacterota*; red: *Firmicutes*; blue: *Bacteroidota*; orange: *Actinobacteriota*; green: *Proteobacteria*; grey: ASVs with <2 % relative abundance.

Shannon-Alpha diversity of the five different caverns ranged from 3.0 to 5.0 (Figure SI-3). Furthermore, all caverns had a different microbial community and abundance composition. While duplicate samples of caverns Cav-A, Cav-C, Cav-D and Cav-E were consistent, sequencing results of Cav-B differed from each other (Figure 2). Sample Cav-B.1 was dominated by *Firmicutes* and *Bacteroidota*, while the other replicate (Cav-B.2) was dominated by one family, *Moraxellaceae*. Sample Cav-A was dominated by *Halanaerobiaceae* (70%), Cav-C was dominated by *Balnaeolaceae* (88%) and in Cav-D and Cav-E, *Halobacteriaceae* were most abundant (52%).

Halobacteriaceae were also found to lesser extents in the other caverns (4-10%), as well as *Desulfohalobiaceae* (3-7%) and *Halanaerobiaceae* (0.1-7%). These findings are supported through constrained analysis of principal components based on weighted unifrac dissimilarity (Figure SI-4).

Microbial core community

In order to investigate the microbiome shared between all caverns, the microbial core community was extracted from the dataset based on a detection threshold of 0.01 and prevalence of 50 %. Members of seven families from five phyla, namely *Bacteroidota*, *Desulfobacterota*, *Halanaerobiaeota*, *Proteobacteria* and *Halobacterota*, were found in all caverns and are displayed in Figure 3. All archaeal ASVs found are *Halobacteria*, seven could be assigned to the genus level and comprise *Halapricum*, *Halodesulfurarchaeum*, *Halanaeroarchaeum*, *Halorhabdus*, *Haloarcula*, *Natronomonas* and *Halomicrobium*. One group could not be further assigned than to the order level *Halobacterales*, and two groups are closely related to so far uncultured members of *Halobacteriaceae* and *Halobacterales*. The bacterial representatives consisted of members of the order *Halanaerobiales* (genus *Halanaerobium*), *Desulfovibrionales* (genus *Desulfovermiculus*) and *Balneolales* (genus *Aliifodinibius*).

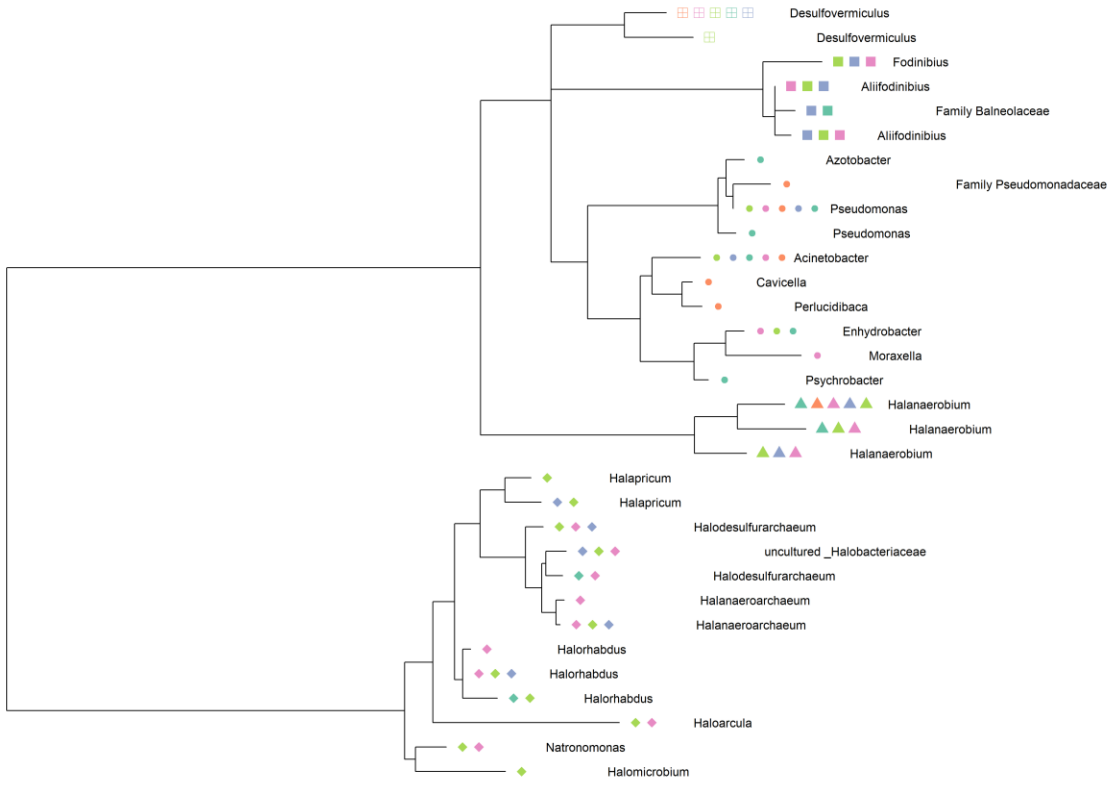

Figure 3: Maximum likelihood tree representation of the microbial core community of Zechstein formation. Colors represent cavern brine origin: ■ Cav-A, ■ Cav-B, ■ Cav-C, ■ Cav-D, ■ Cav-E; shapes represent phylum,

which are from top to bottom: *Desulfobacterota*, *Bacteroidota*, *Proteobacteria*, *Halanaerobiaeota*, *Halobacterota*

Discussion

This study was conducted to investigate the microbiomes of five different salt caverns which are used for natural gas storage, but regarded as potential storage site for green H_2. Various eco-physiologically different microorganisms can consume H_2 as an energy source [36]. It is therefore essential to study the microbiome inhabiting the storage site, as this allows for assessment of possible biogeochemical effects during H_2 storage. In the following sections the characteristics of salt caverns as a microbial habitat and their microbiome itself are discussed.

Habitat characteristics

Main environmental factors determining microbial growth are pH, temperature and osmolarity. The cavern pH values were neutral at 6.2, more acidic than for example average seawater pH, which is at around 8, and alkaline soda lakes, which have a pH value up to 11. The temperature was moderate, too, at 26.4 °C which allows growth of the majority of mesophilic microorganisms.

Salinity was high (4.7 M ±0.05 NaCl), for reference the maximum solubility of NaCl in pure water is 6.2 M (at 30°C). The osmotic pressure resulting from the high NaCl concentration (4.7 M ±0.05 NaCl) in the brines limits microbial activity and growth and is therefore an important factor shaping the inherent community composition. Halophilic microorganisms have been found in other habitats, such as solar salterns and salt and/or soda lakes, with salinities above 25 % w/v [37, 38]. Bordenave and colleagues reported NaCl concentrations of 16 % w/v in brine samples retrieved from a salt cavern in Canada [23]. They found microbial activity when inoculating the samples into salt-free medium [23]. This suggests that microorganisms are able to maintain the integrity of the cell machinery also when salinity temporarily inhibits growth and that salinity determines microbial activity. Osmoregulation is key for extremely halophilic microorganisms, in order to maintain cell integrity and enzymatic activity. Two main strategies are known for microbial adaptation to elevated salt concentrations. One strategy relies on constant excretion of salt ions from the cytoplasm and balances osmotic pressure with synthesis and accumulation of uncharged compatible molecules, such as glycine and ectoine [39, 40]. This requires much energy, but allows the cells to easily react on fluctuating salt concentrations. At very high salt concentrations, the "salt-in" strategy is more likely. In this case, intracellular K^+ and Cl^- concentrations are kept at high levels, while Na^+ concentration is maintained low [41]. The accumulation of KCl reduces the intracellular water availability and has to go along with proteomic adaptations, namely an increased incorporation of the acidic amino acids asparagine and glutamine and a low content of hydrophobic amino acids, allowing for a better

hydration at low water activity whilst proteins are stabilized through high intracellular salt concentrations [42]. This mode of osmoadaptation requires less energy, but cells are more sensitive to reduced salt concentrations in their surrounding environment. K^+ is accumulated from the extra-cellular environment, where Na^+ usually exceeds potassium concentration by 100-fold [41]. In the case of the Zechstein brine samples of this study, the concentration of sodium is 1000 fold higher than the potassium concentration (1.3 mM). However, it was shown for *Halobacterium halobium* that even potassium concentrations starting from 1 mM supported cell growth, until all potassium was cell bound [43]. The trait of "salt-in" strategy is widespread among members of the archaeal class of *Halobacteria*, who made up 50 % of the cavern core microbiome. This strategy is as well reported for *Halanaerobiales*, but no information is found for high salt adaptation modes in the other members of the cavern core microbiome.

Microbial origin & diversity

Mainly, autotrophic extremophiles were expected to thrive in such a nutrient limited environment. However, in this study we found that the brine of the caverns was colonized by various, diverse microorganisms in higher cell numbers than usually observed in groundwater ecosystems [44]. This indicates the presence of live and metabolically active microbial communities. The microorganisms originate from inclusions within the rock formation, or have been introduced either during solution mining, gas charging or together with crude oil, serving as a blanket between brine and gas space. Microorganisms originating from the rock formation might have acquired most efficient strategies to cope with high salinity, as fluctuating formation water levels allowed for adaption of the enzymatic machinery and salt tolerance mechanisms.

Nutrients and microbial nutrient cycling

Aside from extreme salt concentrations, nutrient limitation was considered to be a major limitation to microbial growth. However, different potential carbon sources were found. The brine hydrocarbons and DOC most likely originate from the usage of synthetic fluid blankets, usually glycols or oil, to separate the storage gas from the brine. Additionally, dissolved organic matter (DOM) might leach from the interface of rock salt and brine, expanding the carbon pool. The alcohols, which were also found in all caverns,
i.e. methanol, ethanol and isopropanol, were introduced by cavern installation and maintenance procedures (personal communication). Inorganic carbon is further provided by dissolution of carbonate from the surrounding rock formation. The low concentrations of acetate, formate, lactate and butyrate are thought to have no artificial external origin. They could potentially be linked to microbial activity, mainly to fermentation and homoacetogenesis. Depending on the pathway and initial carbon substrate, fermentation goes along with production of acetate, ethanol, H_2, carbon dioxide and butyrate, lactate, propionate or formate from carbohydrates, amino acids or organic compounds, which in turn are either externally provided or synthesized

by other microorganisms to balance osmotic pressure [45].

Halanaerobium sp., which were found in all caverns, are reported to produce acetate, formate and ethanol (11.8, 0.2 and 1.1 mM) from glucose [46, 47]. *H. praevalens* is additionally capable of producing lactate, propionate and butyrate, the latter in concentrations around 0.68 mM, from complex medium and glucose [48]. This concentration is similar to the butyrate concentrations in the brine samples, which were 0.553 mM (±0.084 mM). In aquitards contaminated with hydrocarbons, accumulation of butyrate up to 28 mM was reported [49]. *H. hydrogenoformans*, found at high pH and salt concentrations, is capable of H_2, acetate, and formate production during fermentation of C5 and C6 sugars [50], while *Haloarcula* sp. is known for amino acid fermentation producing ammonium [51].

With H_2 and CO_2 produced during fermentation, (homo-)acetogenesis is possible [52]. Only few studies investigated homoacetogenesis in saline habitats. Among *Acetohalobium* sp., present in samples from Cav-B, *Acetohalobium arabaticum* was found to be capable of lithotrophic growth and acetate production from CO_2 and H_2 at salinities up 25 % NaCl [53], while *Natroniella acetigena* produces acetate from organic compounds such as lactate and ethanol [54]. Acetate and other metabolites from fermentation, in turn, can serve as electron donors for microbial energy generation via anaerobic respiration.

Methanogenesis, respiration of CO_2 to CH_4, yields only little energy. In saline habitats, methanogenesis is rather performed from formate, acetate or methanol, compounds which were detected in different concentrations of the cavern samples (Table 3). Methylotrophic methanogens were recently enriched from a hypersaline soda and salt lakes by Sorokin and colleagues [55]. The obtained pure cultures were affiliated to a new class *Methanonatronarcheia*. Methane is formed through the reduction of methylated C1-compounds with electrons derived from formate or H_2. Before that, methylotrophic methanogenesis was described for halophilic members of the class of *Methanomicrobia*, in saline sediments with salt concentrations around 4.6 M [42]. Our results support the finding that methanogens can be extremely salt tolerant, as both *Methanomicrobia* and *Methanonatronarchaia* were found in our cavern samples and can be revived in media with lower salt concentrations [56].

Information on anaerobic respiratory processes with nitrate and iron in saline habitats is scarce; generally, nitrate and Fe(III) are the energetically most favorable electron acceptors in anoxic environments. Reduction of Fe(III) was reported for salinities up to 5 M NaCl [57]. In terms of nitrate reduction, both denitrification and ammonification were reported [58, 59] and extensively studied in the model haloarchaeon *Haloferax mediterranei* [60]. However, respiration with nitrate or iron (III) is of lesser importance in this study due the limited availability in the salt caverns. In fact, the limited amount of iron as an important trace element, could even limit microbial growth in the caverns.

Sulfate reduction and the sulfur cycle in general, are well studied in saline habitats. The sulfate concentration in the brine was 40.8 mM and with that, similar to oceanic sulfate concentrations [61]. Sulfate respiration is of major interest for UGS operators. Gas souring, i.e. microbial generation of H_2S from sulfate will reduce gas quality, lead to material corrosion and is highly toxic. The complete sulfur cycle was shown to be carried out at salinities up to 5.1 M [13]. Sulfate reduction in particular, was described in laboratory experiments at salinities up to 3.9 M [62] or 3.1 M coupled to the oxidation of propionate or butyrate [63, 64]. *In situ* sulfate reduction at 5.8 M NaCl could either not be observed [65], or was detected to low extent [66]. Microorganisms mainly associated with reduction of sulfur species in saline habitats are *Desulfovermiculus*, *Halodesulfurarchaeum* and *Halorhabdus* [14, 67]. Species of these genera were found in the cavern samples that were studied. *Halodesulfurarchaea* were previously enriched from anoxic hypersaline sediments expressing a lithoheterotrophic metabolism, using formate/ H_2 as electron donors and sulfur or thiosulfate, but not sulfate, as an electron acceptor [14]. Their optimal salt concentration for growth ranged from 3.5 to 4 M NaCl, with yeast extract as carbon source. Presence of *Halodesulfurarchaea* in the samples presented in this study, indicates an elevated salt tolerance up to 4.7 M. To date, *H. formicicum*, is the sole isolate belonging to the *Halodesulfurarchaea*. Three species have been described for the genus *Halorhabdus*, of which one originated from an anoxic basin of the Red Sea at a similar salinity to our study (4.7 M), while another was found in borehole samples of salt rock [67-69]. The organisms are described to ferment sugars and growth increases with addition of sulfur, resulting in production of sulfide [67, 68]. As of *Desulfohalobiaceae*, only one representative, *Desulfovermiculus halophilus*, was studied more detailed. It was isolated from brine of an oil field, with optimal growth conditions being 10 % NaCl (1.7 M) and a pH value of 7.2. It was capable of sulfate reduction with NaCl concentrations up to 3.8 M and its ability for autotrophic growth with H_2 and CO_2 or formate distinguished it from other genera of the family *Desulfohalobiaceae*. Heterotrophic sulfate-reduction is further described with formate, butyrate, lactate and ethanol, but not with acetate [62, 70]. Actively sulfate-reducing *Desulfovermiculus* sp. at salinities around 4.7 M are not yet reported.

Implications for underground H_2 storage

To date, no incident with sulfide formation during UGS operation with natural gas at our sampling site was reported. The endogenous microbiome, as mentioned above, is presumably capable of sulfate reduction and acetogenesis with H_2 as electron donor. However, if H_2 underground storage triggers metabolic activity of microorganisms in general and sulfate reduction specifically has to be clarified in cultivation approaches. Nevertheless, monitoring of (i) microbial cell numbers, (ii) the fraction of microorganisms closely related to known sulfate reducers and (iii) the fraction of known acetogens throughout the storage process of H_2 is necessary to evaluate the potential for microbially mediated H_2 oxidation processes. We therefore, provide a

field sampling and monitoring guideline for storage facility operators (Figure SI-5) to keep track of the microbiology of their caverns intended for H storage.

Conclusions

Salt caverns are considered as promising option for storing large quantities of hydrogen in the underground due to their specific geological conditions and inertness to chemical reactions as well as for economic reasons (Zivar et al., 2020). Notably, cell numbers as well as structures and functions of microbiomes of subsurface salt formations are poorly investigated; such information is essential to evaluate possible biogeochemical effects caused by microorganisms in salt caverns upon long-term hydrogen storage. Our results demonstrate that the brines of salt caverns located in mid Europe (Germany) are colonized by microorganisms in higher cell counts than usually observed in groundwater ecosystems. The microbiomes were structurally different and consisted mainly of phylotypes affiliated to prokaryotes known to be metabolic active at extremely halophilic conditions, demonstrating that the microorganisms were endogenous and adapted to the harsh environmental conditions. Considering that several ecophysiological different halophilic prokaryotes are capable of utilizing H_2 as energy source, the results suggest that long-term storage of H_2 in salt caverns should be accompanied by biogeochemical monitoring to detect unwanted microbial reactions in time.

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Supplementary Material

The Supplementary Material contains information on the following:

- Evaluation of different DNA extraction methods for cavern brine samples
- Diversity measures of the cavern microbiome
- Monitoring guideline for microbial analysis in salt caverns

The 16S rRNA gene amplicon sequencing data generated for this study can be found in the European Nucleotide Archive (ENA) under accession number PRJEB49822 (http://www.ebi.ac.uk/ena/data/view/PRJEB49822).

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