Microbial hydrogen consumption leads to a significant pH increase under high-saline-conditions– implications for hydrogen storage in salt caverns

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Article

Keywords: Hydrogen underground storage, salt caverns, sulphate reducing microbes, methanogenesis

Posted Date: May 3rd, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2854152/v1

Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at Scientific Reports on June 29th, 2023. See the published version at https://doi.org/10.1038/s41598-023-37630-y.

Abstract

Salt caverns have been successfully used for natural gas storage globally since the 1940s and are now under consideration for hydrogen (H_2) storage, which is needed in large quantities for the Green Shift. Salt caverns are not sterile, and H_2 is a ubiquitous electron donor for microorganisms. This could entail that the injected H_2 will be microbially consumed, leading to a volumetric loss and potential production of toxic H_2S. However, the extent and rates of this microbial H_2 consumption under high-saline cavern conditions are not yet understood. To investigate microbial consumption rates, we cultured the halophilic sulphate-reducing bacteria *Desulfohalobium retbaense* and the halophilic methanogen *Methanocalcus halotolerans* under different H_2 partial pressures. Both strains consumed H_2, but consumption rates slowed down significantly over time. The activity loss correlated with a significant pH increase (up to pH 9) in the media due to intense proton- and bicarbonate consumption. In the case of sulphate-reduction, this pH increase led to dissolution of all produced H_2S in the liquid phase. We compared these observations to an original brine retrieved from a salt cavern located in Northern Germany, which was incubated with 100% H_2 over several months. We again observed a H_2 loss (up to 12%) with a concurrent increase in pH up to 8.5 especially when additional nutrients were added to the brine. Our results clearly show that sulphate-reducing microbes present in salt caverns will consume H_2, which will be accompanied by a significant pH increase, resulting in reduced activity over time. This potentially self-limiting process of pH increase during sulphate-reduction will be advantageous for H_2 storage in low-buffering environments like salt caverns.

Introduction

Many renewable energy systems utilize “Power-to-Gas” technologies, which use renewable electricity to produce hydrogen (H_2), a universal energy carrier. H_2 can be utilized, transported or stored for later use for several industrial sectors like chemical industry, heavy transport and steel production. Storage of H_2 is needed to buffer daily to seasonal variations in energy supply and demand [1–3]. Underground storage in salt caverns is suggested to be the ideal option for large-volume storage of H_2 when the gas can be injected into a cavern and can be withdrawn flexibly for energy generation [4–6]. This process is very similar to the current storage of natural gas or oil. Salt caverns are solution mined large underground cavities inside a salt layer or salt dome by gradually dissolving the salt with freshwater or seawater. The resultant voids are several tens of meters in diameter and several hundreds of meters in length, with volumes ranging from several hundreds of cubic meters and maximum pressures of 200 × 105 Pa (200 bar). They are proposed to be ideal for short- to mid-term storage, with opportunities for rapid injection and withdrawal of gases for energy balancing. Major benefits of salt cavern storage are the available high volumes and relatively low operational costs [5]. As the demand for more storage sites rises, and upfront capital investments for salt caverns are high (~ 30 M€/onshore cavern [7]) there is an increasing need for improved understanding of possible microbiologically triggered subsurface reactions of H_2 within the salt cavern. These potentially can reduce the caloric value as well as leading to

health/safety/environmental relevant generation of H_2S, which constrain the operational window and requires purification measures. Currently there are four salt caverns used for H_2 storage worldwide [5], without reported failures but operational data is not available.

Salt caverns are, like most subsurface environments, not sterile but harbour diverse microbial organisms [8–11]. Specially adapted extremophiles (halotolerant or halophilic) can live in or even require high-salt conditions, as can be found in salt caverns, for their survival [12,13]. Although high osmotic stress is suggested to cause energetic constraints [14] by forcing the microorganisms to spend high amounts of energy for osmoregulation, i.e., production of compatible solutes; a higher salinity in cavern brines or cavern brine sumps does not necessarily lower the risks of microbial presence and/or activity. In case of H_2 storage, the microorganisms will be in direct contact with the stored H_2 for an extended period (up to several months for mid-/long- term storage). H_2, being an excellent and ubiquitous electron donor, is an important driver for microbial activity in living environments [15,16], which is in stark contrast to natural gas (CH_4). Microbial activity in H_2 filled salt caverns could induce a variety of processes and risks related to them: gas volumetric effects, gas composition changes and purity loss by e.g. sulphate-reducing microbes (SRM) forming the toxic gas H_2S (see reaction 1), methanogenic archaea forming CH_4 (see reaction 2), overall causing a reduced energetic value [17,18].

$$1/4\ SO_4^{2-} + H_2 + 1/4\ H^+ \rightarrow 1/4\ HS^- + H_2O\ (1)$$

$$1/4\ HCO_3^- + H_2 + 1/4\ H^+ \rightarrow 1/4 CH_4 + 3/4\ H_2O\ (2)$$

In field trials for H_2 storage in porous reservoirs and aquifers, microbial H_2 consumption and conversion into CH_4 has been described several times [19,20]. It is unclear whether or how these microbial H_2 oxidation processes will occur in salt caverns and if so, to what extent. Some modelling approaches have indicated potential H_2S formation [21,22]. However, these models are based on kinetic rates of general sulphate-reducers, and it can be assumed that a) growth and consumption is different with H_2 as an electron donor, and b) that extremely halophilic strains show different rates due to their energy expenditures on osmoregulation. For better prediction of the microbial risks, it is therefore necessary to study specific halophilic microbial rates to estimate the extent of microbial H_2 oxidation under high-salt conditions.

Therefore, the aim of this study was to investigate known halophilic H_2-consuming microbial strains to obtain not only H_2 consumption rates but also to find some key parameters that influence and/or can be used as indicators for microbial H_2 consumption. Furthermore, we compared our findings with microbial enrichments from a brine sampled in a salt cavern located in Northern Germany. These enrichments were incubated with H_2 for over 150 days at the cavern specific temperature range. Our study clearly shows that microbial H_2 consumption is a relevant topic for salt cavern storage delivers important kinetic data on H_2 consumption of both cultured and environmental samples.

Material and methods

Source of organisms. The two halophilic cultures *Desulfohalobium retbaense* DSM5692 [23] and *Methanocalculus halotolerans* DSM14092 [24] were purchased from the DSMZ (German Collection of Microorganisms and Cell Cultures GmbH). The original cavern brine was sampled at a cavern field located in Northern Germany. Samples were taken at the wellhead. Before taking samples the first brine was discarded (around of 10 minutes flushing) to avoid sampling the brine standing in the well. Afterwards the samples were filled into sterile and anoxic glass bottles under continuous nitrogen flush to preserve anoxic conditions and then immediately shipped to the lab.

Standard culture conditions. Both reference strains were routinely cultured in their specific media given by the DSMZ. For *D. retbaense* DSM5692: 1 g/L NH_4Cl, 0.3 g/L K_2HPO_4, 0.3 g/L KH_2PO_4, 20 g/L $MgCl_2$ x 6 H_2O, 100 g/L NaCl, 2.7 g/L $CaCl_2$, 4 g/L KCl, 3 g/L Na_2SO_4, 1 mL/L trace element solution SL-10, 0.3 g/L Na_2S x 9 H_2O – pH 7.2. For *M. halotolerans* DSM14092: 1 g/L NH_4Cl, 0.3 g/L K_2HPO_4, 0.3 g/L KH_2PO_4, 3.2 g/L $MgCl_2$ x 6 H_2O, 50 g/L NaCl, 0.6 g/L $CaCl_2$, 0.17 g/L KCl, 3 g/L Na_2SO_4, 10 mL/L modified Wolins mineral solution, 0.3 g/L Na_2S x 9 H_2O, 2 g/L $NaHCO_3$ – pH 7.2. Carbon sources and yeast extract were added separately to the bottles depending on the experiment. Both microorganisms were incubated at their respective optimal temperature of 37°C. Standard growth for *D. retbaense* was on 24 mM lactate with 0.1% yeast extract and 0.1% peptone. *M. halotolerans* was routinely cultivated with 20 mM acetate, 20 mM formate, 0.05% yeast extract and 80% H_2, 20% CO_2. Growth was confirmed by gas production (H_2S or CH_4).

H_2 consumption experiments. Bottles (total volume 58.35 mL) were always filled with 25 mL medium. For growth on H_2, *D. retbaense* cultures were amended with 24 mM acetate and 0.35 mL modified Wolins vitamin solution, inoculum 10% (2.5 mL) of a culture grown on lactate for 7 days. The incubations of *M. halotolerans* were amended with 20 mM acetate, 35 mM formate, 0.05% yeast extract, inoculum 10% (2.5 mL) of a culture grown on 80%/20% H_2/CO_2 for 4 days. Different amounts of H_2 were added to headspace to obtain 10% (~ 4 mL), 40% (~ 11 mL) and 100% (~ 25 mL)(rest gas: N_2) of the total headspace volume. Fluid and gas volume was always kept constant. Because *M. halotolerans* requires CO_2 for methanogenesis, 5% of the gas phase was CO_2, which was re-supplied after gas analysis at each sampling point. Incubation temperature was 37°C for both strains. Each experiment was conducted twice independently. The original cavern brine was anoxically filled in sterile bottles and the headspace was flushed with 100% H_2. In some bottles, 20 mM acetate and 0.04% yeast extract were added as growth boosters. Incubation temperatures were 30°, 60° and 80°C. All bottles were stored upside down during incubation to minimize loss of H_2 due to diffusion through the rubber stopper. Still, we observed diffusion through the stoppers especially over longer incubation periods.

Sampling and calculations. Pressure measurements, gas analysis and liquid sampling were performed in regular intervals during the incubation: For *D. retbaense* every week, for *M. halotolerans* every day/every second day and for the original brine enrichment every 50 days. Pressure measurements of the

experiments were conducted before and after each sampling session. When the pressure fell below 25 mbar (detection limit of the microGC), the bottles were re-pressurized with N_2 (for *D. retbaense)* or N_2/CO_2 (for *M. halotolerans)*. At some sampling points 1 mL liquid were withdrawn for pH, HPLC and cell number determination. To calculate the amount of H_2 in the bottles, the ideal gas law (3) was used to correlate temperature (T in Kelvin), pressure (p) in the bottles with atmospheric pressure – water vapor pressure at the given temperature (in Pascal) and the gas constant 8.3144 (J/mol K). The measured composition of the gas phase (%) and the known volume of the bottle (58.3 mL – liquid medium + additions) was used to calculate gas volume of H_2 the bottles containing the medium V (in m^3).

$p \times V = n \times R \times T$ (3)

The resulting mol of H_2 was calculated into mL by assuming STP (Standard temperature and pressure) (1 mol = 22.4 L). For each sample we calculated the volume of H_2 before and after the sampling procedure by measuring pressure at the beginning and at the end of the sampling. The loss of H_2 in between is related to withdrawal of gaseous and liquid samples. This loss through sampling was calculated and subtracted from the calculated first pressure value to obtain the volume of consumed H_2.

Analytical methods. Gas composition was measured with a micro gas chromatography (microGC) 490 (Agilent) by directly measuring the gas in the headspace of the serum bottles. Pressure in the bottles was measured using a pressure sensor from Sensortechnics 0–3 barg Press D/C 2916 with an individual set-up for direct measurement of the headspace of serum bottles. Liquid samples were analyzed by using liquid chromatography of Agilent 1260II UHPLC equipped with a Flexible pump, autosampler, 1260 RI, and 1260 DA HS detectors. All analytes were identified and quantified based on their respective reference standard calibration curves. For determining cell numbers, we isolated DNA from 1 mL of sample which was withdrawn using syringes and centrifuged for 20 min at 13,000 rpm. The pellet was frozen for several hours at -80°C and after that at -20°C. This procedure was necessary because the pellets did not freeze at -20°C due to the high salt content. Prior to DNA isolation, the pellet was ultrasonicated for 5 minutes, frozen for several hours at -80°C, and again ultrasonicated 5 minutes. DNA was isolated using the Blood&Tissue Kit (Qiagen) following manufacturer's instructions. Cell numbers were measured via digital droplet PCR (ddPCR; BioRad) using Dsr1 primer for sulphate-reducing bacteria [25] or standard 16S rRNA Archaea [26]. The ddPCR reactions were run with a total volume of 20 µL on a DX200 instrument (BioRad) using 1x EvaGreen supermix (BioRad) and 250 nM (final concentration) of primers. Complete PCR reactions were emulsified with QX200 Droplet Generation Oil for EvaGreen using the QX200 Droplet Generator and then transferred to a 96-well plate. PCR reactions were performed in a C1000 Touch Thermocycler with deep-well module (BioRad) using the following program: 95 ^oC for 15 minutes, 40 cycles of 95 ^oC for 30 seconds, 57.1 ^oC (sulphate-reducing bacteria) or 63.1 ^oC (archaea) for 1 minute, 4 ^oC for 5 minutes, 90 ^oC for 10 minutes and finally an infinite hold at 4 ^oC. Plates were equilibrated to room temperature for at least 10 minutes before being analysed on a QX200 Droplet Reader (BioRad). Thresholds for positive and negative droplets were manually set using positive (bacteria or archaea cultures) and negative (ultra-pure water) controls.

Results

Hydrogen consumption during halophilic sulphate reduction. The halophilic sulphate-reducing bacteria *Desulfohalobium retbaense* consumed H_2 in the headspace over several weeks. The cells consumed 10% H_2 (around 4 mL H_2) after 20 days while the incubations with 40% (initial 11 mL H_2) and 100% (initial 25 mL H_2) H_2-headspace were only partially consumed (Fig. 1A). We observed maximum rates of 0.31, 0.44 and 0.62 mL/day for 10, 40 and 100% respectively. In the set-ups with 10% H_2, we added 10% H_2 at day 35 after the first consumption but subsequently consumption rate was significantly slower with a maximum rate of 0.09 mL/day. In total *D. retbaense* consumed 3.8 + 1.8 (initial 10% + renewed addition of 10%), 7.5 (40% set-up) and 14.3 (100% set-up) mL of H_2, which correspond to the relative amounts of 99% + 52%, 65% and 57% (Fig. 1B). The experiment was repeated twice with a standard deviation variation of the maximum rates between 7–23% (see all data in suppl. Table 1). In sterile controls 0, 0.9 and 1.5 mL of H_2 was lost for the 10%, 40% and 100%, respectively, through diffusion out of the rubber stopper or by other reactions, which shows that only minimal loss occurs abiotically. During the incubation, no measurable (10%) or only minor amounts of acetate were consumed (0.7 mM with 40% and 1.1 mM with 100% H_2) (Table 1), which shows that acetate was not limiting. From an initial 2.80E + 10 cells/mL, cell numbers did only slightly increase with all H_2 concentrations to around 4E + 10 cells/mL indicating that the strain was not able to build up substantially more cell mass.

Only minor amounts of H_2S were detected in the headspace with a maximum value of around 4000 ppm at day 32 in one duplicate of the 40% bottles. However, at the next sampling stage the H_2S concentration was again back to the background level (suppl. Figure S1). This indicates a changing H_2S/HS^- dissolution in the liquid, which was confirmed by pH measurements, showing a significant increase in pH over time. Starting from pH 7.5 at day 0 and resulting in a final pH of 8.9 for 10% H_2 and pH 9.1–9.2 for the 40% and 100% H_2 bottles (Fig. 1A & Table 1, all pH values over time can be found in supplemental table 2). Sulphate was not quantified but was in excess (21 mM) in the media allowing for theoretical consumption of 47 mL H_2. We therefore assume that sulphate was never limiting.

Hydrogen consumption during methanogenesis. The halophilic methanogen *Methanocalculus halotolerans* consumed H_2 much faster compared to *D. retbaense* but still slow compared to other reported methanogenic growth [27,28]. In all set-ups all H_2 was consumed (Fig. 2A) with maximum rates of 1.1, 4.1 and 4.7 mL/day for the 10%, 40% and 90% set-ups. Re-addition of 10% H_2 in the 10% bottles led to an increased growth with a consumption rate of 1.7 mL/day (Fig. 2B). The experiment was conducted twice with a standard deviation between maximum rates between 15–37%. CH_4 was produced accordingly in all set-ups with consumption of CO_2 (suppl. Figure S2). CO_2 was always re-added when values dropped below 1.5%, to not limit growth. Formate was completely consumed after 1 day in all set-ups also the non-hydrogen controls. Acetate was consumed only in low concentrations for the 10% (0.2 mM acetate consumed) and 40% bottles (0.5 mM acetate consumed). 2.2 mM acetate were consumed in the 90% H_2 bottles (Table 1). Cells numbers increased from 7.2E + 09 cell/mL to 4E + 10, 4.5E + 10 and

5.1E + 10 cells/mL for the 10%, 40% and 90% set-ups. Similar to *D. retbaense*, pH increased in all the *M. calculus* cultures. Starting from a pH of 7.2, it increased to pH 8.6, 8.5 and 8.8 (Table 1, Fig. 2A, Supplemental table 2).

Hydrogen consumption of a real salt cavern brine community. The sampled cavern is located in Northern Germany in the Permian Zechstein Group salt layer. It has been filled with brine since the initial leaching several decades ago. The brine has a salinity of 27%, pH 7.4 and a high sulphate content of 4190 ± 57 mg/L. The pure cavern brine was incubated in serum bottles with a headspace of 100% H_2. We incubated for 176 days at 30°C which is relevant for this specific cavern. We added acetate and yeast extract to one set of bottles to trigger microbial activity, the other set was pure brine without any additional supplements. Sterile water with 100% H_2 lost 1.6 mL of H_2 during the incubation of 211 days. The brine sample incubated only with 100% H_2, consumed 1.7 mL H_2 on average (Fig. 3A). One bottle showed significantly lower total H_2 loss compared to the duplicate, although initial consumption rate was similar. In contrast with addition of acetate and yeast, 5.3 mL H_2 (corresponding to ~ 11%) (Fig. 3B) was consumed in both bottles with the maximum rate of 0.069 mL/day and a total of 0.9 mM acetate was consumed after 136 days. Interestingly, at day 100 no acetate was consumed although sulphate-reduction was already visible. This indicates that the first carbon from either the brine or the yeast extract was used before acetate was consumed. H_2S was first detected after 94 days with a maximum of 1505 ppm at day 136 in the H_2 + acetate + yeast bottles and declined afterwards due to the increase in pH. In the pure H_2 bottles, H_2S was not detected in the headspace, but black precipitates were visible on the glass wall, which indicates possible FeS formation caused by H_2S reacting with ferrous iron ions. pH first decreased to around 7 and afterwards increased to 8.5 in the H_2 + acetate + yeast bottles and to pH 7.8 in the pure H_2 bottles. Interestingly at the last sampling point (after 176 days), trace amounts of CH_4 (< 0.1%) were detected in one incubation of the acetate + yeast + H_2 experiment. CO_2 concentrations were always below 0.1%. We therefore assume that the CH_4 production is attributed to acetogenic or methylotrophic methanogens. Further investigations are needed to confirm this observation.

Cultures containing 10% CO_2 + 90% H_2 were established to investigate activities of hydrogenotrophic methanogens and acetogens, but no CH_4 or acetate production was observed at any time. It must be mentioned that the addition of CO_2 led to a pH decrease to pH 6.5 because of the lack of buffering compounds in the brine, which might have affected the microbial community.

Table 1: Measured and calculated results for hydrogen consumption and growth of *D. retbaense* (incubation at 37C for 71 days, starting pH 7.5, initial cell number 2.80E+10 cells/mL), *M. halotolerans* (incubation 37°C for 10 days, starting pH 7.4, initial cell number 7.2E+09 cells/mL) and an original salt brine sample (incubated at 30°C, starting pH 7.4). All experiments were performed in 58 mL glass bottles containing 25 mL medium/brine. n.d. = not determined

	Volume H_2 in headspace day 0 [mL]	Hydrogen consumed/lost [mL]	Acetate consumed [mM]	Cell number at end of incubation [cells/mL]	pH at end of incubation
Desulfohalobium retbaense	3.9 ± 0.1 3.5 ± 0.1	3.9 ± 0.0 1.8 ± 0.0	0 ± 0.6	4.10E + 10	8.9 ± 0
Desulfohalobium retbaense	11.5 ± 0.7	7.5 ± 0.9	0.7 ± 0.1	4.13E + 10	9.2 ± 0.1
Desulfohalobium retbaense	24.9 ± 0.1	14.3 ± 2.4	1.1 ± 3.7	4.12E + 10	9.1 ± 0
Sterile medium	3.4 ± 0.2	0	-	-	7.3 ± 0.1
Sterile medium	11.3 ± 0.2	1 ± 0.2	-	-	7 ± 0
Sterile medium	24.5 ± 0.4	1.5 ± 0.2	-	-	6.9 ± 0.1
Methanocalculus halotolerans	3.4 ± 0.2 4.1 ± 0.5	3.4 ± 0.6 4.1 ± 0.5	0.2 ± 0	4.04E + 10	8.6 ± 0.1
Methanocalculus halotolerans	11.9 ± 0.9	11.9 ± 0.9	0.5 ± 0.1	4.45E + 10	8.5 ± 0.1
Methanocalculus halotolerans	23.6 ± 1.4	22.8 ± 2.2	2.2 ± 0.6	5.08E + 10	8.8 ± 0.1
Sterile medium	3.6 ± 0.3	0.4 ± 0.1	-	-	7.4
Sterile medium	10.5 ± 0.3	0.1 ± 0.1	-	-	7.8
Sterile medium	22.5 ± 0.2	2.3 ± 0.3	-	-	7.7
Salt cavern brine – 30C	54.1 ± 1.3	2.2 ± 0.9	-	n.d.	8.5 ± 0.1
Salt cavern brine – 30C + acetate/yeast extract	57.3 ± 0.4	5.8 ± 0.1	0.9 ± 0.3	n.d.	7.8 ± 0.1
Sterile water	56.2	1.6	-	-	-

Discussion

Hydrogen consumption of halophilic microbes. With the increased interest in storing H_2 in the subsurface and especially in salt caverns, it becomes important to understand the risks of microbial H_2 consumption during storage within high-salt environments. Many Bacteria and Archaea can live and even strive under

high salt concentrations. Although high salinity environments are hostile for many groups of microorganisms [13,29], diverse microbial communities can still be found in high-saline environments like salt lakes and also salt caverns [30]. However, a distinction between halophilic and halotolerant must be made. The former ranging from slightly to moderate to extreme) require a certain amount of salt to grow and show their optimum growth behavior typically at salinities above 100 g/L (Oren, 2011) and can tolerate up to extreme ranges (> 200 g/L). The latter, do not need high salt concentrations but can tolerate it to a certain degree. Salinity is a common stress factor and microorganisms have different strategies to adapt to the osmotic pressure imposed by the ionic strength of the surrounding environment [31]. Both strategies, the “salt-in”- or the “osmotic solutes” strategy, require a certain amount of energy (ATP) to uphold cell activity. Either ATP is used directly in the production of compatible solutes (Oren, 2006), or ATP is required to uphold the correct sodium and potassium gradient between the inside and the outside of the cell. This is also the reason why many halophilic microbes living at the “energetic edge”, using much energy for upholding their cell stability, tend to grow slower compared to non-halophilic counterparts. Therefore, it is very important to specifically assess H_2 oxidation by halophilic H_2-oxidizers because the relative kinetic rates in such environments are largely unknown.

To estimate the H_2 consumption rates and associated effects in high-salt subsurface environments, we investigated and cultured two halophilic microorganism which belong to the two most relevant metabolic groups regarding H_2 consumption: i) Sulphate reduction is a very widespread metabolism with many different microbial groups potentially using H_2 as electron donor in the presence of sulphate as electron acceptor [32], resulting in the production of the toxic and corrosive gas H_2S; ii) Methanogenesis from H_2 and CO_2 is another highly relevant H_2 consumption process, which recently received major attention for potentially producing “green” methane (biomethanation) in the subsurface after injecting renewable H_2 with captured CO_2 [33]. The two investigated strains in our study are halophilic with *Desulfohalobium retbaense* at a salt optimum at 12% growing up to 24% [23] and *Methanocalculus halotolerans* with the optimum of 5% growing up to 12.5% salinity [24]. Both strains were originally described to be able to use H_2 for growth, both requiring acetate as an additional carbon source. To estimate the potential H_2 consumption kinetics in the high-saline subsurface we carefully cultured the strains with different volumes of H_2 in the headspace. We clearly observed H_2 consumption over time, with an increased H_2 loss relative to the initial concentration when growing with 10% H_2 instead of 40% or 100%. H_2 is normally a very scarce electron donor in the environment, typically in the nmol range. So high amounts of H_2 seem to have a negative effect on cellular H_2 uptake or involved hydrogenase enzymes. It is known from H_2-producing cultures that increased H_2 partial pressure has a product-inhibition effect on the enzymes [34]. A similar effect could be at play also during H_2 oxidation, but details need to be further investigated. *D. retbaense* was not able to oxidize all the provided H_2 in the 40% and 100% H_2 set-ups although acetate and sulphate was provided in excess. This lack of continued activity or consumption can be explained by an intense pH increase during H_2-oxidation. It can be seen in the reaction rate of H_2-dependent sulphate reduction (1) that this reaction is a proton consuming process leading to an increase

in surrounding pH [35,36]. This also explains the near complete absence of gaseous H_2S throughout our study. At higher pH H_2S will be in its highly soluble form HS^- ($HS^- + H^+ \leftrightharpoons H_2S$). HS^- is a weak acid which should partly counteract the pH increase but clearly the intense H_2 oxidation is the main driver and overrides the HS^- effect. In biogas plants and biocorrosion studies it is commonly reported that intense sulphate-reduction can lead to a pH increase [37,38]. As biogas and corrosion involves also intermediate H_2, this is also a direct effect of H_2 oxidation. Although it has been long known that H_2 is an electron donor for SRMs, it was never clearly described that the significant pH effect limits the microbial activity when growing on pure H_2. As the pH approached pH 9, which is beyond the acceptable limit for *D. retbaense*, its activity ceased. Another inhibiting effect could be significant amounts of dissolved HS^- (theoretically based on the amount of H_2 consumed: 1.4, 1.9 and 3.6 mL for the 2x10%, 40% and 100% H_2 respectively), which might have an additional inhibitory effect. When growing on lactate as an electron donor, pH did not rise significantly with an end value of pH 7.8 after 7 days with H_2S concentrations far beyond 15% of the headspace. So, the observed pH effect is purely associated to H_2 oxidation.

We were however not able to correlate the overall volume of H_2 consumed with the pH. In the 40% set-up the strains consumed 6.6 mL (consumption minus the diffusion loss observed in the sterile controls) and in the 100% set-up it consumed almost double with 12.4 mL and both reached a pH 9.1–9.2. One possible explanation could be that under 100% H_2 atmosphere more H_2 is dissolved in the media. H_2 has a low solubility in water (0.0014 g gas per kg water at 37°C) and even lower in saline brine. So, growth will be limited by H_2 dissolution, which will be increased with higher H_2 concentration or pressure. Given the optimal growing conditions in the beginning (optimal pH and nutrients) and a high initial cell number, 100% H_2 gives the cells more electron donor to be active in the initial growth phase compared to 40%. Another possibility are concurrent chemical reactions of the H_2 gas with the slightly alkaline media, leading to additional abiotic H_2 loss. Further careful investigations combined with chemical and biological kinetic modelling will help to answer understand the H_2- and proton consumption.

The H_2 electron chain towards sulphate is well understood and relatively conserved in members of the Deltaproteobacteria [39], therefore it can be assumed that the observed effects of pH increase when grown on H_2 is a general phenomenon and not strain specific.

Similarly, methanogenesis is a proton consuming process (reaction rate 2). Also, during methanogenesis the Archaea are consuming bicarbonate/CO_2 from the media, which reduces buffer potential and increases pH as described previously [40]. We observed in our set-ups with *M. halotolerans* a pH increase, although not as intense as with *D. retbaense*. All H_2 was consumed in all cases although activity rates decreased during the end of incubation for the 100% H_2 bottles probably because pH increased and was close to the upper pH limit of this strain. Again, the strain had much higher relative consumption rates with 10% H_2 compared to 40 and 100%. Renewed addition of 10% gave an even increased rate which is related to an increased cell number.

Potential risks for souring of salt caverns. Halophilic Archaea and Bacteria have been isolated from pure halite crystals in salt mines [41] and it is therefore expected that that also salt caverns will be habitat to a diverse set of microbes. Recent studies of salt cavern microbiology showed microbes in all studied caverns [11]. Also, our studied salt cavern brine was microbiologically active and after several months of incubation with and without nutrient addition we observed H_2 consumption. With addition of the growth booster yeast extract (supplying microbes with a variety of vitamins and trace elements) and the carbon source acetate, a significant H_2 consumption was measurable together with acetate consumption, H_2S production and also a significant pH increase. At the end of the incubation the pH increased 1 log factor from an initial 7.5 to 8.5. This shows that H_2 oxidation by halophilic SRMs does indeed lead to a significant pH increase similarly to what we observed with our type strain *D. retbaense*. In the pure brine bottles without addition of carbon source or yeast extract, H_2 consumption was less pronounced. This shows that the natural community is limited by a certain growth factor, trace element, vitamin or similar. No H_2S formation was measurable but again a pH increase indicating sulphate-reduction. We hope to be able to identify and enrich the sulphate-reducing community in future studies to better understand the growth behavior.

If we assume that the studied sulphate-reducers will also be active in the salt cavern when in contact with H_2, there is indeed a real risk for H_2 loss and souring. The sampled cavern brine has a very high sulphate content of over 4000 mg/L (~ 44 mM), which gives sulphate-reducing organisms sufficient electron acceptors. A back of the envelope calculation of a typical salt cavern with a volume of 600.000 cubic meters (most volume will be occupied with gas but roughly 3000 m^3 will be brine in the sump together with insoluble minerals) contains 12.6 metric tons of sulphate in the cavern to be potentially converted by sulphate-reducing organisms. However, in case of 100% sulphate conversion without any additional input, the cavern could contain around 3E + 06 L liters of H_2S or only 0.6% of the total cavern volume. Based on the equilibrium (1), four times the amount of H_2 would be needed as electron source (1.2E + 07 L), a loss of around 2% of the total volume. As we observed in our enrichments, another limiting factor will be the amount of available carbon source and/or growth factors, which could lead to a H_2 loss of maximum 5–13% under laboratory conditions. The consumption rates will be dependent on the surface area of the brine-gas interphase and therefore the cavern shape. A higher surface area will allow for a higher H_2 availability for the microbes. Dissolved organic carbon has been detected in the cavern brine of around 85 mg/L and was most likely introduced by the leaching process using diesel oil as a leaching blanket or by different operational procedures. Still, additional growth enhancers were necessary for significant sulphate reduction to occur, which significantly slowed down when pH approached 9. This means that during the first H_2 storage cycles, some volumes of H_2 will be converted by microbes until the pH value will be outside of the optimum for these microbes to strive. As a cautionary note, that the derived laboratory enrichments can only give hints about the metabolic potential in extreme environments since most microbes do not grow under lab conditions. A much more complex and chemolithoautotrophic community with higher cell numbers might be present and active in the cavern leading to a) faster H_2 consumption and b) longer and more intense H_2 consumption. For example, it

could be that alkali-tolerant strains will take over H_2 oxidation as soon as neutrophilic strains reached their limit or a certain adaptation to higher pH will occur. Indeed, given the production of methane after 170 days when pH was seemingly too high for the sulphate-reducing community, methanogenesis is inferred.

Our data shows that artificially leached salt cavern can contain H_2-consuming microbes, especially sulphate-reducers which seem to be nutrient limited and additionally will significantly increase pH. Field tests are now required to understand if the observed effects regarding H_2 consumption and pH will also occur in the cavern itself

Conclusions

- Halophilic hydrogen-consuming sulphate-reducers and methanogens are able to consume significant volumes of H_2 over time.
- H_2 oxidation leads to a significant pH increase in both cases. In case of SRMs pH exceeds growth limits and H_2 is not completely consumed.
- Original salt cavern brine contains active SRMs which are able to consume parts of the available H_2 especially when carbon and nutrients were added.
- The pH increase caused by H_2 oxidizing-SRMs might limit microbial H_2 consumption over long-term and might therefore be a self-limiting process.

Declarations

Acknowledgements

We want to thank Equinor for funding this project and providing salt cavern brine for the experimental studies. We also want to thank Carsten Reekers, Nils Kruse and Michael Mollenhauer for their help in the project and the good discussions.

Data availability

All main data generated or analysed during this study are included in this published article (and its Supplementary Information files).

Competing interests

The author(s) declare no competing interests.

Author contributions

ND: conceptualization, methodology, experiments, writing. KM: cell number analysis. AK: water and HPLC analysis. DB: sampling. EA: writing and editing. BAS: writing and editing. KD: lab support. JB:

conceptualization. SH: conceptualization, writing, acquiring internal grant.

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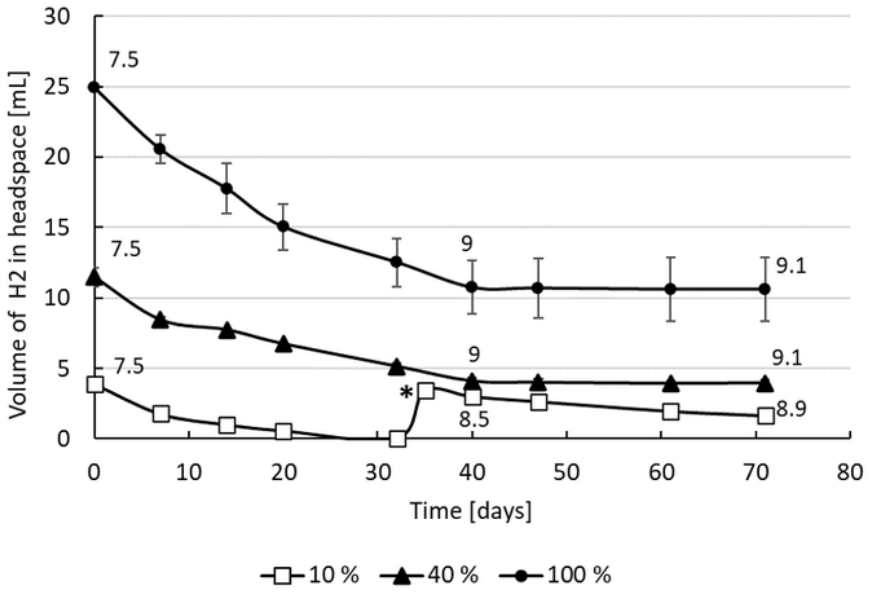
Figures



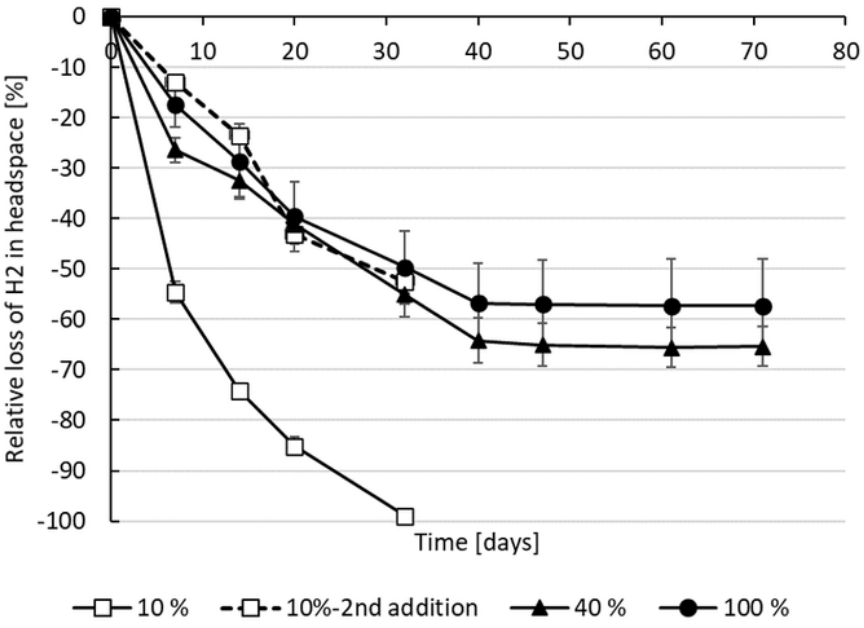


Figure 1

1A: Consumed hydrogen of *Desulfohalobium retbaense* in ml over time measured in the headspace of bottles incubated at near atmospheric pressure and 37 °C. Bottles were amended with 100% H_2 (●, solid line), 40% H_2 (▲, solid line) and 10% H_2 (□, solid line). The star * at 35 days indicates re-feeding the bottles with 10% H_2. pH measured at the day are given above the line. 1B: Values plotted in loss in % over

time. Error bars indicate deviation from the mean of duplicates. At day 35, 10% of H_2 was again added to the 10% bottles (dashed line - 2nd addition).

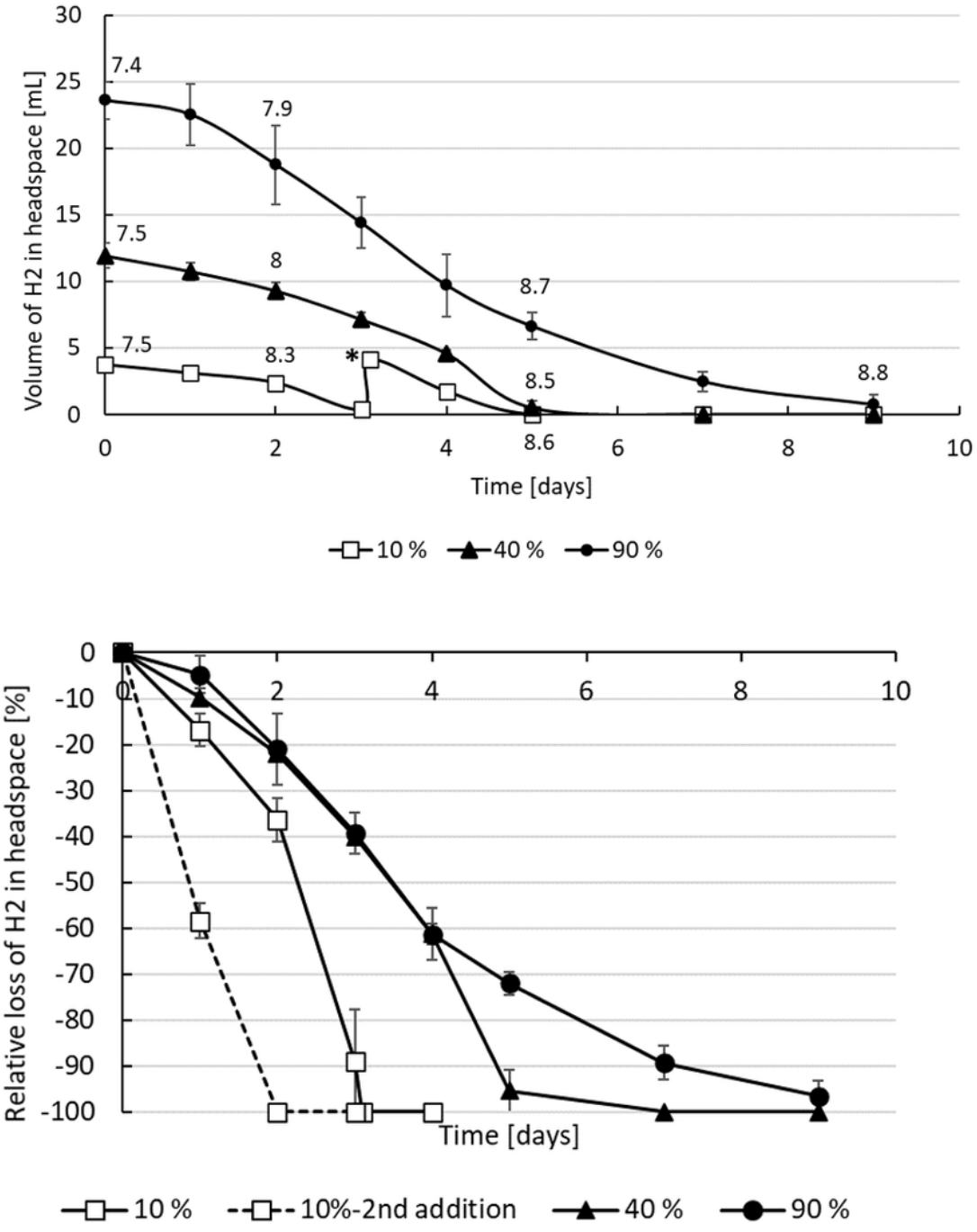
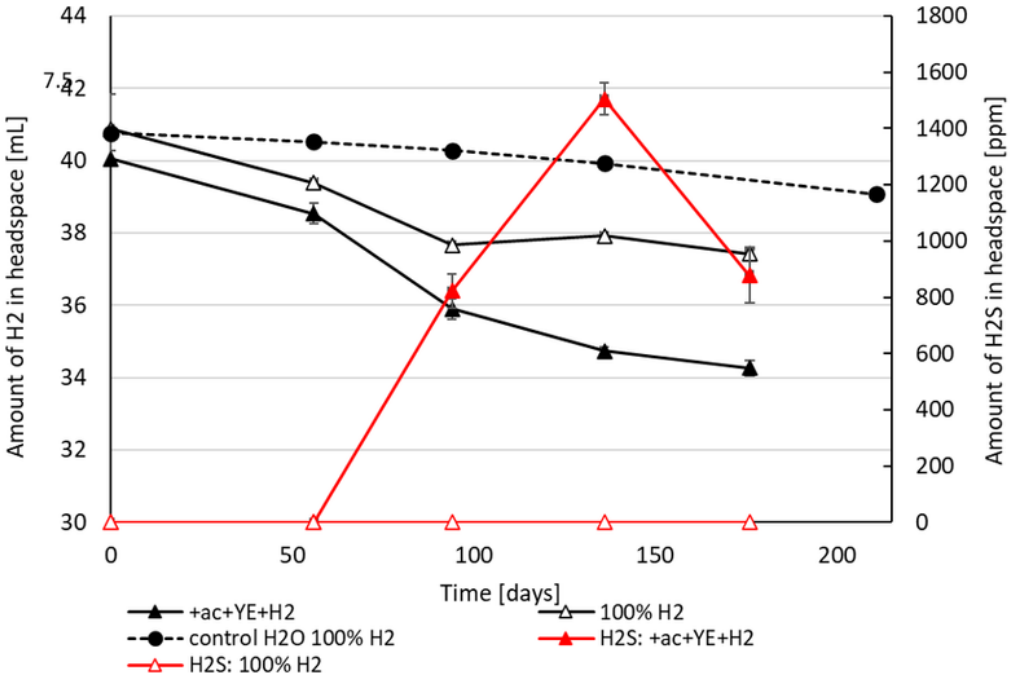


Figure 2

2A: Consumed hydrogen of *Methanocalculus halotolerans* in mL over time measurable in the headspace of bottles incubated at near atmospheric pressure and 37°C. Bottles were amended with 90% H_2 (●, solid

line), 40% H_2 (⊠, solid line) and 10% H_2 (□, solid line). The star * at 35 days indicates re-feeding the bottles with 10% H_2. pH measured at the day are given above the lines (10% day 5 is under line). 2B: Values plotted in loss in % over time. At day 3, 10% H_2 was re-added in the 10% set-ups (dashed line- 2nd addition). Error bars indicate deviation from the mean of duplicates.



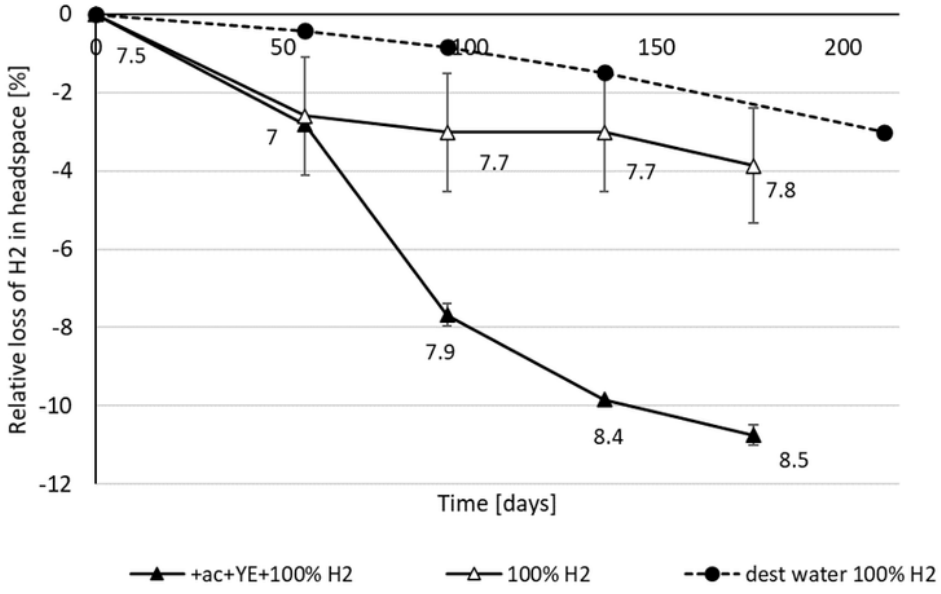


Figure 3

3A: Consumed hydrogen of an original salt cavern brine in mL over time measurable in the headspace of bottles incubated at near atmospheric pressure and 30°C. Bottles were amended with 100% hydrogen (△, solid line) or with addition of 20 mM acetate and 0.04% yeast extract (▲, solid line). H_2S measured in ppm in the headspace is given on the secondary axis for the acetate+yeast extract bottles (red line). Diffusion loss in an only sterile lab water bottle is shown with ●, solid line. 3B: Values plotted in % loss. pH values are given at the single sampling points in the graph. Error bars indicate deviation from the mean of duplicates.

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