hystories
Hydrogen Storage in European Subsurface

Changes in gas composition in micro-habitat experiments as a result of microbial gas consumption

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1. Introduction

1.1. Potential microbial processes in hydrogen-loaded porous reservoirs

The scarcity of fossil fuel resources as well as the negative environmental impact of its utilization has raised the demand for clean and renewable energy sources. However, renewable energy generation is commonly challenged by geographical constraints and seasonal fluctuation, which make it difficult to satisfy energy demand. Energy storage is essential for renewable energy to avoid energy shortage during periods of high energy demand. According to the United Nations Industrial Development Organization, hydrogen is "a true paradigm shift in the area of more efficient energy storage, especially for renewable energy". In addition, hydrogen storage can enable reduction of CO_2 emission and transition towards low carbon industry. Compared to surface storages in tanks or pipelines, underground storage in geological formations such as salt caverns, depleted oil and gas reservoirs or aquifers are considered to be a promising technology for large-scale hydrogen storage and as a result balance energy supply and demand. Salt caverns are often used to store natural gas and have already used for hydrogen storages in United Kingdom and United Sates and Germany (Panfilow 2016). While our knowledge and practical experience on hydrogen storage in porous structures remain limited. Experiences with underground hydrogen storage in porous geological formations have so far been limited to the storage of town gas with a maximum hydrogen content of 60 %.

Since decades, studies have shown that microorganisms actively inhabit not only the surface but also subsurface environments (Zobell 1936). Porous storages facilities such as depleted oil/gas reservoirs and aquifers have a broad range of fluid salinity, temperature and pressure. Despite the extreme conditions and unique physicochemical characteristics these reservoirs offer habitat for microorganisms. Microorganisms have been detected in porous media storages with the densities up to 10^6 cells per ml formation water (Ivanova 2007, Smigan 1990, Kleinitz 2005; Nazina 2023). Microbial activities were reported for town gas storage in aquifers in France (Beynes), Czech Republic (Lobodice) and Germany (Engelborstel, Bad Lauchstaedt, Kiel) (Panfilow 2016, Wagner 1985, Wagner 1988, Buzek 1994).

In the subsurface, hydrogen is generated and consumed through both biotic and abiotic processes. However, hydrogen consumption is mainly depicted as biotic process rather than abiotic (Gregory 2019). Various metabolic pathways are possible in anaerobic environments (Figure 1), and important physiological groups of the deep biosphere such as sulfate-reducing prokaryotes, iron reducers, methanogenic archaea and heterotrophic microorganisms are

associated with hydrogen. Sulfate reduction, methanogenesis and acetogenesis are the most important hydrogen-consuming processes found in subterranean structures (Hagemann 2016, Dopffel 2021, Heinemann 2021).

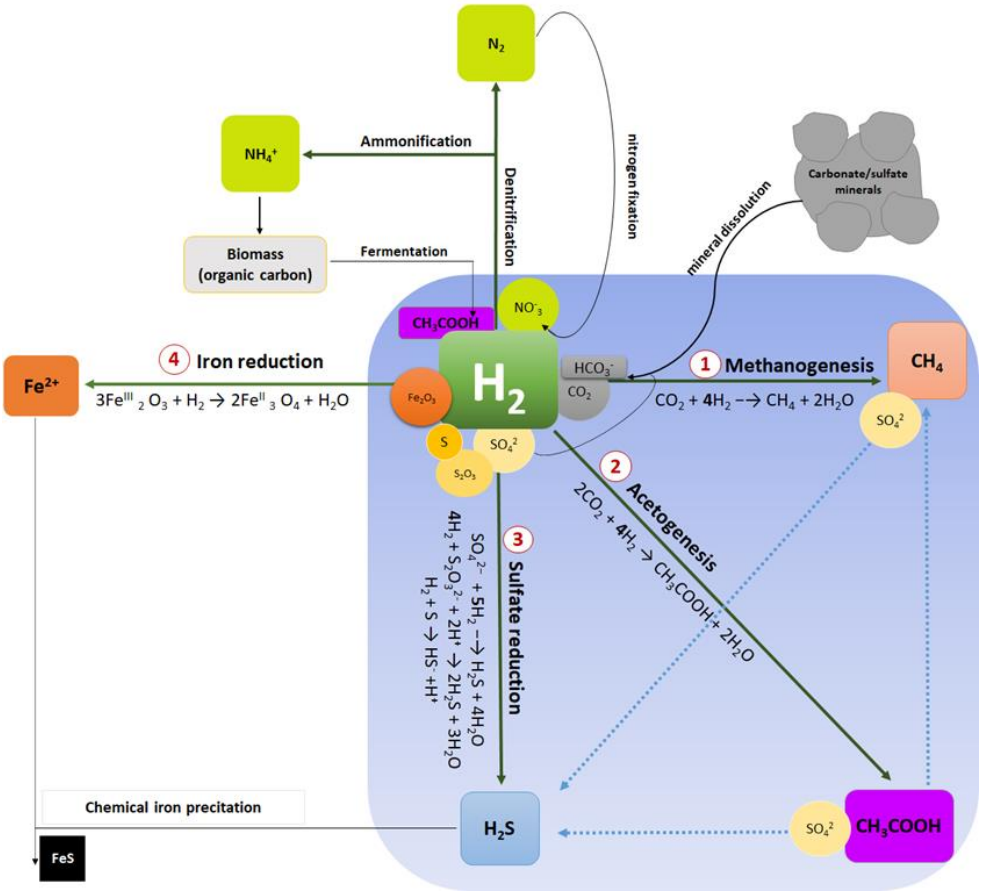

Figure 1: Most relevant hydrogen-related metabolic pathways in anaerobic environments

Sulfate reduction: Sulfate-reducing prokaryotes (SRP) include sulfate-reducing archaea (SRA) and sulfate-reducing bacteria (SRB). SRP can use organic compounds or hydrogen as energy sources under anaerobic conditions and simultaneously reduce sulfate to sulfide (S^{2-}). In addition, SRP are often very tolerant of extreme living conditions and can, for example, grow optimally in a wide temperature range (30°C - 60°C) as well as at a salinity (< 100 g/l) (Kleinitz 2005, Heinemann 2021). Microbial formation of H_2S from sulfate reduction (reactions 1 and 2 below) poses a serious technical problem for the operation of underground hydrogen storage facilities. H_2S gas can cause damage such as acidification, corrosion or plugging in the storage tanks and above ground facilities. The problem of microbial hydrogen sulfide formation occurs, among other things, in the course of flooding measures at gas and oil storage sites or as

a result of chemicals (oxygen scavenger, glycol, methanol) introduced into the storage facility. (Gieg 2011) For sulfate reduction, a sulfate concentration of more than 10 mg/l must be present in water in addition to suitable electron donors (Kleinitz 2005).

If the generated sulfides react with metals, the formed metal sulfides can additionally lead to plugging in rock pores or filter material as precipitation (reaction 3 below). With the formation of H_2S and CO_2 as a result of the microbial sulfate reduction process, there is not only a loss of gas but also a reduction in gas quality (Dopffel 2021, Dieterich 2011).

$$SO_4^{2-} + 5\ H_2 \rightarrow H_2S + 4\ H_2O \quad (1)$$

$$2\ CH_3CH(OH)COOH + 2\ SO_4^{2-} \rightarrow 4\ CO_2 + 2\ H_2S + 4\ H_2O + CH_3COOH \quad (2)$$

$$Fe_2^{+} + H_2S \rightarrow FeS \downarrow + 2\ H^{+} \quad (3)$$

Methanogenesis: Another metabolic pathway relevant to underground gas reservoirs is methanogenesis, i.e. the biogenic formation of methane. Methane is produced by most orders of methanogens (hydrogenotrophic methanogens) from the oxidation of H_2 and carbon dioxide/carbonate (reaction 4 below), from formate or from H_2 and methanol. Only one order can produce methane from acetate (acetoclastic methanogens) (reaction 5 below) or from disproportionate C_1 compounds (methylotrophic methanogens) (Wagner 2018). Methanogenic microorganisms are exclusively classified as archaea (Boone 1993). Similar to SRP, methanogens are able to evolve over a wide temperature range (Thaysen 2021). However, their tolerance to high salinity is limited. Hydrogenotrophic methanogens can tolerate salt concentrations of up to 3.4 M (Zhilina 2013). Methanogenesis can also lead to gas losses and negatively affect gas quality, as the conversion of hydrogen to methane reduces the stored energy and, as a result of microbial growth, the concentration. The gas storage tank of the town of Lobodice (Czech Republic) is a well-described study of the conversion of H_2 to methane by methanogens, where a loss of almost 17 % of the hydrogen was observed over a storage cycle of seven months (Smigan 1990). In addition, the involvement of methanogenesis in microbially induced corrosion has also been reported (Kip 2017), which can become a risk factor for infrastructure integrity associated with underground storage (Libert 2014).

$$4\ H_2 + HCO_3^{-} + H^{+} \rightarrow CH_4 + 3\ H_2O \quad (4)$$

$$CH_3COO^{-}\ (Acetate) + H_2O \rightarrow CH_4 + HCO_3^{-} \quad (5)$$

Acetogens: A competing process with methanogenesis in anaerobic environments is acetogenesis. Homoacetogenic microorganisms (homoacetogens) utilize hydrogen and carbon dioxide and/or CO to produce acetate through acetogenesis process (6). Acetogens are obligate anaerobic bacteria and have optimum growth temperatures from 20 °C to 66 °C

(Bengelsdorf 2018). Acetogens are also considered to be one of the contributors of steel corrosion (Mand 2014).

$$2\ CO_2 + 4\ H_2 \rightarrow CH_3COOH + 2\ H_2O \quad (6)$$

Besides the three main processes of hydrogen consumption mentioned above, iron (III) reduction is also a relevant process in subsurface environment (7). Iron reducers have been reported to be able to grow at temperature as low as 4 °C and as high as 121 °C (Finneran 2003, Kashefi and Lovley 2003). The activity of iron reducers might lead to the reduction of gas volume and storage capacity due to hydrogen consumption as well as water production.

$$3\ Fe_2^{III}O_3 + H_2 \rightarrow 2\ Fe_3^{II}O_4 + H_2O \quad (7)$$

In the consortia, microorganisms from various metabolic pathways can compete for nutrients and energy but they can also have syntrophic interactions which enable mutual developments. Acetate produced by acetogens can be utilized as carbon source for SRP growing with hydrogen (Mand 2014). In the presence of sulfate, sulfate-reducing- bacteria easily outcompete methanogens for hydrogen while acetoclastic methanogens can compete reasonably well with acetate-degrading sulfate reducers (Stams 2005). Some SRPs are also able to survive by cooperating with methanogens and syntrophic bacteria when sulfate is limiting (Muyzer 2008).

In addition to hydrogenotrophic microorganisms, other anaerobic microorganisms such as fermentative microbes, nitrate-reducing species might also have negative impacts for underground hydrogen storages. Fermentation of organic matter is an important process in subsurface environments when organic carbon is available. The products of fermentation such as hydrogen, alcohols, organic acids, carbon dioxide, methane can be utilized by sulfate-reducing microorganisms. In contrast, nitrate reducing microorganisms could compete against SRP for available electron donors (Lovley and Chapelle 1995). This microbial group could reduce the impact of SRP on storage facilities due to their competition. However, the involvement of nitrate-reducing bacteria in iron corrosion and steel corrosion has been reported (Liu 2021). As a result, their effects on storage facilities must not be underestimated.

In principle, any microbial activity in porous underground reservoirs is detrimental to technical operations, as the proliferation of microorganisms in the pore space of the rock matrix leads to the formation of complex organic substances in the form of extracellular substances (e.g. slime), single cells or biofilms. Even if the cells should be destroyed by treatment measures, the cell residues can serve as a substrate for other microorganisms and thus set further processes in operation in the long term. For this reason, any microbial activity in the reservoir must be critically evaluated.

1.2. Objective of the conducted research focusing on stimulation of microbial hydrogen consumption within the framework of work package 3.

The understanding of microbial activities in the deep underground is crucial for an economically successful operation of underground gas storage in general and underground hydrogen storage in particular. Microbial life in geological storage systems is determined by reservoir conditions, mainly temperature, salinity, composition of formation water and rock matrix, availability of energy sources, electron acceptors and inorganic nutrients. (Wagner 2013, Thaysen 2021, Gregory 2019). But also, production rates, work-over measures or injection of chemicals can influence the microbial population, especially in the near-wellbore zone. The injection of hydrogen into porous structures potentially stimulates hydrogen consuming microorganisms as hydrogen is an energetic electron donor, supplying energy source for many microbial groups. Microorganisms that use hydrogen as electron donor additionally require carbon sources to grow and form biomass. Therefore, microbial development based only on pure hydrogen is not possible. Certain reservoir constellations, where e.g. the carbon source is limited and the rock matrix contains hardly any carbonates, represent an unsuitable environment for hydrogenotrophic microorganisms. Similarly, limited availability of electron acceptors (e.g. sulfate) may limit the microbial activity of sulfate-reducing prokaryotes. Accurate knowledge of real reservoir conditions and factors influencing microbial processes, as well as knowledge of the composition of the indigenous microflora, their metabolism and activity, are essential for a comprehensive understanding of the current situation and possible future developments in deep subsurface environments.

Although numerous studies have investigated the microbial diversity in underground gas storage facilities, the potential impact of hydrogen injection on the microbial communities in the subsurface is still poorly understood. This study addresses the extent to which previous experience with natural gas storage facilities and pure hydrogen storage can be transferred to hydrogen storage in porous underground storage facilities. While work package D3.1 deals with the characterisation of microbial diversity from borehole water samples of different underground facilities, work package D3.2 investigates hydrogen conversion rates and turnover rates of microorganisms under real storage conditions (pressure, temperature, rock materials and pure hydrogen atmosphere). The aim is to highlight possible technical risks of hydrogen storage and thus provide guidance for the selection, construction and operation of these storage facilities.

2. Materials and methods

2.1. Sample material

A dozen of representative formation water samples of selected porous reservoirs were used for the microbiological investigations and hydrogen consumption tests of work package D3.2. For this purpose, depth samples were taken from the boreholes of the reservoirs to be investigated. The reservoir overpressure of the samples was monitored on site and slowly released at a maximum of 4 bar/min).

In order to avoid contamination and any chemical reactions of the samples prior to laboratory analysis, the samples were filled into gas-tight transport containers under oxygen exclusion by flushing with nitrogen immediately after sampling and transported directly to the laboratory under cooling.

Some reservoir samples were transported directly from the reservoir to the laboratory in pressurized steel cylinders. In these cases, the pressure was released slowly and in a controlled manner in the laboratory, and the fluid was extracted under flushing with nitrogen. By preparing the samples under a protective gas atmosphere, contact with atmospheric oxygen was avoided until the samples were cultivated.

Figure 2: Acquisition of deep formation water samples and decompression procedure in the laboratory

2.2. Enrichment cultures and hydrogen consumption tests

2.2.1. Enrichment of microorganisms

In order to be able to carry out the hydrogen consumption tests in acceptable periods of time and with measurable conversion rates, enrichment cultures had to be created from the formation water samples beforehand, as some of the original samples only had very low microbial contents. The enrichment culture for the respective underground storage was obtained by first cultivating the different microbial groups separately in specific growth media until they reached the necessary minimum cell content for subcultures. Subsequently, mixtures of these enrichment cultures were adapted to original formation water with 100 % hydrogen in the gas phase and in combination with core material for 1 - 2 weeks before inoculation for the experiments (Table 1). The microbiological characterisation of formation water samples is described in the project report Deliverable 3.1.

Table 1: Formation water samples and microorganisms used to test potential stimulation of microbial hydrogen consuming processes

Sample	Salinity of formation water (% NaCl)	Incubation temperature (°C)	pH of formation water	Pressure at high-pressure tests	Hydrogen-consuming microbial groups*
1	1.5	50	6.8	30 bar	SRP, methanogens, acetogens
2	1.7	60	5.8	70 bar	SRP, acetogens
3	0.1	60	6.2	100 bar	SRP, acetogens
4	0.1	30	7.5	45 bar	SRP, methanogens, acetogens
5**	1.5	50	6.8	30 bar	SRP, methanogens, acetogens
6	2.8	40	6.5	35 bar	SRP, methanogens
7	4.9	45	6.7	35 bar	TRP

* SRP=Sulfate reducing prokaryotes; TRP=Thiosulfate reducing prokaryotes
** Sample #5 is a repeated test of storage sample #1

2.2.2. Low pressure tests

Hydrogen consumption tests at slightly increased pressure were performed in 125 mL glass bottles (unless otherwise specified) sealed with airtight rubber/butyl stoppers and crimp caps.

Each bottle contained a total of 60 mL of formation water and a mixed enrichment culture. Core cuttings or an artificial carbon source ($NaHCO_3$ or $CaCO_3$) was added to the experimental mixture to stimulate microbial growth. Pure hydrogen gas was injected to an initial absolute pressure of 1,500 - 2,000 mbar. For control purposes, abiotic batches without the addition of microorganisms were also prepared in the same way.

The cultures were incubated at a specific temperature corresponding to the respective reservoir conditions. During the experiment, the absolute pressure was monitored regularly. When the pressure dropped below atmospheric pressure, either H_2 or N_2 gas was added. Any changes in gas composition (H_2, CH_4, CO_2) in the gas phase were determined by gas chromatography. Liquid samples were taken at regular intervals for cell count, pH measurement and chemical analysis. In addition, liquid samples were taken at the beginning and end of the hydrogen consumption test to quantify specific groups of hydrogen-consuming microorganisms by qPCR (quantitative polymerase reaction).

Figure 3: Low pressure experiments for hydrogen consumption tests

2.2.3. High pressure tests

The hydrogen consumption tests at high pressure were carried out with high-pressure reactor units (Berghof Products, Instruments GmbH). The reactors are teflon-lined, temperature-controlled and pressure-resistant up to 150 bar. Pressure and temperature are recorded and controlled in real time. For the experiments, each reactor was prepared with formation water, a mixed enrichment culture and core material (if available).

A requirement for the measurement of microbial hydrogen conversion is the presence of a correspondingly active microbial population. Therefore, the high-pressure experiments were only carried out for those reservoirs for which certain hydrogen conversions were detectable in

the previous experiments. Before the experiment, hydrogen was added to the reactors in such a way that each reactor eventually contained 100 % hydrogen gas at the desired absolute pressure for the test (minimum bottom hole pressure of each underground storage site). First, the reactor was purged with hydrogen at least seven times by adding hydrogen with an overpressure of 10 bar and then discharging the reactor gas again. Then a defined volume of hydrogen was added to reach the desired absolute pressure. In addition, the rate of addition or release of the gas was kept below 4 bar/min to avoid disturbing the microorganisms. The pressure and temperature were continuously monitored and controlled during the experiment. The composition of the gaseous phase, the pH and microbial composition in the liquid phase were determined at least twice, at the beginning and at the end of the high-pressure test.

Figure 4: High pressure bioreactor units

2.3. Analytical methods

2.3.1. Microscopy

The microscopic examination of original fluid samples was carried out directly after the arrival of the samples in the laboratory using phase contrast microscopy (Zeiss AxioScope A.1, magnification: 1:1,600). This is a simple and very efficient method for an initial evaluation of the samples with regard to microbial content, variance, cell morphology and status of the

microorganism cells present as well as the content of particles and hydrophobic phases. Since the sample does not have to be manipulated for this examination, it provides a direct and unbiased picture of the current state of the samples. In addition to the original samples, the enrichment cultures, dilution series and the samples from the model experiments were also examined microscopically with regard to microbial growth and the state of the cells.

For samples in which the methodical minimum microbial count of 10^6 cells/ml was reached, the cells were counted using a THOMA cell counting chamber (Depth: 0,01 mm) in addition to this microscopic examination.

2.3.2. Gas analysis

The alteration of the composition of the gas phase in sealed test batches, together with other analytical data, is a very good and precise indicator of microbial activities. Biochemical reactions and turnover rates can be determined by gas chromatic measurements of the individual components of the gas phase and by manometric measurement of pressure changes. Thereby, gas depletion can occur as a result of e.g., the conversation of hydrogen and carbon dioxide to methane, as well as gas generation, e.g. as a result of microbial methane formation from organic substances. Pure cultures or group-specific enrichment cultures, where only individual substrates are available, usually convert these according to stoichiometric equations, so that the underlying biochemical reactions can often be clearly assigned.

The concentration of the gases most relevant here, such as N_2, H_2, CO_2 and CH_4, was determined from gas samples taken from the headspace gas phase of the gas-tight culture bottles and high-pressure reactors using gas chromatography.

2.3.3. Chemical analysis

In addition to the gaseous carbon and energy sources (CO_2, H_2) that are particularly relevant here, various cations and anions, as well as dissolved organic compounds, are also of decisive importance for the development of microorganisms. The availability and concentration of some anions (SO_4^{2-}, PO_4^{3-}, NO_2^-, NO_3^-, F^-, Cl^-, Br^-, BrO_4^{2-} , ClO_4^{2-}), in combination with cations (e.g. Na^+, Mg^{2+}, Ca^{2+}, K^+), can be decisive for the survival of certain groups of microorganisms (e.g. sulfate-reducing prokaryotes, halophiles).

Volatile fatty acids (VFA), such as lactate, acetate, formate, propionate, butyrate, iso-butyrate, are easily utilisable substrates, but also intermediates whose concentration provides information about the presence or activity of certain groups of microorganisms. Concentration of anions and volatile fatty acids were determined by ion chromatography.

2.3.4. Molecular biological analysis

The molecular biology technique, quantitative real-time polymerase chain reaction (qPCR), was used to quantify microbial diversity in formation water samples and laboratory cultures. The focus of these qPCR analyses was on those microbial groups whose occurrence in samples from the deep biosphere is known and which are particularly relevant for microbial conversion reactions of hydrogen. The DNA extraction and qPCR analyses were optimised by MicroPro to detect genes from eubacteria and archaea, methanogens, sulfate-reducing archaea, sulfate-reducing bacteria and acetogens from partly highly saline formation water samples. For the molecular biological analysis of the hydrogen-utilising microorganisms, the water samples were filtered through a sterile 0.2 µm membrane filter (Sartorius Stedim Biotech). Depending on the number of microorganisms in the sample, a certain volume of formation water was used for filtration. The cell-containing filters were stored at -20 °C before DNA extraction. DNA extraction was performed using the DNeasy® PowerSoil® kit (Qiagen) according to the manufacturer's protocol. Details of applied specific forward and reverse primers for targeted species are shown in Table 2.

Table 2: Primers used in the study for quantification of hydrogen-consuming microorganisms

Primers	Sequence (5'-3')	Target gene	Target species	Product size (bp)	Reference
arch806_f	ATTAGATACCCSBGTAGTCC	*16S rDNA*	Archaea	144	Takai et al., 2000; Yu et al., 2005
arch_r	CCCGCCAATTCCTTTAAGTTTC				
eu338_f	ACTCCTACGGGAGGCAGC	*16S rDNA*	Bacteria	558-569	Øvreås et al., 1997; Morales and Holben, 2009
eu907_r	CCGTCAATTCMTTTGAGTTT				
dsr1_f	ACSCACTGGAAGCACG	*dsrAB*	Sulfate-reducing bacteria	434	Wilms et al., 2006; Dhillon et al., 2003
dsr-500r	CGGTGMAGYTCRTCCTG				
archdsr1830_f	TGCTGTCNAAACATGTGTG	*dsrB*	Sulfate-reducing archaea	200	Wagner et al., 1998
dsr4far_r	GTGTAGCAGTTACCGCA				
a189gc	GGNGACTGGGACTTCTGG	*pmoCA*	Methanogens	470	Holmes et al., 1995; Costello and Lidstrom, 1999
mb661	CCGGMGCAACGTCYTTACC				
aceFTHFS_1250F	TGGGMDAARGGYRGHBWDGGYGG	*fhs*	Acetogens	250	Henderson et al., 2010
aceFTHFS_1500R	GTATTGDGTYTTRGCCATACA				

3. Results

3.1. Analysis of storage #1

The enrichment cultures required to perform the hydrogen consumption tests were prepared on basis of a mixture of the cultures of methanogens/acetogens, sulfate-reducing prokaryotes and anaerobic heterotrophs enriched from the formation water. Active enrichment cultures with the required minimum bacterial content served as inoculum for the hydrogen consumption experiments.

The aim of the hydrogen consumption tests was to simulate microbial processes under conditions that are as close to reality as possible in order to be able to estimate the possible effects. The formation water of the underground reservoir had a salt content of 1.5 % NaCl (w/v), a pH value of 6.8 and a temperature of 49 °C (Table 1, sample 1). Accordingly, the hydrogen consumption tests were carried out at 50 °C. The carbon source was either core material (10 % w/v) from the deposit or artificial carbonate in the form of 30 mM HCO_3^-. The test conditions (pressure, carbon source) are summarised in Table 3.

Table 3: Hydrogen consumption tests under low and high-pressure of hydrogen

Test	Carbon source	Pressure	Gas volume/liquid volume	Liquid phase	Gas phase
Low pressure test (Addition of Carbonate)	30 mM HCO_3^-	1,500 – 2,000 mbar	65 ml/60 ml	formation water + enrichment cultures	100 % H_2
Low pressure test (Core material)	10 % (w/v) core	1,500 – 2,000 mbar	65 ml/60 ml		
High pressure test (Core material)	10 % (w/v) core	30 bar	125 ml/125 ml		

3.1.1. Hydrogen consumption at low pressure

The experimental batches inoculated with pre-cultures at slightly increased pressure showed significant turnovers of hydrogen (H_2) with simultaneous formation of methane (CH_4). The microbially induced changes in the gas phase are shown in Figure 5. In the experiments with carbonate as sole carbon source, the microorganisms actively consumed hydrogen at a maximum rate of 4.2 mol/m^3/day within the first 16 days of incubation, depleting about 50 % of the available hydrogen. In parallel to the hydrogen consumption process, the formation of methane took place. Although carbonate was added after 16 and 30 days, no significant additional hydrogen consumption or methane formation was observed.

During the tests, carbon dioxide (CO_2) was not detected in the gas phase in any of the approaches (data not shown).

In the experimental series with core materials as the only carbon source, microbial hydrogen consumption was observed during the first 16 days of incubation, with a maximum rate of 2.5 mol/m^3/day and almost 35 % hydrogen consumed. Hydrogen consumption occurred only during the first 16 days of the experiment, and the hydrogen concentration remained unchanged until day 30. However, hydrogen consumption resumed when soluble carbonate (HCO_3^-) was added. An analogous pattern was observed for methane formation: Methane concentration increased significantly in the first 16 days, then remained stable and continued to increase with the addition of carbonate. In the abiotic control series at low overpressure, no change in hydrogen concentration was observed, regardless of the carbon source (core material or HCO_3^-) (Supplementary data, Figure 9).

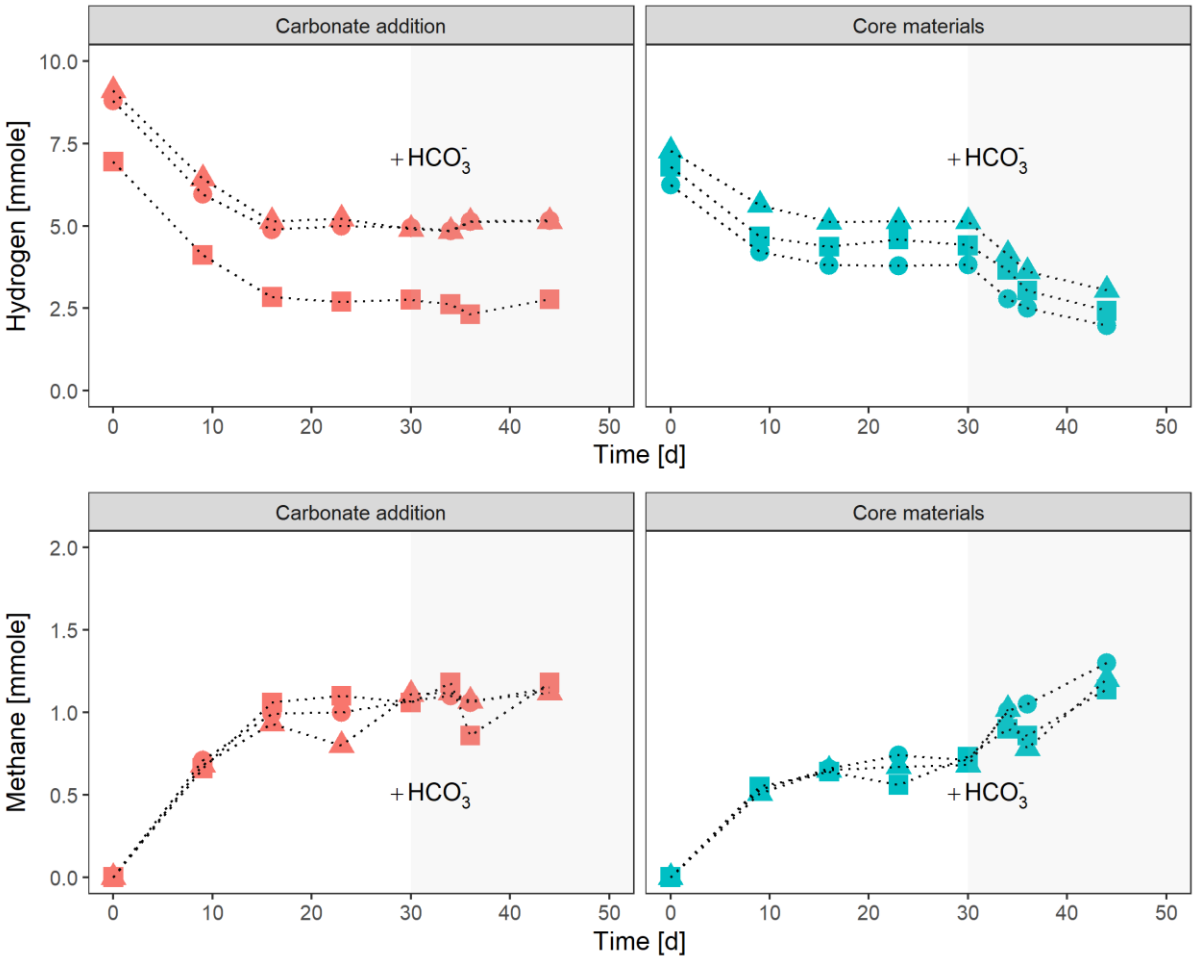

Figure 5: Hydrogen consumption and methane formation due to microbial activities in the presence of pure hydrogen at low pressure (1,500 - 1,900 mbar), temperature 50°C, salinity 1.5 %. Triplicates indicated by point shapes.

Table 4: Hydrogen consumption by microorganisms with carbon source from core materials or from carbonate addition in the presence of pure hydrogen at low pressure (1,500 - 1,900 mbar), temperature 50°C, salinity 1.5 %.

Assays (carbon source)	Replicate	Maximum H_2 consumption rate (mole/m³/day)	H_2 consumed after 30 days (%)	H_2 consumed after 44 days after extra carbonate addition (%)
Carbonate addition	1	4.13	43.80	41.18
	2	4.17	46.21	43.58
	3	4.33	60.29	60.14
Core materials	1	2.59	38.78	68.43
	2	2.29	29.53	58.10
	3	2.59	35.05	64.36

During the hydrogen consumption phase, changes in pH and total number of microbial cells were recorded (Figure 6). In biotic experiments with addition of core materials, a significant change in pH was observed, increasing from 8.2 to 9.0 - 9.2 within 9 incubation days. The pH remained at about 9.0 - 9.2 from day 9 to day 30 and further increased to about 10 after addition of carbonate. In biotic trials with carbonate addition, the pH increased from 8.2 at the beginning of the trial to 9.5 - 9.9 on day 16. The addition of carbonate did not lead to a further increase in pH as observed in the core materials, but to a slight decrease.

Microscopic analysis of the microorganism cells, showed a slight increase in cell numbers. Sulfate and acetate were detected in both biotic tests at the beginning of the incubation. The sulfate concentration with core material was twice as high as the sulfate concentration in tests with carbonate addition. The concentrations of sulfate and acetate did not change during the experiment (Supplementary data, Table 6).

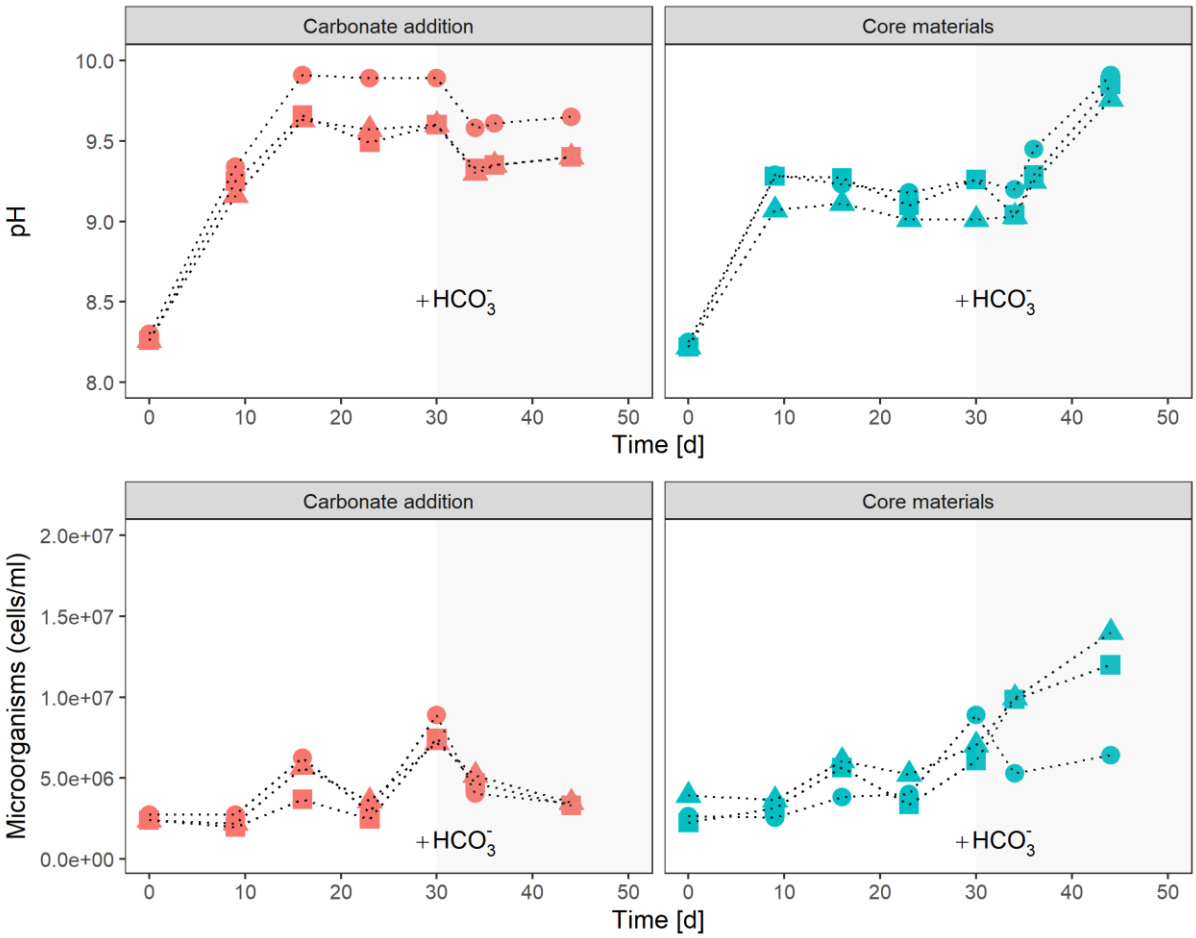

Figure 6: Changes of pH and total number of microbes during the hydrogen consumption process under the presence of pure hydrogen at low pressure (1,500-1,900 mbar), temperature 50°C, salinity 1.5 %. Triplicates indicated by point shapes

3.1.2. Hydrogen consumption at high pressure

In addition to the stimulation experiments with hydrogen at low pressure, the microbial hydrogen consumption at high pressure was also investigated using high-pressure bioreactor units. The pressure in the bottom of the reservoir is 30 – 160 bar. For the hydrogen consumption test, a lower pressure was chosen for the simulation tests to allow observation of microbial activity (if any) in the available test time. Therefore, the hydrogen consumption test was carried out in the high-pressure unit at 30 bar. Pressure and temperature in the bioreactors were monitored during the experimental periods and are shown in Figure 7. The temperature was kept relatively constant during the test.

As shown, there was no significant pressure drop in either bioreactor. The gas analysis showed that hydrogen was not converted during the periods examined and no other gases were produced.

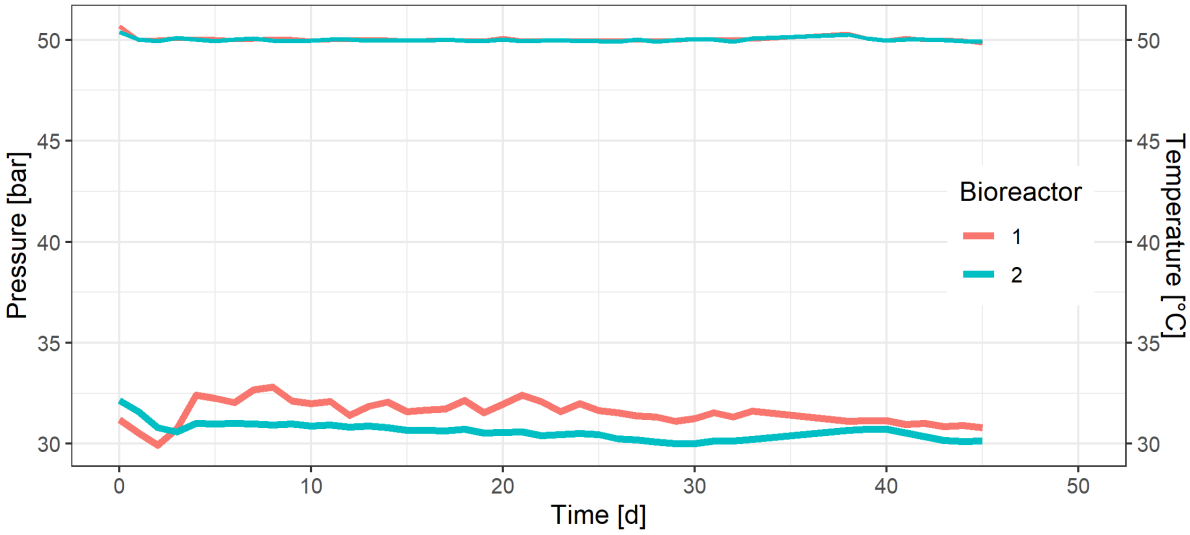

Figure 7: Visualization of pressure (thick lines) and temperature (thin lines) monitored during hydrogen consumption test under high pressure condition.

Changes in concentrations of sulfate, acetate, pH, and total cell numbers were shown in Table 5. Similar to observations in the biotic low-pressure tests, concentration of sulfate and acetate remained relatively unchanged over the experimental duration. A decrease in the total cell count was observed during the course of the experiment. In both bioreactors the increase in pH was noticed after 3 days of incubation, from pH 7.8 to pH 9.1 and a pH of ~ 8.8 was recorded the end of the test. (day 45)

Table 5: Hydrogen consumption test with core materials in the presence of hydrogen at high pressure (30 bar): changes in concentration of sulfate, acetate, pH, and cell numbers

Bioreactor	Sulfate (mg/l)		Acetate (mg/l)		pH			Cell numbers (cells/ml)		
	Day 3	Day 45	Day 3	Day 45	Day 0	Day 3	Day 45	Day 0	Day 3	Day 45
1	181.04	178.68	55.87	56.48	7.8	9.15	8.82	3.5e+06	1.2e+06	1.5e+06
2	245.41	253.76	54.48	56.23	7.8	9.09	8.75	3.5e+06	1.1e+06	1.4e+06

3.1.3. Microbial composition

At the beginning and the end of the hydrogen consumption tests, molecular analysis for quantification of hydrogen-consuming microbial groups (acetogens, methanogens, SRA and SRB) were performed. The results are shown in Figure 8.

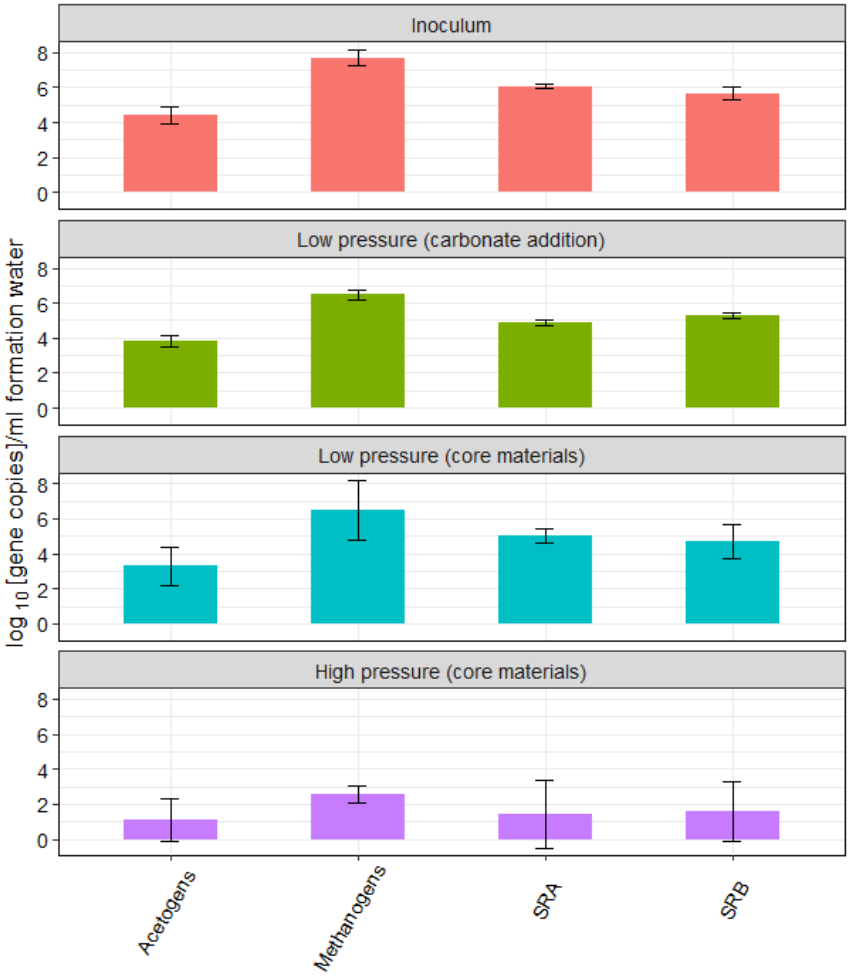

Figure 8: Quantification of hydrogen-consuming microbial groups at beginning and the end of hydrogen consumption tests; Same inoculum for high and low pressure; Error bars show standard deviation of triplicate experiments (low pressure) and triplicate qPCR measurements.

Acetogens, methanogens, SRA, and SRB were present in the cultures at the beginning of the hydrogen consumption tests in both the low pressure and high-pressure experiments. Methanogens were the dominant group in the consortium (6.2×10^7 copies/ml), followed by sulfate-reducing microorganisms (1.7×10^6 copies/ml) and acetogens (3.4×10^4 copies/ml).

The qPCR analysis showed a decrease in the number of specific genes for various hydrogenotrophic microorganisms at the end of the assay, both in the low-pressure and high-pressure assays. The assays in the high-pressure test showed a much greater decrease than in

the low-pressure test. At the end of the test, methanogens in the high-pressure test were about five orders of magnitude lower than at the beginning of the test, while the reduction in the low-pressure test with core materials was only about one order of magnitude. In the low-pressure test, the number of quantified microorganisms was similar between the low-pressure test with carbonate additive and the low-pressure test with core materials. Despite the reduction, methanogens remained the dominant groups in all tests.

3.1.4. Discussion

The consumption of hydrogen by the activities of the microorganisms enriched from the reservoir is clearly demonstrated by the hydrogen consumption tests at low pressure. Molecular analysis confirmed the presence of various hydrogen-consuming microorganisms in the inoculum, with hydrogenotrophic methanogenic microorganisms predominating in number. During the study period, the activity of methanogens was clearly detected on the basis of methane formation, while significant activity of acetogens (acetate formation) or sulfate reducers (sulfate reduction) was not evident in the tests, neither in the low-pressure nor in the high-pressure tests. When H_2/CO_2 is the only substrate present, methanogens are expected to compete successfully with homoacetogens based on growth kinetics (Weijma 2002). Furthermore, according to a study by STAMS (1994), methanogens could be expected to dominate in environments with low sulfate concentrations, even though sulfate reduction is thermodynamically the most efficient hydrogen consuming reaction. It can be concluded that hydrogen was metabolised mainly by methanogens, although different groups of hydrogen-consuming microorganisms were present. In other words, of the three common microbial reactions associated with subsurface hydrogen storage (methanogenesis, acetogenesis and sulfate reduction), only methanogenesis occurred under the conditions studied.

Hydrogenotrophic methanogens couple the oxidation of H_2 with the reduction of CO_2 to produce CH_4 ($4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$). The energy source hydrogen was present without limitation in the hydrogen consumption tests and the contents of dissolved organic carbon in the investigated formation water sample were neglectable, the limit concentration of available soluble inorganic carbon is likely responsible for the reducing activities of microorganisms. As hydrogen consumption and methane formation recovered after the addition of HCO_3^- in the assays with core materials, the limit concentrations of HCO_3^-/CO_2, which dissolved from core materials, could be the reason for almost no microbial activities after the first two weeks of the experiments (Figure 6).

However, in the hydrogen consumption tests with HCO_3^- addition, it is observed that no further microbial activities occurred despite the addition of carbonate after day 30. Therefore,

carbonate seems not to be the only limiting factor for microbial hydrogen consumption. Bicarbonate could be converted to CO_2 by methanogens for use in methanogenesis or biomass generation. The conversion reaction consumes protons ($HCO_3^- + H^+ \rightarrow CO_2 + H_2O$), resulting in an increase in pH (Figure 6). At high pH levels protons for the conversion reaction are limited. In our other investigation, compared to hydrogen consumption at neutral pH microbial hydrogen consumption by a methanogens-dominated enrichment culture reduced by 90 % at pH 10 (refer to Hystories report D3.4 for more information). Consequently, it is very likely that the increased pH inhibits methanogenesis from $H_2 + HCO_3^-/CO_2$.

In the hydrogen consumption tests at high pressure, a significant reduction in hydrogen pressure as well as the formation of methane were not recorded. Nevertheless, the presence of microorganisms in the high-pressure bioreactors were indicated by molecular analysis at the end of the experiment. The increase of pH in the high-pressure tests at the end of the experiment could also be the indication for microbial activities. Besides, microorganisms were still microscopically visible, and their viabilities were confirmed in a recultivation test with the presence of H_2/HCO_3^- at low pressure (data not shown). It could be possible that concentration of H_2/HCO_3^- dissolved from core materials was low and therefore the amount of generated methane by microorganisms was too low to be detected within investigated periods. Therefore, although microbial hydrogen consumption or methane production were not clearly observed in high-pressure test, microbial risks especially due to methanogenesis for hydrogen storage should not be excluded.

It should be noted that the content of carbon sources in our hydrogen consumption tests (10 % core material) is technically much lower compared to its availability in the storage. In contrast to the laboratory tests, the ratio of rock matrix (80 - 90 %) to water-filled pore space (10 - 20 %) is inverted. If carbon sources are available in the storage reservoir, e.g., a constant dissolution of carbonate rocks occurs in porous medium, which continuously generates HCO_3^-/CO_2, microbial hydrogen-consuming process will be inevitable. Further investigation, for example, with higher contents of core materials and/ or longer investigation duration should be considered.

3.1.5. Supplementary data

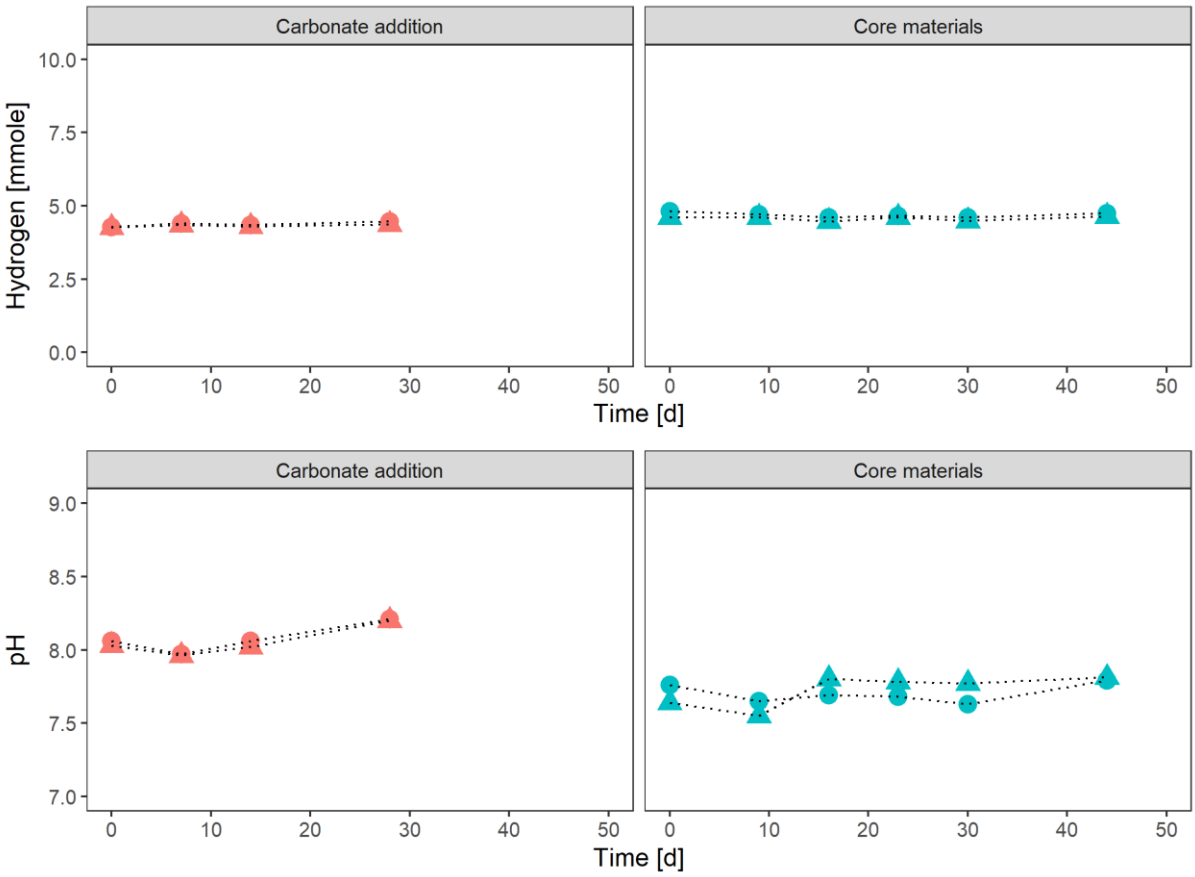

Figure 9: Abiotic control tests; H_2 and pH with core materials and with carbonate addition in the presence of hydrogen at low pressure (1,500 – 2,000 mbar), temperature 50°C, salinity 1.5 %. Duplicates indicated by point shapes.

Table 6: Concentration of sulfate and acetate during hydrogen consumption test with core materials and with carbonate addition in the presence of hydrogen at low pressure (1,500-1,900 mbar), temperature 50°C, salinity 1.5 %.

Assays (carbon source)	Replicate	Sulfate (mg/l)			Acetate (mg/l)		
		Day 0	Day 30	Day 44	Day 0	Day 30	Day 44
Carbonate addition (Biotic assays)	1	29.47	29.05	22.31	41.93	39.72	39.65
	2	24.57	23.27	25.2	44.2	42.02	41.37
	3	n.a	25.2	n.a	41.75	41.44	39.9
Core materials (Biotic assays)	1	179.76	170.3	173.14	51.62	46.01	47
	2	178	173.08	162.64	50.07	49.94	50.52
	3	177.11	185.08	178.75	49.22	46.36	45.09
Core materials (Sterile control, abiotic assays)	1	154.29	160.48	140.24	42.22	44.76	44.73
	2	194	207.2	207.61	45.35	44.45	47.16

n.a: no data available.

3.2. Analysis of storage #2

The formation water sample used for hydrogen consumption test was collected from a storage site with salinity of 1.7 % NaCl (w/v), pH 5.8 and temperature of 67 °C.

The mixed cultures of acetogens, sulfate-reducing prokaryotes enriched from the formation water used as inoculum for the subsequent hydrogen consumption experiments. Temperature for cultivation of microorganisms as well as for hydrogen consumption test was at 60 °C. Carbon source was either provided core material (10 % w/v) originated from the storage site or from the addition of $CaCO_3$ (3 %). The pressure and carbon source for each assay were summarized in Table 7.

Table 7: Hydrogen consumption tests under low and high-pressure of hydrogen.

Test	Carbon source	Pressure	Gas volume/liquid volume	Liquid phase	Gas phase
1. Low pressure test (Carbonate addition)	3 % $CaCO_3$	< 1,500 – 2,000 mbar	65 ml/60 ml	formation water + enrichment cultures	100 % H_2
2. Low pressure test (Core material)	10 % (w/v) core	< 1,500 – 2,000 mbar	65 ml/60 ml		
3. High pressure test (Biotic: core)	10 % (w/v) core	70 bar	125 ml/125 ml		

3.2.1. Hydrogen consumption at low pressure

Over the experimental period of about 7 weeks, there was no significant microbial hydrogen consumption in the biotic experiments compared to abiotic control experiments without microorganisms. Also, different carbon sources do not influence this result. The pressure curves during the experimental period are shown in Figure 10. The slight decrease in the recorded pressure is caused technically by the measurement.

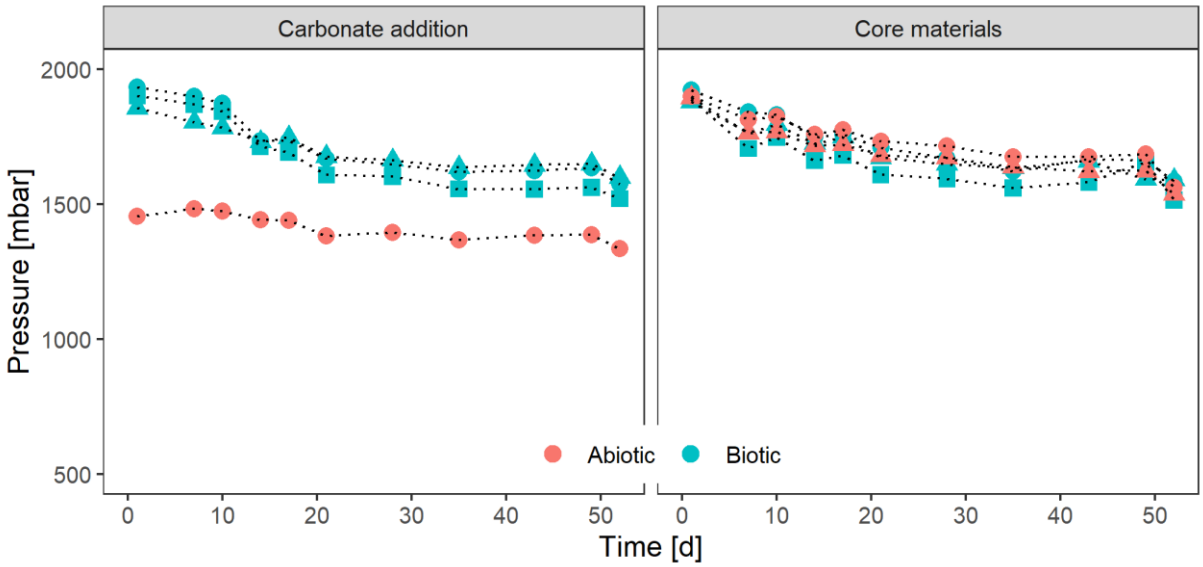

Figure 10: Pressure changes during hydrogen consumption test at low pressure (1,500-2,000 mbar), salinity 1.7 %, temperature 60 °C. Biotic assays (blue) were done in triplicate while abiotic assays (red) were performed one or in duplicate.

In both the abiotic and biotic tests, only H_2 was present in the gas phase at the beginning of the experiment. However, after a day of incubation the release of CO_2 was detected (Table 8). At the end of the test, both H_2 and CO_2 were present in the gas phase and no other gas was detected.

Table 8: Release of CO_2 (in %) into the gas phase during hydrogen consumption tests.

Assays (carbon source)		Biotic test			Abiotic test		
	Replicate	Day 0	Day 1	Day 52	Day 0	Day 1	Day 52
Carbonate addition (low pressure test)	1	0	0.51	1.1	0	1.38	n.a
	2	0	0.51	0.86	0	n.a	n.a
	3	0	0.53	0.98	0	n.a	n.a
Core materials (low pressure test)	1	0	0.51	0.86	0	1.03	9.33
	2	0	0.50	0.86	0	1.05	n.a
	3	0	0.72	0.98	0	n.a	n.a

n.a no data available

During hydrogen consuming process, changes in pH and total microbial cell numbers were recorded. In both biotic and abiotic assays with core materials and carbonate addition, an increase of pH was recorded, from 5.43 to 6.5 - 7.0 within 52 days of experiment (Table 9).

For biotic test, microorganisms were visually observed under microscopy. There were approximately 1.23×10^6 cells/ml at the beginning of the experiment. At the end the cell number decreased.

Table 9: Changes in pH during hydrogen consumption tests at low hydrogen pressure (1,500-2,000 mbar)

Assays (carbon source)		Biotic test			Abiotic test (sterile control)		
	Replicate	Day 0	Day 1	Day 52	Day 0	Day 1	Day 52
Carbonate addition (low pressure test)	1	5.43	6.56	6.99	5.8	6.77	7.27
	2	5.43	6.6	7.09	5.8	n.a	n.a
	3	5.43	6.7	7.09	5.8	n.a	n.a
Core materials (low pressure test)	1	5.43	5.93	6.54	5.8	6.3	6.63
	2	5.43	5.88	6.51	5.8	6.3	6.58
	3	5.43	5.94	6.44	5.8	n.a	n.a

n.a no data available

Volatile fatty acids and anions were also measured at beginning and the end of the tests. The change in concentration of sulfate and acetate were shown in Figure 11. Sulfate was present in the liquid phase of both abiotic and biotic assays with the initial concentration of approximately 90 – 94 mg/l. In comparison to abiotic assays, no significant reduction of sulfate occurred due to microbial activity. In contrast, compared to abiotic assays, a slight increase in acetate concentration was noticed in biotic assays. Acetate raised from 634 mg/l at day 1 to 897 mg/l at day 52 of the experiment for biotic assays with carbonate addition and from 666 mg/l at day 2 to 793 mg/l at day 52 for biotic assays with core materials.

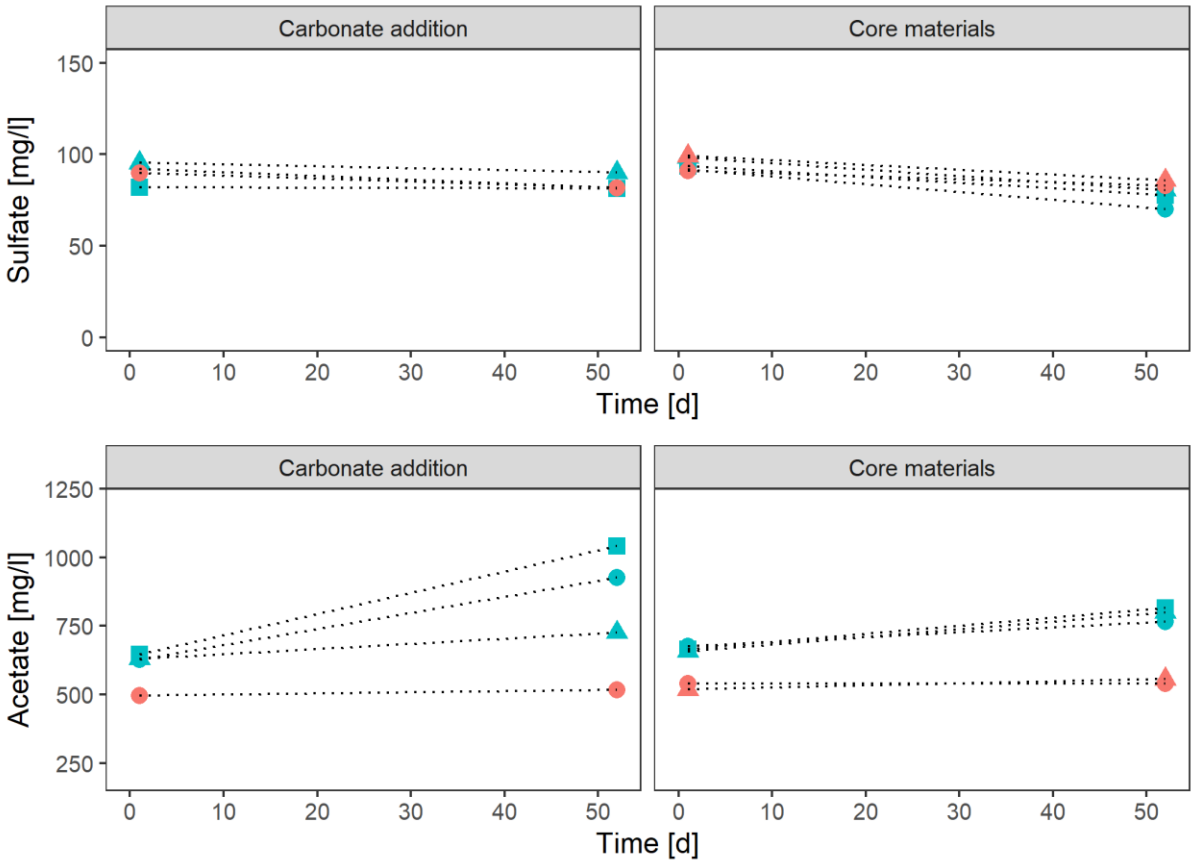

Figure 11: Concentration of sulfate and acetate at the beginning and the end of the hydrogen consumption tests at low pressure (1,500-1,900 mbar) with core materials and with carbonate addition, temperature 60°C, salinity 1.7 %.

3.2.2. Hydrogen consumption at high pressure

The hydrogen consumption test in high-pressure units was performed at 70 bar and a temperature of 60 °C. Pressure and temperature in bioreactors were monitored during the experimental periods and are shown in Figure 12. As observed, approximately 5 bar of hydrogen was loss in both bioreactors during the experimental periods. Gas analysis indicated that no other gas than H_2 was in the gas phase of the bioreactors.

Changes in concentrations of sulfate, acetate, pH, and total cell numbers at beginning and the end of the tests were monitored and shown in Table 10 . Similar to observation in the biotic low pressure tests sulfate concentration remained relatively unchanged while acetate concentration slightly increased over the experimental duration. A reduction in total cell numbers was observed. In one bioreactor a slight decrease in pH was observed, from 6.25 to 5.78 after 52 days of the experiment. No significant change in pH was noticeable in another bioreactor.

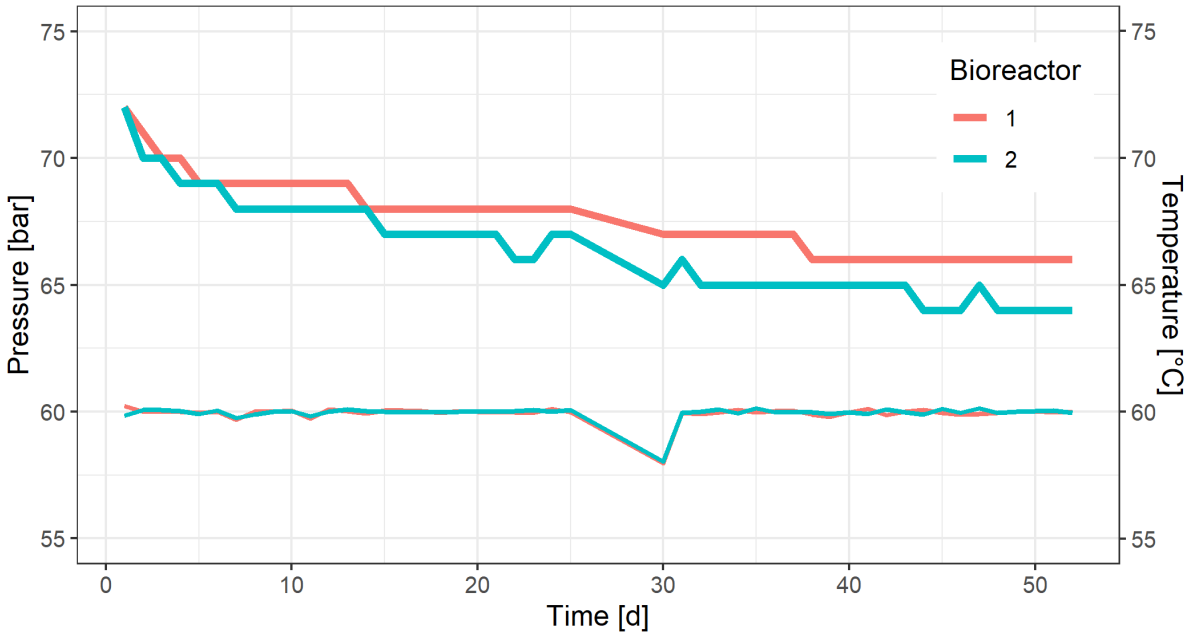

Figure 12: Pressure (thick lines) and temperature (thin lines) monitored during hydrogen consumption test under high pressure condition.

Table 10: Hydrogen consumption test with core materials in the presence of hydrogen at high pressure (70 bar): changes in concentration of sulfate, acetate, pH, and cell numbers

Bioreactor	Sulfate (mg/l)		Acetate (mg/l)		pH		Cell numbers (cells/ml)	
	Day 1	Day 52	Day 1	Day 52	Day 1	Day 52	Day 0	Day 52
1	167.16	152.47	683.85	1742.88	6.25	5.78	1.23e+06	1.07e+06
2	87.74	89.42	659.07	846.49	6.08	6.12	1.23e+06	5.87e+05

3.2.3. Microbial composition

Hydrogen-consuming microbial groups including acetogens, methanogens, SRA and SRB present in the biotic assays were quantified by molecular analysis at the beginning and the end of the hydrogen consumption tests. As shown in Figure 13, acetogens and sulfate-reducing microorganisms (SRB, SRA) were present in the cultures at the beginning of the hydrogen consumption tests (low pressure and high pressure). Based on the numbers of gene copies, in the inoculum sulfate-reducing microorganisms were nearly 2.5 times higher than acetogens (≈5.07 x 10^4 copies/ml and 1.86 x 10^4 copies/ml, respectively). The qPCR analysis showed a decrease in the number of specific genes for acetogens and sulfate-reducing microorganisms at the end of the experiment in both low pressure and high-pressure test. For low pressure test,

there were only 7.6 x 10^1 copies/ml of acetogens and 1.6 x 10^2 copies/ml of sulfate-reducing prokaryotes in biotic assays with core materials. The assays with carbonate addition showed a reduction with much higher extent than assays with core materials. For high pressure test, 7.5 x 10^2 copies/ml of acetogens and 2.7 x 10^2 copies/ml of sulfate-reducing prokaryotes were recorded.

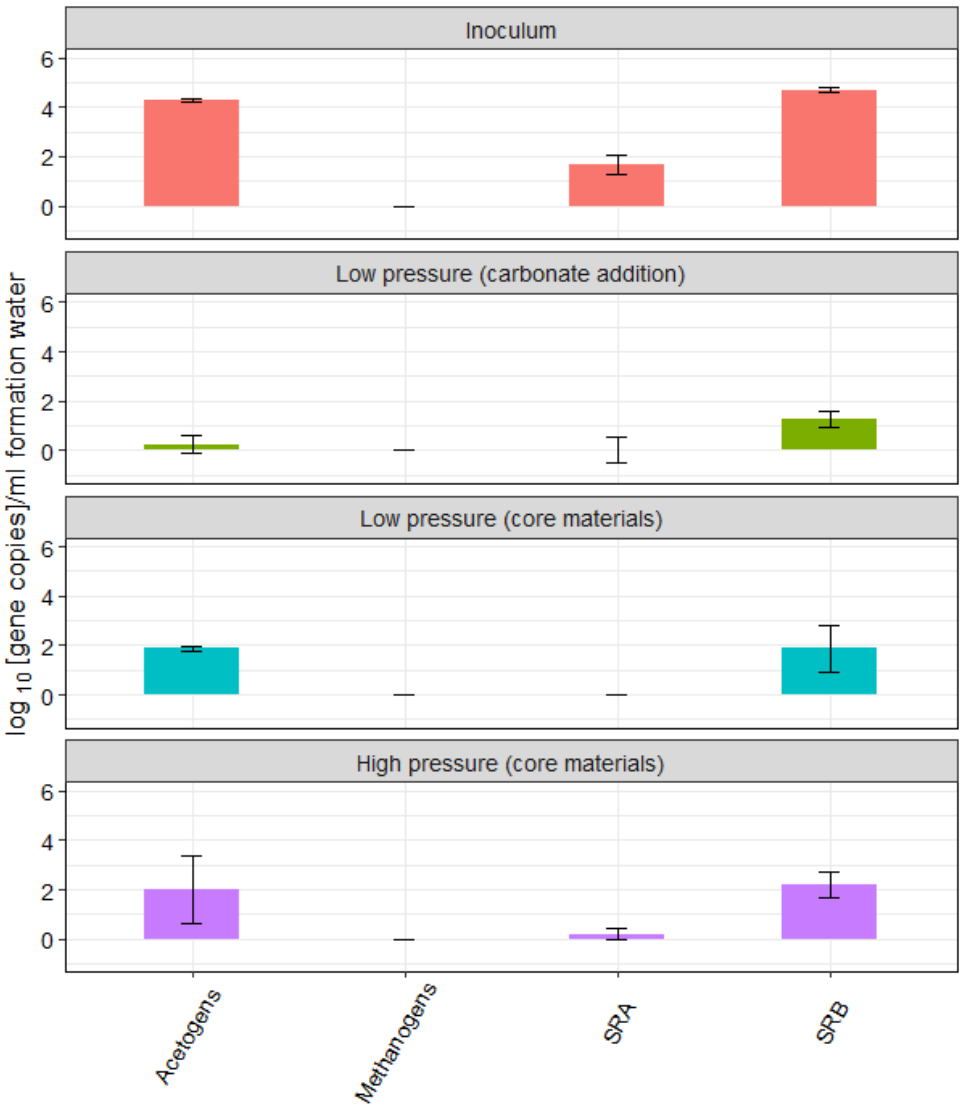

Figure 13: Quantification of hydrogen-consuming microbial groups at beginning and the end of hydrogen consumption tests. Same inoculum for tests at high pressure and low pressure) Error bars show standard deviation of triplicate experiments (low pressure) or duplicate experiments (high pressure) and duplicate qPCR measurements.

3.2.4. Discussion

Hydrogen-consuming microorganisms, namely acetogens and sulfate-reducing microorganisms, successfully enriched from original formation water, were used for hydrogen consumption tests at low hydrogen pressure (1,500 – 2,000 mbar) and high hydrogen pressure (70 bar). However, no obvious reduction in pressure due to microbial activity was observed under the chosen test conditions. Furthermore, the cell numbers in all experiments were significantly reduced at the end of the experiments compared to the inoculum.

Due to the proven increase in acetate concentration at the end of the test, a certain activity of acetogens in the low-pressure tests can be assumed. However, the conversion rates are very low. At high hydrogen pressure, acetogenesis appears to occur at a higher rate than at low pressure. Studies have indicated that maximum microbial hydrogen consumption rates were lower at low pH than at neutral pH (Goodwin 1988). It could be possible that microorganisms were inhibited by the relatively low pH of formation water (pH 5.8). Furthermore, at pH as low as pH 6, CO_2 is the dominant species in the carbonate system, while solubility of CO_2 at 60 °C is very low. As a result, CO_2 eventually releases into gas phase. This process would reduce the available carbonate for acetogens.

There was no evidence for the occurrence of sulfate reduction in the experiments although sulfate-reducing microorganisms were also present in the inoculum. Hydrogen and organic compounds like acetate can be utilized as electron donors by sulfate-reducing microorganisms (Muyzer and Stams 2008). However, compared to methanogens, sulfate reducers are usually more susceptible to elevated volatile fatty acid (James 1998) and the inhibition of sulfate reduction by organic acids at low pH, has also been reported (Reis 1990; Voskuhl 2022; Koschorreck 2008).

In addition, low pH environments might contain elevated concentrations of dissolved metals that could be toxic to microorganisms. There was unfortunately no current chemical data available regarding dissolved metals or toxic compounds in the formation water or the core materials. It is possible that microbial activities were inhibited by toxic chemical compounds potentially present in the used formation water and core materials.

The hydrogen consumption tests suggested that the microbial risks for the storage could be low despite the presence of acetogens and especially sulfate-reducing microorganisms. Nevertheless, it should be mentioned that the concentration of carbon sources used in the tests might be far lower than the actual available carbonate in the reservoir. Furthermore, the duration for the laboratory tests is only limited to around 8 weeks. Therefore, it may be that microorganisms can later adapt to environments, develop and increase their activities. Further

details studies on chemical composition in formation water and core materials, effect of toxic compounds, carbon concentration, pH on microbial activities as well as extended investigation duration are recommended.

3.3. Analysis of storage #3

The formation water sample used for hydrogen consumption test was collected from a storage site with salinity of < 1 % NaCl (w/v), pH 6.2, temperature of 65 °C, and bottom hole pressure of 105 bar (Table 1, sample 3).

The mixed cultures of methanogens/acetogens, sulfate-reducing prokaryotes and anaerobic heterotrophs enriched from the formation water used as inoculum for hydrogen consumption experiments. Temperature for cultivation of microorganisms as well as for hydrogen consumption test was at 60 °C. Carbon source was either from core materials (10 % w/v) originated from the storage site or from the addition of HCO_3^- (30 mM). Biotic assays were done in triplicate. In addition, duplicate abiotic tests were similarly performed for sterility control without the addition of microorganisms. The pressure and carbon source for each assay are summarized in Table 11.

Table 11: Hydrogen consumption test under low and high hydrogen pressure

Test	Carbon source	Pressure	Gas volume/liquid volume	Liquid phase	Gas phase
1. Low pressure test (Carbonate addition)	30 mM HCO_3^-	< 1,500 - 2,000 mbar	65 ml/60 ml	formation water + enrichment cultures	100 % H_2
2. Low pressure test (Carbonate addition with pH adjustment)	30 mM HCO_3^-	< 1,500 - 2,000 mbar	65 ml/60 ml		
3. Low pressure test (Core materials)	10 % (w/v) core	< 1,500 - 2,000 mbar	65 ml/60 ml		
4. High pressure test (Biotic: core)	10 % (w/v) core	100 bar	125 ml/125 ml		

Four pieces of core materials were received and prepared for H_2 consumption tests. Details of the core cuttings used and their compositions are given in Figure 12 and Figure 14. The core material was first cut and crushed into smaller size (Figure 15) to enhance to contact surfaces. Due to the limited amount of available core material, four provided core sections were combined. The core materials mixed in this way were used for the experiment.

Table 12: Core materials used for hydrogen consumption test

Core materials	Length (mm)	Width (mm)	High (mm)	Weight (g)	Calcite (%)	Dolomite (%)	Quartz (%)	Shale (%)	Pyrite (%)
MA	40.3	30.6	10.3	33.2	84	6	11	0	0
MB	40.2	31.2	10.7	33.9	64	< 5	11	22	0
M1	42.2	31.1	10.8	36.2	58	< 5	14	17	8
M2	40.8	30.7	10.8	35.1	74	0	10	10	7

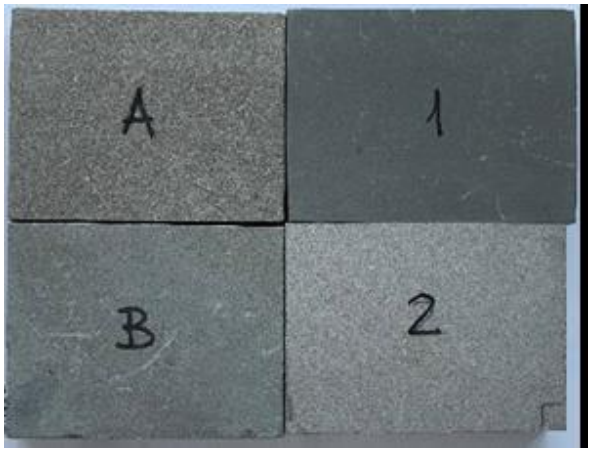

Figure 14: Original core materials used for H2 consumption test before processing

Figure 15: Preparation of core cuttings for H2 consumption tests
A, B) cutting of core material; C) rock material after crushing

3.3.1. Hydrogen consumption at low pressure

At low pressure, microbial hydrogen consumption was observed in biotic tests with both core materials and carbonate addition (Figure 16). In all biotic tests, the microorganisms actively consumed hydrogen within the first 2 weeks, with a total of about 21 % of the hydrogen being oxidised during this time. Compared to the biotic experiments with core materials as carbon

source, the microorganisms oxidised hydrogen at higher rates in the experiments with carbonate as carbon source. After 2 weeks of incubation, the microbial oxidation of hydrogen decreased significantly. At the end of the experiment, the total hydrogen consumption by microorganisms was about 29 % in the biotic test with core materials and in the biotic test with carbonate addition plus initial pH adjustment. Under the conditions with carbonate addition, almost 22 % of the hydrogen was consumed by microbial activity. In the abiotic tests, no hydrogen conversion occurred. (Figure 21).

In addition to hydrogen, carbon dioxide was also detected in the gas phase during the course of the experiment, whereby a higher CO_2 concentration was formed in experiments with carbonate addition than in experiments with core material. A gradual decrease in CO_2 was observed in all biotic experiments regardless of the carbon sources (Figure 16). In contrast to biotic tests, CO_2 concentration remained relatively constant in abiotic control tests (Figure 21).

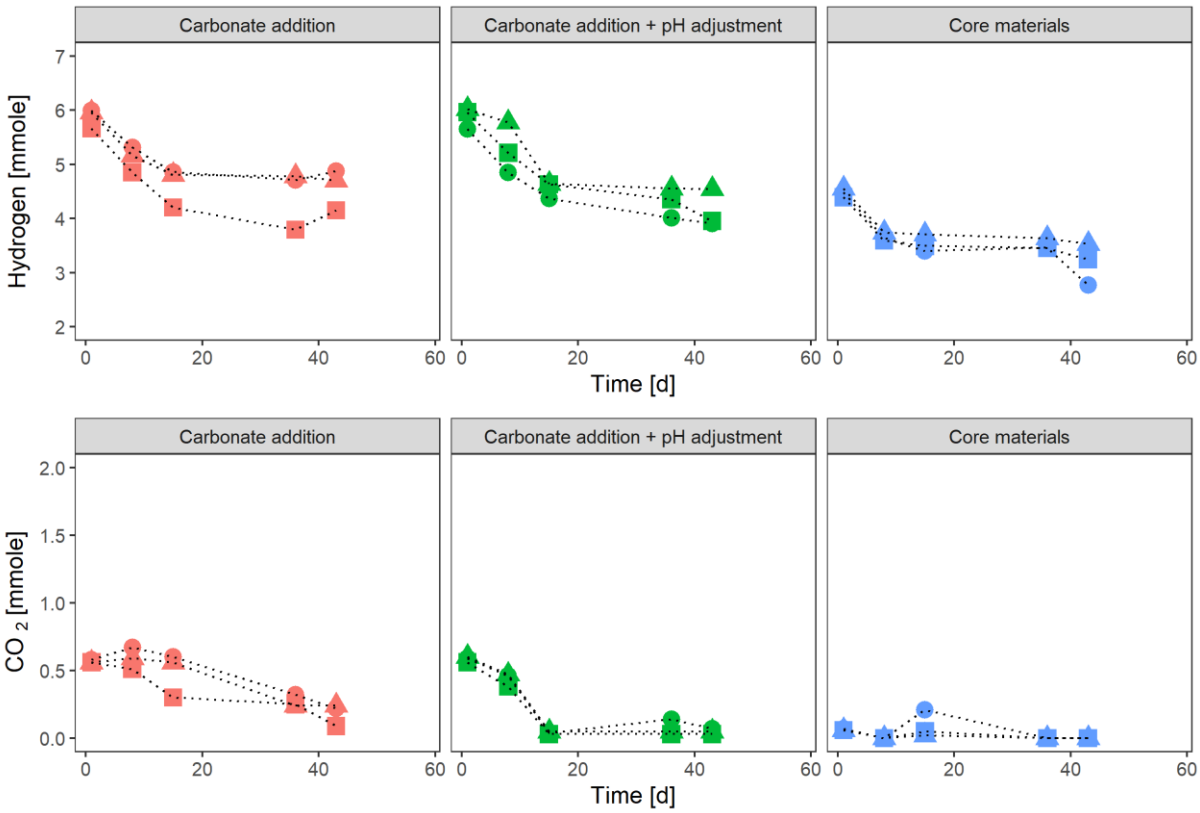

Figure 16: Changes of total hydrogen and CO_2 over time due to microbial activity at low hydrogen pressure (1,500-2,000 mbar), salinity < 1 %, temperature 60 °C. Triplicates indicated by point shapes.

In the three biotic tests, there was a slight increase in the total number of planktonic cells in the first two weeks. However, no further growth was observed thereafter. In addition, there was an increase in pH value in all biotic tests, with the pH increasing from about 6.5 at the beginning to 7.7 at the end of the test with addition of core material.

In order to keep the pH value in the range between 7.0 and 8.0, which is most favourable for microorganisms, an additional test series "pH adjustment" was established. If the pH value fell below 6.0 or rose above 9.0 during the test periods, it was readjusted accordingly. Since the pH changes in this trial still remained within the desired range, no further pH adjustment was made after the first adjustment on day 1. (Figure 17).

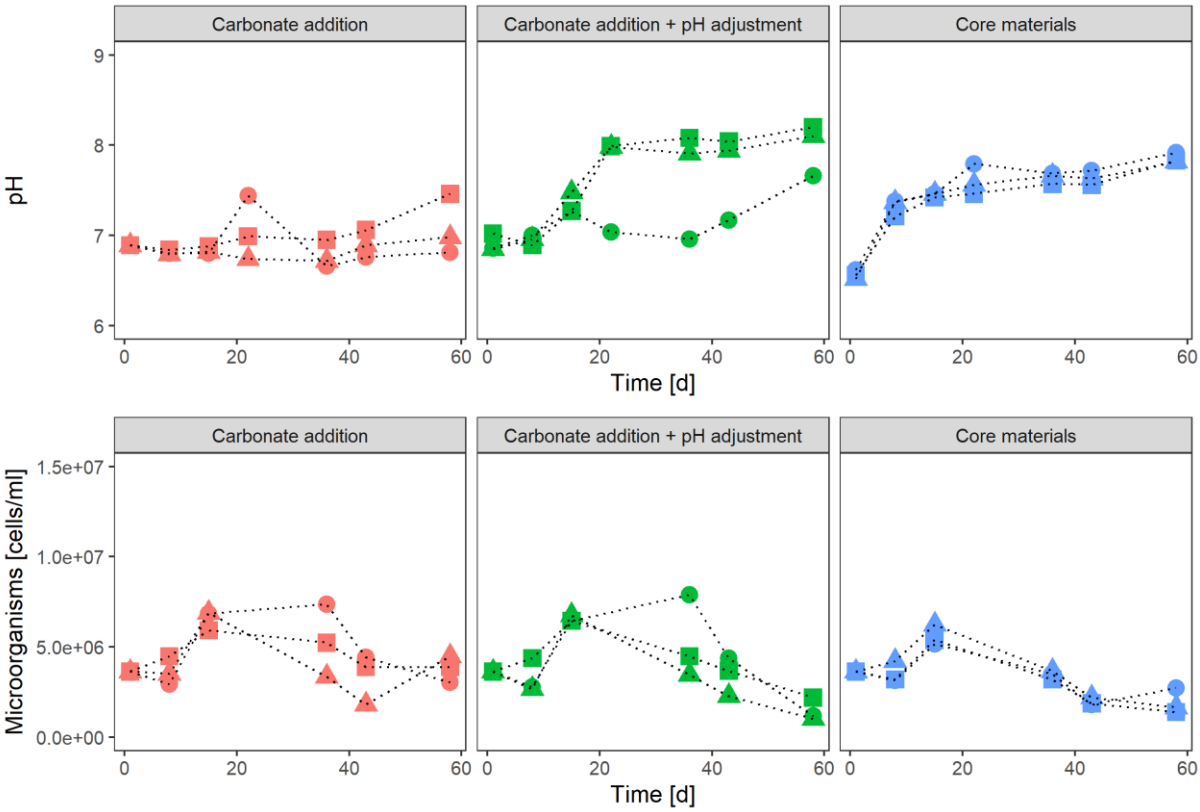

Figure 17: Change of pH and cell numbers during microbial hydrogen consumption test at low pressure. Triplicates indicated by point shapes

Concentration of volatile organic acids and anions were monitored during the experiments. As shown in Figure 18 and Figure 22, sulfate was only present in assays with core materials. The sulfate concentration decreased after two weeks in the biotic tests from 64 ± 22 mg/l on the

first day to below the detection limit (Figure 18) while concentration of sulfate in abiotic tests remained constantly above 100 mg/l throughout the entire duration of the experiment.

The acetate concentration remained above 4,000 mg/l in all tests. A slight increase in acetate concentration was observed in the biotic tests where HCO_3^- was used as a carbon source. When core materials were used as a carbon source, the acetate concentration decreased slightly.

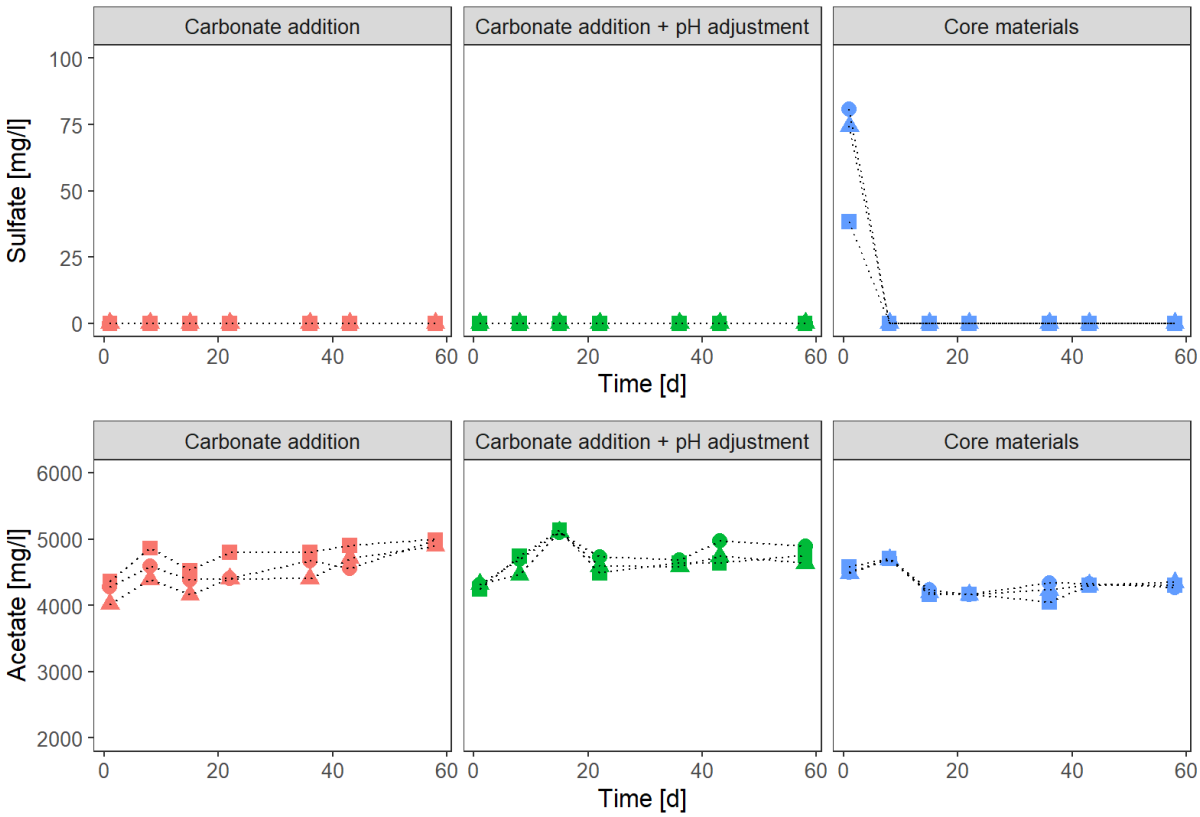

Figure 18: Change of sulfate and acetate concentrations in biotic assays during hydrogen consumption test. Triplicates indicated by point shapes

3.3.2. Hydrogen consumption at high pressure

Pressure and temperature in the bioreactors of the high-pressure experiments were monitored during the experimental periods and are shown in Figure 19. In bioreactor 1, a temporary drop in pressure and temperature occurred on the 14th and 17th day of the experiment due to a technical malfunction. The registered steady pressure drop in the reactors is due to the non-avoidable diffusion of hydrogen. Gas chromatographic analysis showed that there was no alteration in the gas atmosphere during the test period.

The change in concentration of anions and volatile organic acids, pH and total cell count are shown in Table 13. Similar to the biotic experiments with core materials tested at low pressure, the sulfate concentration decreased in the high-pressure experiments and was no longer detectable at the end of the experiment, while the acetate concentration remained high. In addition, an increase in pH was observed in both bioreactors.

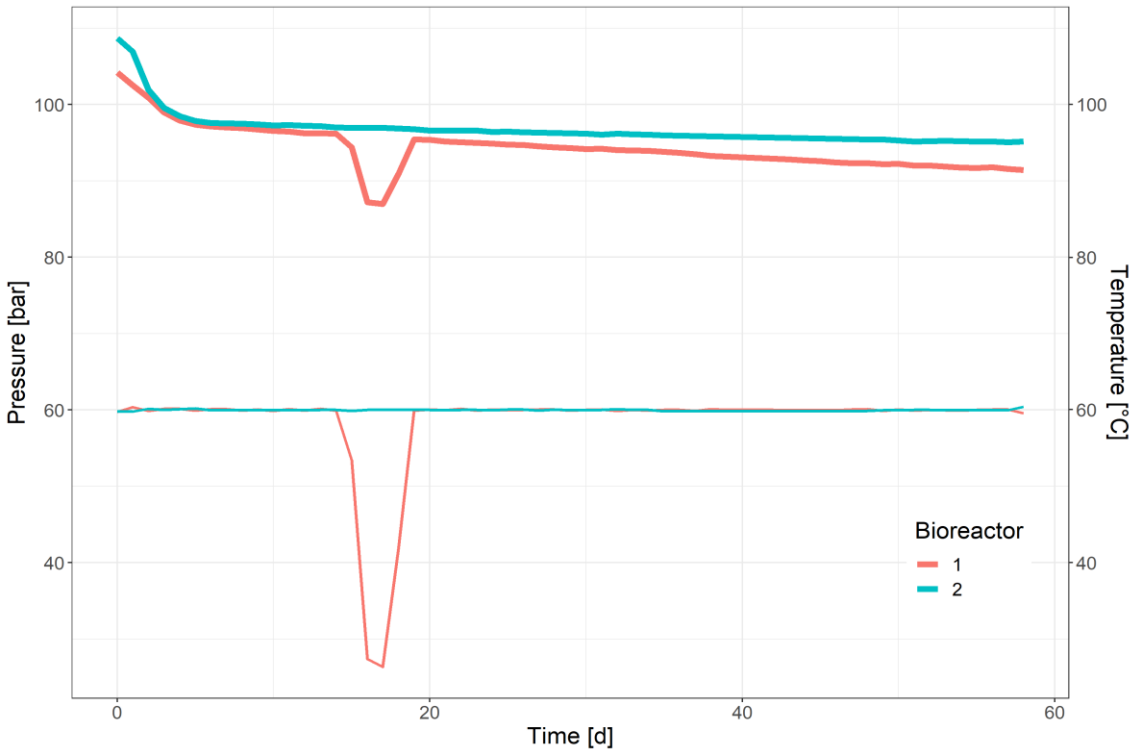

Figure 19: Pressure (thick lines) and temperature (thin lines) monitored during hydrogen consumption test under high pressure condition

Table 13: Concentration of sulfate, acetate, pH, and cell numbers during hydrogen consumption test with core materials at high pressure (100 bar)

Bioreactor	Sulfate (mg/l)		Acetate (mg/l)		pH		Cell numbers (cells/ml)	
	Day 1	Day 58	Day 1	Day 58	Day 1	Day 58	Day 1	Day 58
1	92.82	n.d	4,746.29	4,540.04	6.94	7.87	4.3e+06	1.1e+06
2	95.41	n.d	4,767.06	4,445.70	6.97	7.68	3.3e+06	6.4e+05

n.d: not detectable (below detection limit)

3.3.3. Microbial composition

The results of the molecular biological analyses of the hydrogen-consuming microorganism groups (acetogens, methanogens, SRA and SRB) at the beginning (inoculum) and at the end of the hydrogen consumption test at high and low pressure are shown in Figure 20.

As shown, the hydrogen-consuming microorganisms in the enrichment cultures were acetogens, SRA and SRB, with SRB being the dominant group (5.9×10^4 copies/ml). Methanogens were below the detection limit. A reduction in microbial counts was observed at the end of the series of experiments. An exception was the test with carbonate addition, in which there was an increase in the microbial counts of acetogens and SRB compared to the inoculum.

In the high-pressure test (Figure 20, Biotic, Core, High Pressure), the total microbial count, represented by eubacteria and archaea, decreased significantly at the end of the high-pressure test compared to the inoculum. At the end of the test, only SRB were detected among the hydrogen-consuming microbial groups, and these were about three orders of magnitude lower than in the inoculum. In the low-pressure test, acetogens and SRB were still detected as hydrogen-consuming groups in experiments with the addition of core materials or carbonate with initial pH adjustment (Figure 20, Biotic, Core and Biotic, Carbonate, pH). The amounts of microorganisms in these experiments were significantly lower at the end of the test compared to their initial inoculum. In the experiments with core materials, the reduction was much more significant.

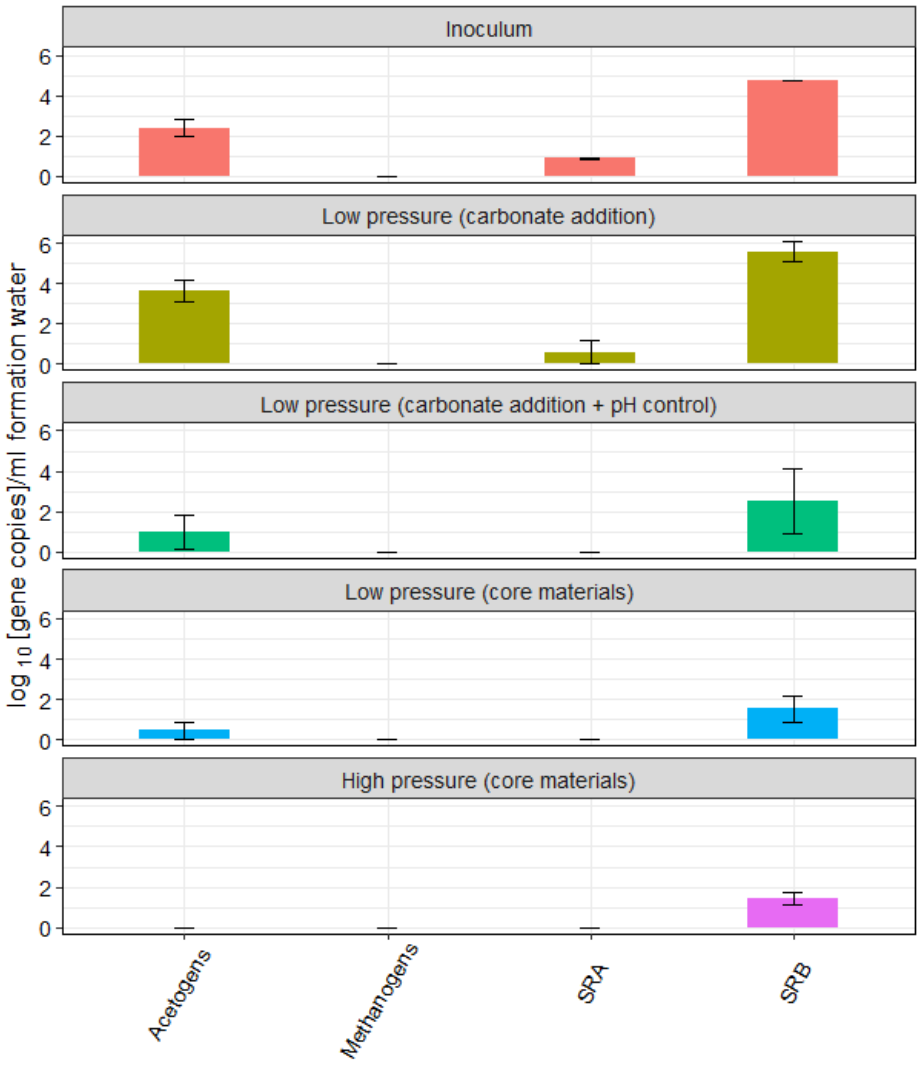

Figure 20: Quantification of hydrogen-consuming microbial groups at beginning (inoculum, same for tests at high pressure and low pressure) and the end of tests. Error bars show standard deviation of triplicate experiments (low pressure) or duplicate experiments (high pressure) and duplicate qPCR measurements

3.3.4. Discussion

In the low-pressure tests, the microorganisms actively consumed hydrogen in both experiments with core materials and with added carbonate ($NaHCO_3$), whereby a higher hydrogen consumption rate was observed in the experiments with carbonate. This is possibly due to the fact that more soluble carbon was available to the microorganisms in these experiments at the beginning of the experiment than in experiments with core materials.

Based on available nutrients as well as microbial composition from initial inoculum, acetogens were likely to be responsible for hydrogen consumption in the assays with $NaHCO_3$ addition.

In the tests with addition of core material, sulfate was detected in both low-pressure and high-pressure tests, being approximately 64 mg/l and 94 mg/l after 1 day of incubation, respectively. In contrast, in assays without addition of core materials, concentration of sulfate remained below detection limit. It should be noticed that during the experiment no external soluble sulfate was added and the concentration of sulfate in the formation water was very low (< 0.1 mg/l). Therefore, the soluble sulfate is very likely from the dissolution of the core material. In the abiotic tests of the low-pressure test, a sulfate concentration of almost 103 mg/l was measured after 8 test days. In the biotic tests, sulfate was completely converted into sulfide by the activity of the sulfate-reducing microorganisms. This suggests that not only acetogens but also sulfate-reducing microorganisms are involved in hydrogen consumption in experiments with cuttings.

Noticeably, for all the assays tested under low-pressure conditions a significant decrease in microbial hydrogen consumption was observed after about two weeks of the experiment. During the experiment, pH values remained within 6.5 – 8.0 which are still considered to be favourable for microorganisms (Thaysen 2021). The limited availability of carbonate and/or sulfate in the test tubes, on the other hand, limit finally the microbial activity. Acetogens usually do not tolerate high acetate concentrations (Baronofsky 1984). As a result, homoacetogens could be inhibited because the formation water from the reservoir contains a very high acetate concentration (> 4,000 mg/l).

Analyses of sulfate concentration in the high-pressure tests show a decrease of sulfate concentration from 94 mg/l at beginning to non-detectable levels in both high-pressure bioreactors at the end of the experiment. Moreover, inoculated microorganisms were still very active when they were re-grown in specific cultivation medium with essential nutrients after the high-pressure experiment. Therefore, it can be postulated that sulfate-reducing microorganisms remain active not only at low pressure but also at high-pressure conditions.

According to the results obtained, it can be concluded that microorganisms enriched from the investigated reservoir are able to consume hydrogen at 60 °C and a pressure up to 100 bar. It is evident that if hydrogen is available in unlimited quantities, the availability of carbon sources and sulfate may become crucial factors for microbial hydrogen consumption. In addition to limiting the availability of soluble carbon sources from core materials, a high acetate concentration in formation water could further minimise the high risk of acetogens.

However, as can be seen from the experimental data, the core materials not only provided a carbon source for the microorganisms, but also sulfate. In the low-pressure experiments, about 5.9 mg of sulfate was dissolved in 60 ml of formation water from an initial 6 g of core material within an experimental period of 58 days. Since the sulfate content of the formation water was

very low, the risks associated with sulfate-reducing microorganisms are strongly dependent on the sulfate content and the dissolution of sulfate from the core material.

As a result of this study, microbial contamination and activity was clearly identified for the deposit. Based on the identified hydrogen consumption by various microbial groups, a general hydrogen conversion process is very likely. This microbial process could be limited in time and location by the above factors and is highly dependent on the specific composition of the rock matrix in each case, which is likely to vary within the geological structure. Further chemical and microbiological analyses are recommended to further assess the identified risks for possible hydrogen storage in the reservoir.

3.3.5. Supplementary data

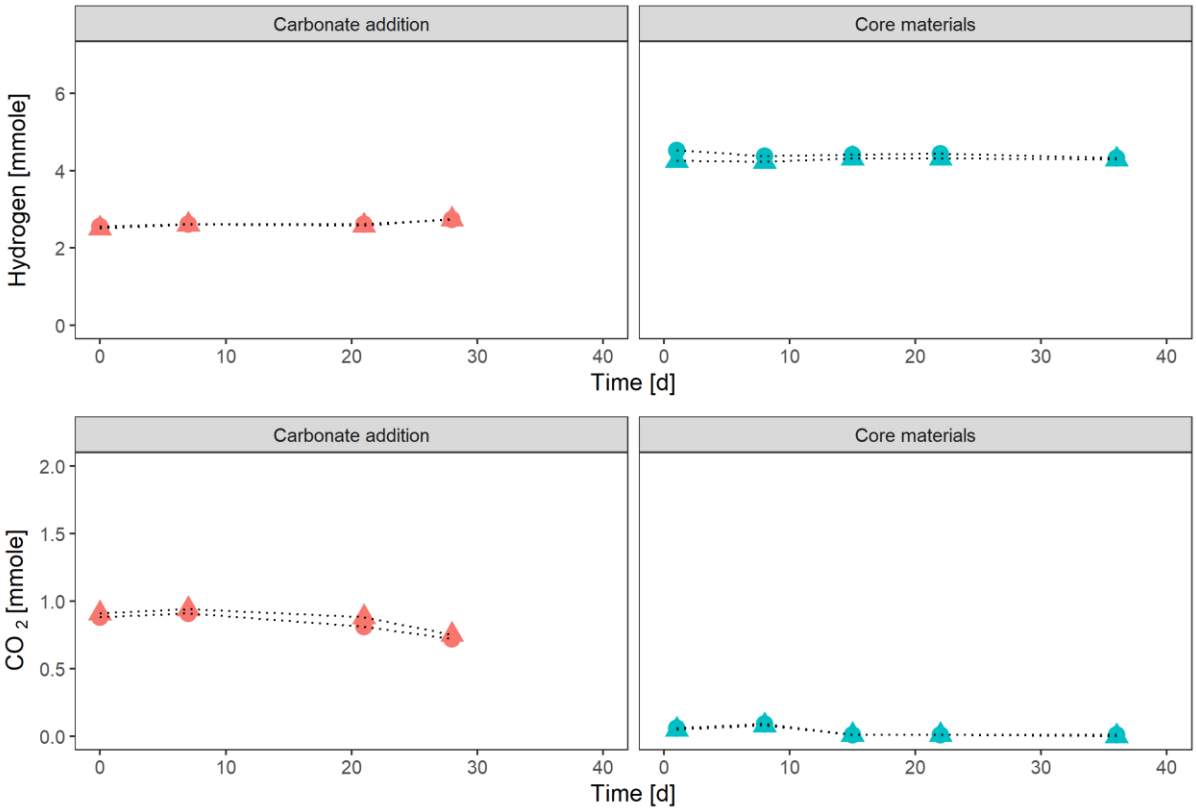

Figure 21: Abiotic control tests; H_2 and CO_2 during hydrogen consumption test with core materials and with carbonate addition in the presence of pure hydrogen at low pressure (1,500-1,900 mbar), temperature 60°C, salinity < 1 %. Duplicates, indicated by point shapes

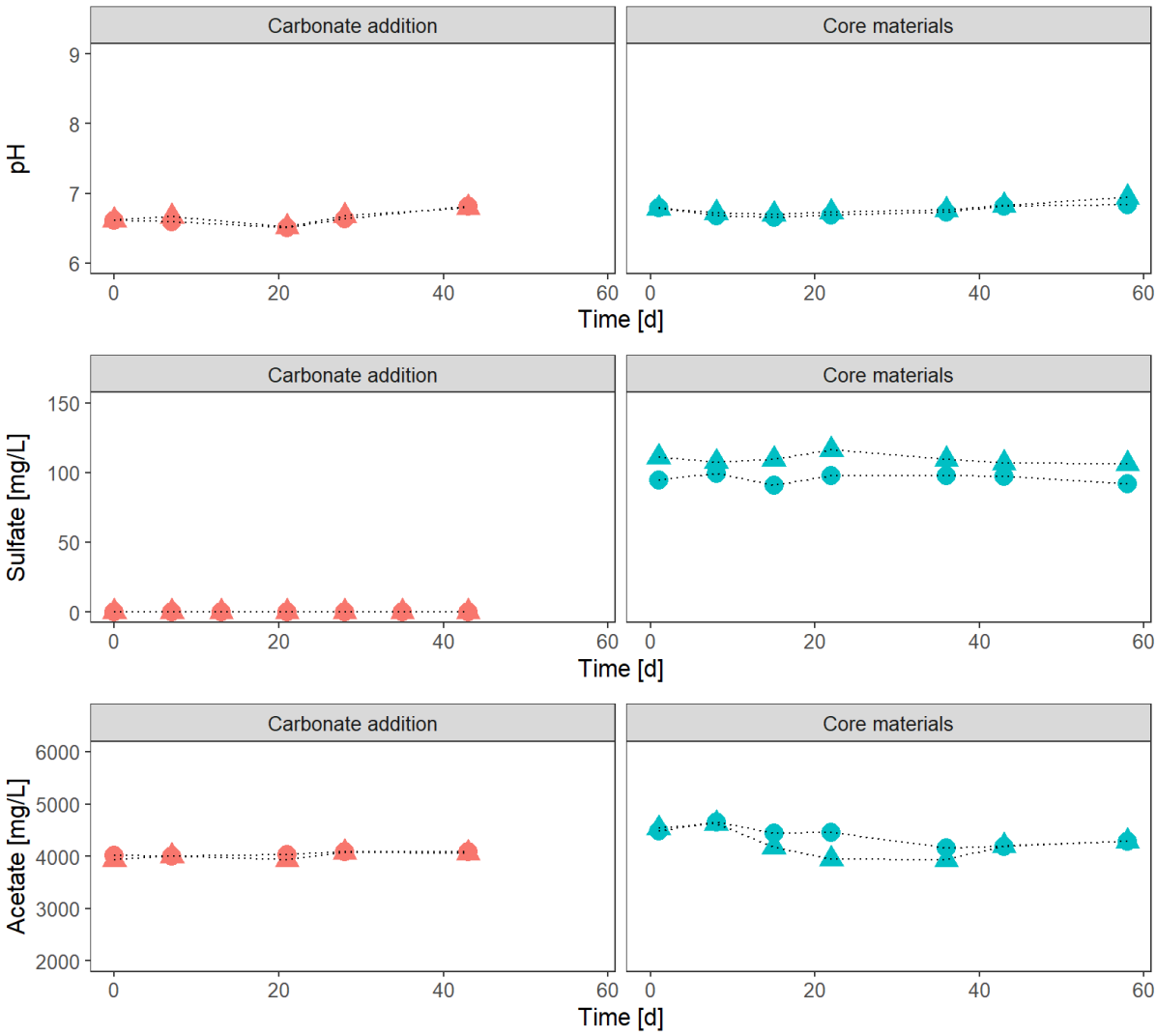

Figure 22: Abiotic control tests; pH, sulfate and acetate concentrations with carbonate addition and with core materials during hydrogen consumption test at low pressure. Duplicates, indicated by point shapes

3.4. Analysis of storage #4

The formation water sample used for the 4th analyses of hydrogen consumption was collected from a storage site with salinity of < 0.1 % NaCl (w/v), pH 7.8, temperature of 26 °C, and bottom hole pressure of 46 – 60 bar (Table 1, sample 4).

The mixed cultures for hydrogen consumption experiments containing of methanogens/acetogens, sulfate-reducing prokaryotes, thiosulfate-reducing microorganisms and anaerobic heterotrophs were prepared on basis of enrichments from the original formation water sample. Temperature for cultivation of microorganisms as well as for hydrogen consumption test was 30 °C.

Since no core material was available from this storage site, an artificial mineral mixture was used for the experiments on the basis of the known mineral composition of the reservoir rock. This mineral mixture consisted of 45 % calcite ($CaCO_3$), 35% pyrite (FeS_2), 5 % barite ($BaSO_4$), and 15 % iron oxide (FeO). As carbon source for the consumption tests either the artificial mineral mixture (10 % w/v) or $NaHCO_3$ (60 mM) was used.

Biotic assays were done in triplicate. In addition, hydrogen consumption tests under low pressure, duplicate sterile control assays without addition of microorganisms were also done in a similar manner. The pressure, carbon source, condition for each assay were summarized in Table 14.

Table 14: Hydrogen consumption tests under low and high-pressure of hydrogen

Test	Carbon source	Sulfate	Pressure	Volume Gas/ Liquid	Liquid phase	Gas phase
1. Low pressure test (Carbonate + sulfate)*	60 mM HCO_3^-	100 mg/l (~1 mM, day 0) 650 mg/l (~7 mM, day 36)	1,500 - 2,000 mbar	65 ml/ 60 ml	formation water + enrichment cultures	100 % H_2
2. Low pressure test (Carbonate addition)	60 mM HCO_3^-		1,500 - 2,000 mbar	65 ml/ 60 ml		
3. Low pressure test (Artificial mineral mixture)	10 % (w/v) artificial mineral mixture		1,500 - 2,000 mbar	65 ml/ 60 ml		
4. High pressure test (Artificial mineral mixture)	10 % (w/v) artificial mineral mixture		45 bar	125 ml/ 125 ml		
5. High pressure test (Carbonate + sulfate) *	60 mM HCO_3^- (day 0) 60 mM HCO_3^- (day 15)	100 mg/l (~1 mM, day 0) 4,328 mg/l (~45 mM, day 15)	45 bar	125 ml/ 125 ml		

* stimulation of worst-case scenario

3.4.1. Hydrogen consumption at low pressure

For the hydrogen consumption test at low hydrogen pressure, it was observed that microorganisms actively oxidized hydrogen regardless of carbon source and presence of sulfate in the liquid phase. (Figure 23).

Microbial hydrogen consumption rates were significantly higher in assays with carbonate (HCO_3^-) than in the assays with artificial mineral mixture. In assays with soluble carbonate, microorganisms oxidized hydrogen with the rate of approximately 3 mole/m^3/day while in the assays with artificial mineral mixture the microbial hydrogen oxidation rates were only 1.7 mole/m^3/day (Table 15). Microorganisms consumed almost 50 % of injected hydrogen in the assays with carbonate and nearly 35 % in the assays with core materials after cultivation for 40 days. In the biotic approaches with carbonate addition, methane was detected by gas chromatography after 15 days, while methane formation in the batches with artificial mineral mixture was only detectable after 20 days (Figure 23). Analogous to the hydrogen consumption, a significantly higher methane formation was observed under the conditions with carbonate addition.

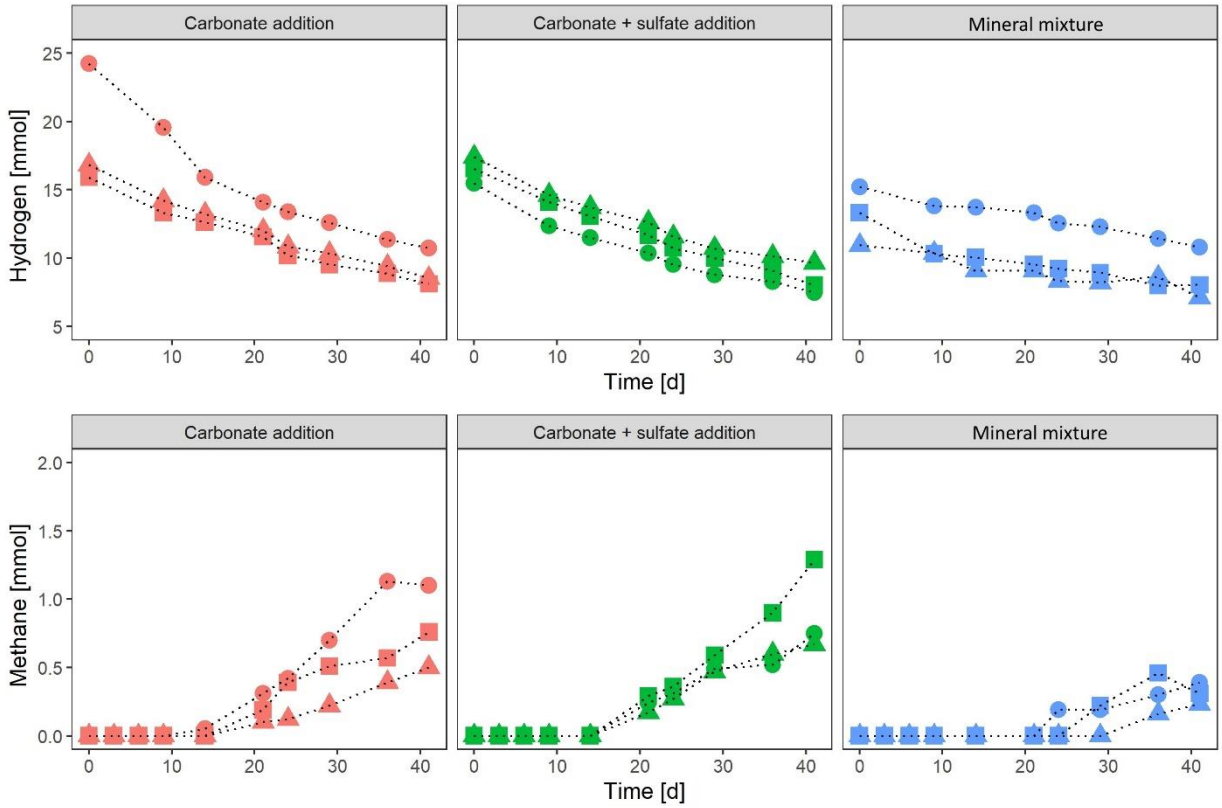

Figure 23: Hydrogen consumption and methane formation over time due to microbial activity at low hydrogen pressure (~ 2 bar), salinity < 0.1 %, temperature 30 °C). Triplicates, indicated by point shapes

Changes in pH and total planktonic cell numbers during the microbial hydrogen consumption process were monitored. It should be noted that pH was adjusted to a value of about pH 7 - 8 using HCl 5 % each time after it was measured. The Figure 24 shows the pH values measured before and after pH adjustment. As shown, a continuous increase of pH was recorded in all the assays, with pH increasing from 7 - 7.5 up to 9 - 9.5. Microbial cell counts were determined microscopically during the experiment, showing an increase in cell numbers in all samples. Significantly faster cell growth was observed in the experimental batches with carbonate addition.

Table 15: Hydrogen consumption by microorganisms with carbon source from core materials or from carbonate addition in the presence of hydrogen at low pressure, temperature 30°C, salinity < 0.1 %

Assays (carbon source)	Replicate	Maximum H_2 consumption rate (mole/m^3/day)	H_2 consumed after 40 days (%)	Gas composition after 40 days (H_2: CH_4: N_2)
Carbonate addition	1	5.28	55.7	83: 9: 8
	2	3.28	49.3	91: 8: 1
	3	3.12	49.1	85: 15: 0
Carbonate + sulfate addition	1	3.07	51.8	93: 3: 4
	2	3.10	44.6	75: 3: 22
	3	3.38	51.5	88: 4: 8
Artificial mineral mixture	1	1.70	29.1	88: 12: 0
	2	1.38	35.1	89: 6: 5
	3	1.91	39.6	88: 9: 3

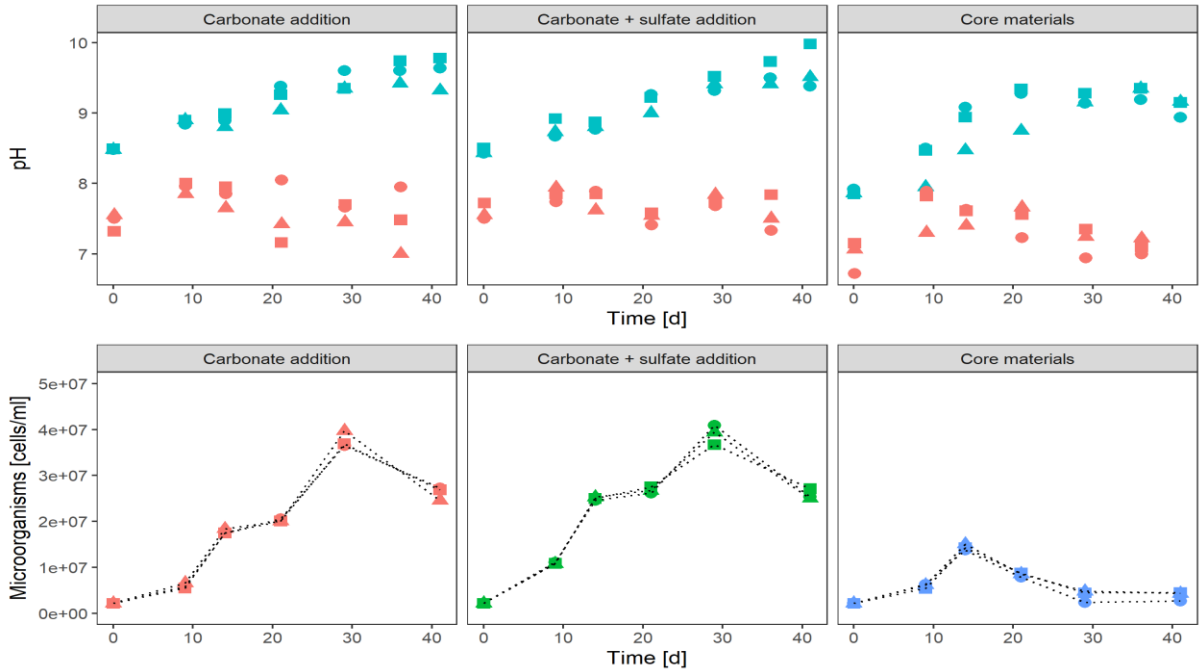

Figure 24: Hydrogen consumption test at low pressure, pH and cell numbers. Triplicates, indicated by point shapes. ***Blue:*** *pH values before adjustment* ***Red:*** *pH values after adjustment*

The formation water used for the experiments with carbonate contained about 40 mg/l of sulfate at the beginning and about 10 mg/l of sulfate at the end. In the experiments with artificial mineral mixture, the sulfate concentration was about 100 mg/l at the beginning, due to the amount of barite in the mixture. After 20 days of cultivation, sulfate could no longer be detected.

In the test series with carbonate and sulfate addition, the initial sulfate concentration was similarly high as in the test series with artificial mineral mixture. The decrease in sulfate concentration was observed here after only 14 days. However, with the second addition of sulfate (650 mg/l, day 36), there was no further decrease in the sulfate concentration (Figure 25).

The acetate concentration, on the other hand, increased very significantly in trials with carbonate addition (with and without sulfate). At the end of the experiment, more than 1,000 mg/l acetate were detected in these tests. Acetate was also formed in the trials with artificial mineral mixture, but to a lesser extent than in the trials with carbonate addition (Figure 25).

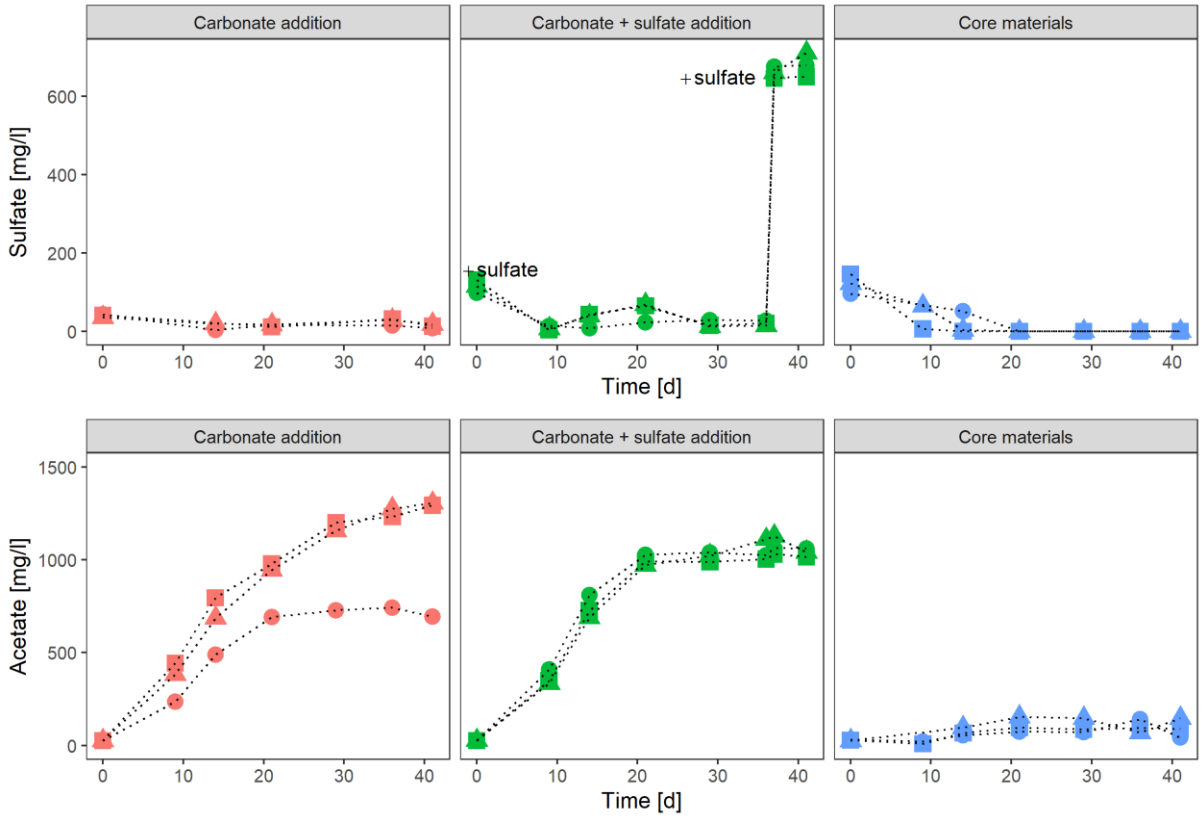

Figure 25: Sulfate and acetate concentrations in biotic assays during hydrogen consumption test. Triplicates indicated by point shapes

3.4.2. Hydrogen consumption at high pressure

In addition to the consumption of hydrogen at low pressure, microbial hydrogen consumption under reservoir conditions at a pressure of 45 bar and a temperature of 30 °C was also carried out. Three bioreactors were used for the hydrogen consumption tests at high pressure. Bioreactor 1 and 2 stimulated hydrogen consumption under the original conditions and contained an artificial mineral mixture, formation water and microorganisms. In bioreactor 3, soluble carbonate and sulfate were added to the formation water to stimulate hydrogen consumption under the worst conditions. Pressure and temperature were monitored during the experiment and are shown in Figure 26. The pressure drop on day 15 is due to sampling and not to microbial activity. Only hydrogen was detected in the gas phase of the bioreactors by gas chromatography throughout the duration of the experiment.

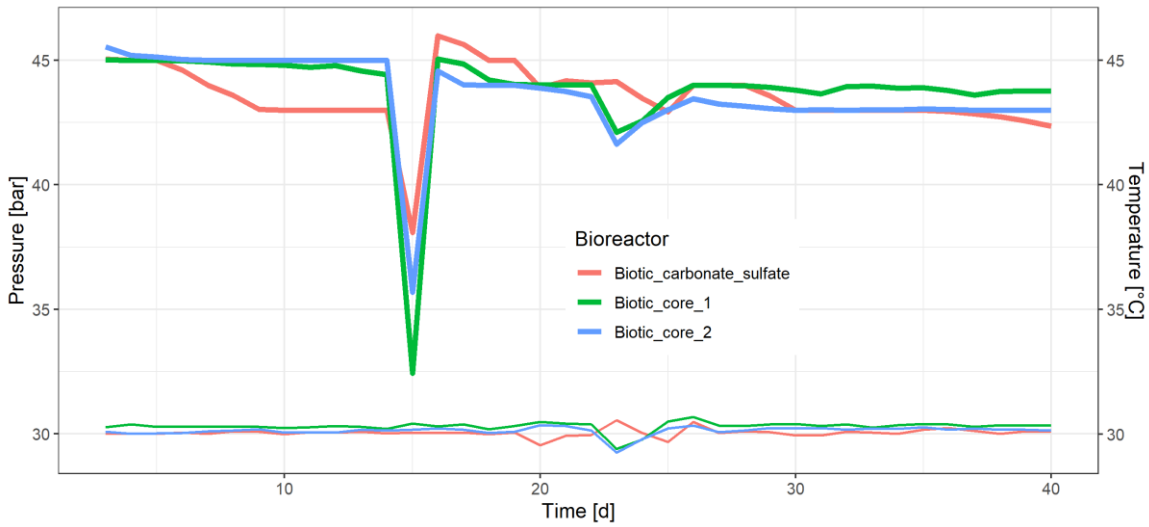

Figure 26: Pressure (thick lines) and temperature (thin lines) monitored at high-pressure experiments

The changes in pH, sulfate and acetate concentrations during the high-pressure tests are shown in Figure 27. This shows that the pH value increased significantly during the test in all three test variants investigated.

In the tests with artificial mineral mixture, the initial sulfate concentration from the $BaSO_4$ used was almost completely converted within 10 - 14 days. This effect was already observed in the sulfate-containing test mixtures of the low-pressure tests. When soluble carbonate and sulfate were added, the sulfate concentration dropped from about 97 mg/l to 22 mg/l from day 0 to day 15 of the experiment. However, after the addition of sulfate on day 15, no further reduction in sulfate was apparent.

The acetate concentration in the batches with mineral mixture increased slightly, but remained below 50 mg/l. In the bioreactor with carbonate and sulfate addition, the acetate concentration increased significantly and amounted to 2,580 mg/l at the end of the experiment.

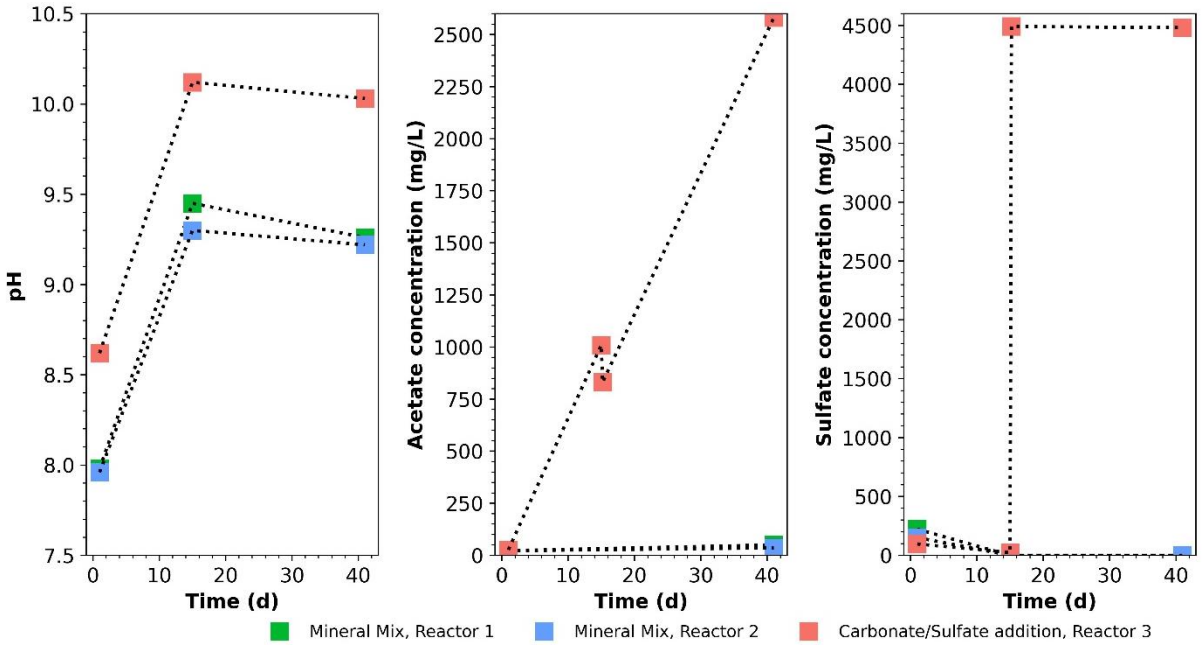

Figure 27: pH value, acetate and sulfate concentration in low-pressure tests

Microscopic measurement of microbial growth in the three bioreactors showed that cell growth occurred only in bioreactor 3. The microbial content increased from 2.4 x 10^6 cells/ml at the beginning to 5.4 x 10^7 cells/ml at the end of the experiment. In the bioreactors with artificial mineral mixture, there was no obvious growth or a slight reduction in the planktonic cell count.

Table 16: Hydrogen consumption test with core materials and with carbonate plus sulfate addition in the presence of hydrogen at high pressure (45 bar): changes in concentration of sulfate, acetate and pH

Day	Bioreactor 1 (Biotic_ mineral_1)			Bioreactor 2 (Biotic_mineral_2)			Bioreactor 3 (Biotic_carbonate_sulfate)		
	Sulfate (mg/l)	Acetate (mg/l)	pH	Sulfate (mg/l)	Acetate (mg/l)	pH	Sulfate (mg/l)	Acetate (mg/l)	pH
1	222.74	22.69	7.98	150.86	22	7.96	96.92	26.39	8.62/7.48**
15	n.d	n.d	9.3/7.2**	n.d	n.d	9.45/6.9	22.53/ 4,328*	1,009.47	10.12/7.7**
41	n.d	50	9.26	n.d	35.83	9.22	4,491	2,581.38	10.03

n.d: not detectable (below detection limit)
* addition of soluble sulfate; ** pH adjustment using HCl 5 %

3.4.3. Microbial composition

The composition of hydrogen-consuming microbial groups (acetogens, methanogens, SRA, and SRB) at beginning (inoculum) and the end of hydrogen consumption test at high and low pressure analysed by molecular analysis confirmed the presence of methanogens, acetogens and sulfate-reducing prokaryotes, with 4.4×10^6, 1.4×10^5 and 2.1×10^6 copies/ml, respectively (Figure 28).

For low pressure tests with the carbonate addition and with both carbonate and sulfate addition, an increase in cell numbers was observed for all investigated hydrogen-consuming groups at the end of the experiment. In assays with carbonate addition, methanogens were the dominant group with 8.3×10^8 copies/ml, followed by acetogens with 9.9×10^7 copies/ml and sulfate-reducing microorganisms with 2.5×10^7 copies/ml.

In assays with carbonate and sulfate addition, methanogens also had the highest abundance with 1.0×10^9 copies/ml, followed by acetogens with 6.3×10^8 copies/ml. Sulfate-reducing prokaryotes in these assays were approximately twice as high as their numbers in the assays with only carbonate addition. In contrast to the assays with addition of carbonate or addition of carbonate and sulfate, at the end of the test the number of microorganisms in the low-pressure tests with core materials decreased significantly in comparison to their initial cell numbers. Methanogens were reduced to 6.0×10^3 copies/ml. Sulfate-reducing microorganisms and acetogens were present with an amount of 7.0×10^4 copies/ml and 9.2×10^3 copies/ml.

Similar to the assays with artificial mineral mixture tested at low pressure, a reduction of microorganisms was observed in the bioreactors with core materials at high pressure. Only 2.4×10^3 copies/ml were recorded for methanogens, 1.7×10^4 copies/ml for acetogens and 2.2×10^5 copies/ml for sulfate reducers. However, at high pressure microbial growth was recorded in the bioreactor with addition of carbonate and sulfate. In this condition, at the end of the experiment, acetogens became the dominant group with 7.7×10^7 copies/ml. Methanogens and sulfate reducers were quantified with 1.1×10^7 copies/ml and 4.5×10^6 copies/ml, respectively.

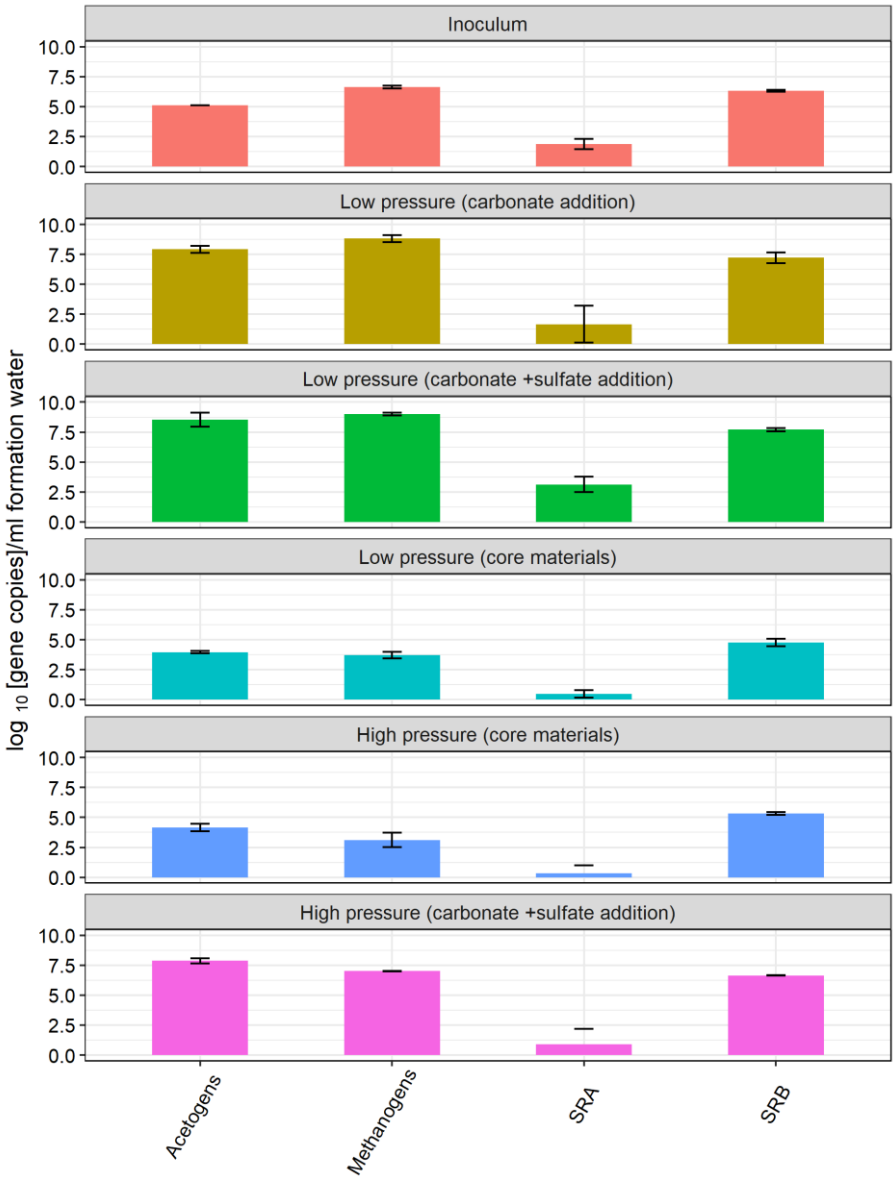

Figure 28: Quantification of hydrogen-consuming microbial groups at beginning (inoculum, same for tests at high pressure and low pressure) and the end of hydrogen consumption tests. Error bars show standard deviation of duplicate qPCR measurements

3.4.4. Discussion

The results of the hydrogen consumption test show that microorganisms actively consume hydrogen. Microorganisms were active not only under low pressure conditions but also under high pressure. Three main groups of microorganisms capable of oxidizing hydrogen, namely acetogens, methanogens, and sulfate-reducing microorganisms were present in the investigated hydrogen consumption tests and their activities were clearly indicated through acetate formation, methane generation and sulfate reduction.

Compared to abiotic assays without microorganisms, the increase in pH could also be a good indicator for microbial activities because the consumption of HCO_3^- by homoacetogens or methanogens by uptake of H_2 causes a reduction in protons and leads to an increase in pH ($4\ H_2 + 2\ HCO_3^- + H^+ \rightarrow CH_3COOH + H_2O$).

In assays with artificial mineral mixture, microbial activities were slower in comparison to assays with the soluble carbonate. The mineral mixture used in the experiments contained only calcite as a carbon source, which has very low solubility in water. Therefore, only a very limited amount of dissolved carbon is available to the microorganisms. This is likely the reason for the low microbial activities in the experiments with the artificial mineral mixture.

In the presence of an excess of sulfate and limited H_2, due to higher affinity and lower threshold values for hydrogen, hydrogen-utilizing SRB outcompete methanogens and acetogens for common substrate hydrogen (Muyzer 2008). Growth kinetics suggest that methanogens can successfully compete with homoacetogens when H_2/CO_2 is present as sole substrate (Thauer 1977). However, in our experiments under low pressure it was observed that acetogenesis and methanogenesis dominated sulfate reduction. Moreover, hydrogen was rapidly consumed by homoacetogenesis rather than methanogenesis within the first 20 days (Figure 23, Figure 25). Homoacetogens thus seem to outcompete sulfate-reducing microorganisms and methanogens under our experimental conditions. This indicates that the interaction or competition is not kinetically determined or cannot be explained by growth kinetics. In experiments with carbonate and sulfate addition, more acetate was produced at high pressure than at low pressure. The availability of hydrogen could increase the activity of acetogens.

It should be noted that both sulfate reducers and methane producers can use acetate as the sole source of organic carbon and energy (Muyzer and Stams 2008; Buan 2018). As sulfate was reduced completely within the first 10 days of the experiments and sulfate reduction did not take place after the second supplement of additional sulfate concentration at day 37, the consumption of acetate by sulfate reducers after 10 days of the experiment could be excluded.

Although studies reported that methanogens grow better with H_2/CO_2 than with acetate (Pan 2016), acetate may also be co-metabolized under H_2 oxidation and CO_2 reduction (Zeikus 1975). It is very likely that acetate produced by acetogens will be utilized by methanogens. Therefore, the recorded acetate concentration could be the net concentration from acetogenesis and acetoclastic methanogenesis.

The results obtained suggest that the microorganisms enriched from the reservoir are able to consume hydrogen at 30 °C and a pressure of up to 45 bar. In the experiments, the activity of sulfate reducers was not evident in the presence of high sulfate concentrations. The reason for the inhibition of sulfate reduction in the mixed cultures is not fully understood. Further studies are needed to fully evaluate the factors affecting the sulfate reduction process. Nevertheless, due to the complexity of microbial interactions, their risks should not be ignored. Microbial activities could alter the reservoir environments and secondary microbial processes can be stimulated. Therefore, it is vital to monitor microbial activities in the formation to recognise and minimize microbial risks.

3.5. Analysis #5 - repeated test of storage sample #1

As part of the fifth analysis program, formation water sample #1 from a reservoir with a temperature of 49 °C and a pressure of 30 bar was analysed again with different test parameters (Table 1, sample 1). In contrast to the first analysis (Chapter 3.1, page 18), the aim was to generate turnover rates for model calculations in underground storage (see also deliverable 3.5) using larger volumes and by using a larger proportion of core material.

The methanogen/acetogen cultures enriched from the original formation water sample served as inoculum for hydrogen consumption experiments (biotic tests). The temperature for culturing the microorganisms and for the hydrogen consumption test was 50°C. The carbon source for this series of experiments was original core material (40%, w/v) from the deposit. The initial pressures and conditions for each test are summarized in Table 17.

Table 17: Hydrogen consumption test under low and high pressure of hydrogen

Test	Carbon source	Pressure	Volume Gas/ Liquid	Liquid phase	Gas phase
Low pressure test A (1.25L bottle)	40 % (w/v) core	1,500 - 2,000 mbar	1,040 ml/ 175 ml	Sterile formation water	100 % H_2
				Formation water + methanogens	100 % H_2 (N_2 addition during sampling)
				Formation water + methanogens	100 % H_2 (H_2 addition during sampling)
Low pressure test B (125 ml bottle)	40 % (w/v) core	1,500 - 2,000 mbar	65 ml/ 52 ml	Sterile formation water	100 % H_2
				Formation water + methanogens	100 % H_2 (N_2 addition during sampling)
				Formation water + methanogens	100 % H_2 (H_2 addition during sampling)
High pressure test	40 % (w/v) core	30 bar	55 ml/ 160 ml	Formation water + methanogens	100 % H_2

3.5.1. Hydrogen consumption at low pressure

A significant microbial hydrogen consumption was observed in the inoculated test mixtures at low pressure, in contrast to the sterile control mixtures. In the gas phases of these tests, methane formation was detected by gas chromatography, whereas no methane was detected in the abiotic controls.

In the hydrogen consumption tests conducted in small bottles (125 ml), the microorganisms consumed hydrogen at a rate of about 4 mol/m^3/day during the first 10 days of the experiment with concomitant methane formation (Figure 29, low pressure B). There was no significant

difference in the hydrogen consumption rate between the biotic experiments with H_2 only (1) and with top-up dosing of N_2 (2). In the following 20 days, the hydrogen oxidation rate decreased to below 1 mol/m^3/day and almost stopped after 30 days (Table 18).

Hydrogen consumption in the 1.25 L bottles followed a very similar pattern (Figure 29, Low pressure A). An initial high rate of hydrogen consumption decreased sharply after about 10 - 14 days and dropped almost completely to zero after about 30 days of incubation. Since the overpressure in the gas analysis remained high enough throughout the course of the experiment due to the large volume, a subsequent dosing of N_2 was not necessary. The two experimental set-ups Biotic_H2 (1) and Biotic_N2 (2) in the low-pressure bottle A are therefore duplicates.

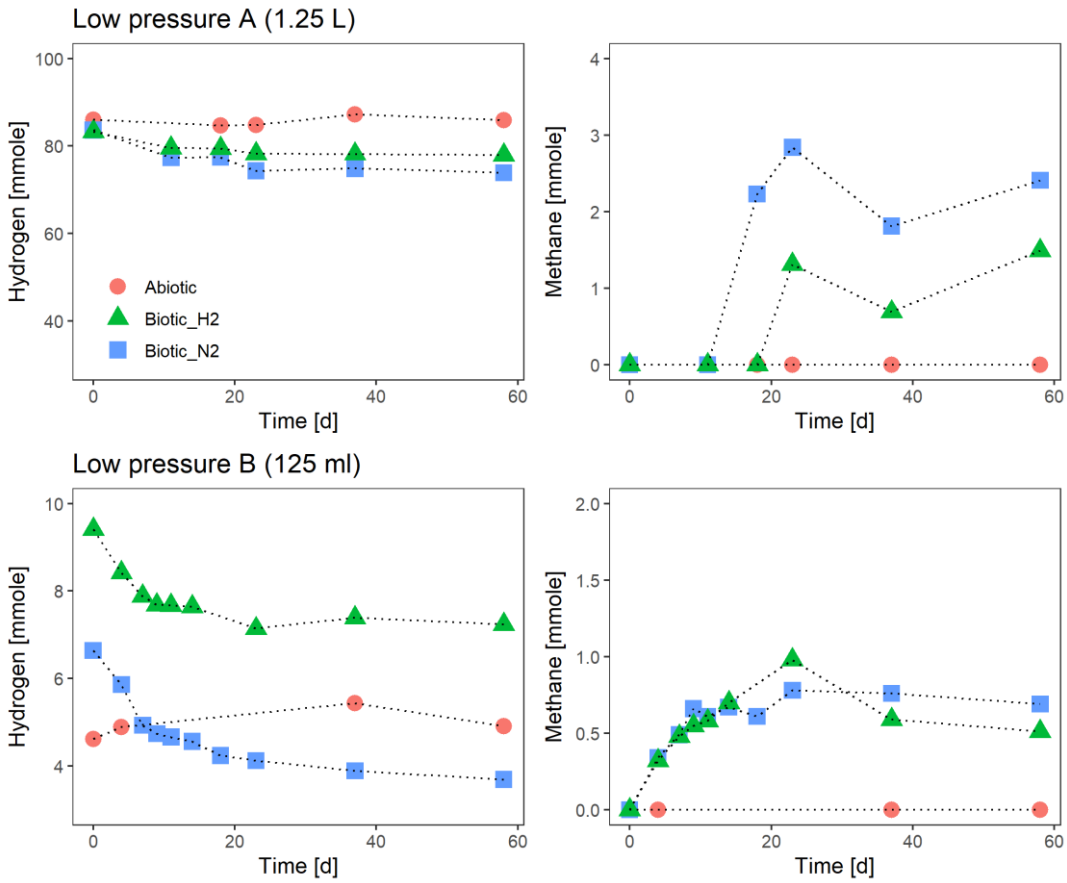

Figure 29: Hydrogen consumption and methane formation due to microbial activities in the presence of pure hydrogen at low pressure (1,500 - 1,900 mbar), temperature 50 °C, salinity 1.5 %.
Biotic_H2: *Biotic assays included assays with 100 % H_2;*
Biotic_N2: *assays with initial 100 % H_2 and addition of N_2 to maintain over pressure*

Table 18: Hydrogen consumption by methanogens dominated culture with carbon source from core materials in the presence of hydrogen at low pressure, temperature 50 °C, salinity 1.5 %

Condition		Maximum H_2 consumption rate (mole/m^3/day)	H_2 consumed after 30 days (%)	Gas composition after 30 days of experiment (H_2: CH_4: N_2)
Low pressure A (1.25 L)	1	1.05	5.0	99: 1: 0
	2	1.82	9.4	96: 4: 0
Low pressure B (125 ml)	1	4.08	18.5	95: 5: 0
	2	4.53	33.4	60: 10: 30

The microbial hydrogen turnover in the biotic tests was accompanied by an increase in the total cell count within the first two weeks of the tests (Figure 30). However, in both experimental series (125 ml and 1.25 l), growth stagnated after about 14 days. Of note is the marked increase in pH within the first two weeks in al inoculated experimental batches in both all low-pressure test A low pressure test B, with pH increasing from about 7.5 at the beginning to 8.7 at the end of the test. In the abiotic tests, the pH remained relatively stable during the test period.

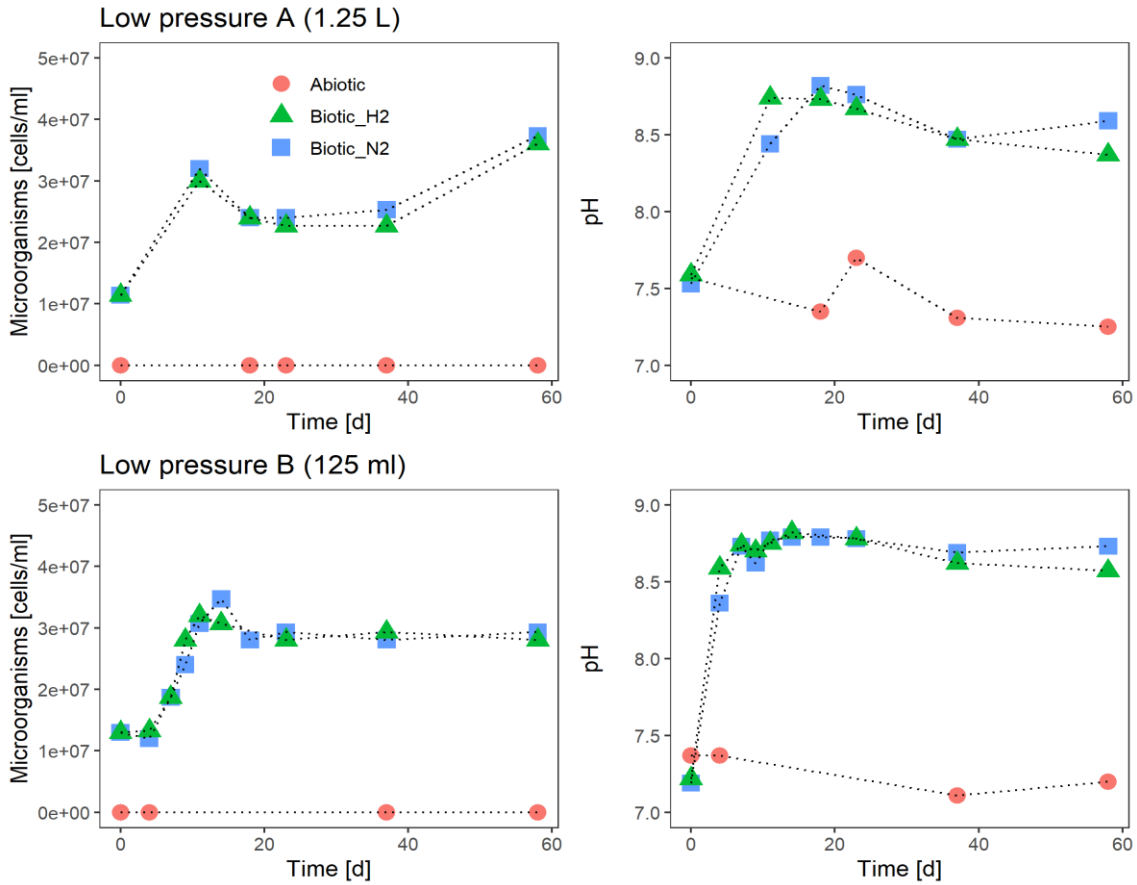

Figure 30: Cell numbers and pH during microbial hydrogen consumption tests, temperature 50°C, and salinity 1.5 %
***Low pressure B:** small volume of gas/liquid; **Low pressure A:** high volume of gas/liquid.*
***Biotic_H2:** Biotic assays included assays with 100 % H*
***Biotic_N2:** Biotic assays with initial 100 % H_2 and addition of N_2 to maintain over pressure*

At the beginning of the experiment, the sulfate concentration in the liquid phase was between 600 and 900 mg/l. In the inoculated 125 ml test batches (Figure 31, low pressure B) a reduction of approximately 300 mg/l sulfate was observed within the first 10 days of the experiment. Thereafter, no further sulfate reduction took place. Also, in the 1.25 L gas consumption tests (low pressure A) the sulfate content dropped significantly within the first 10 - 20 days and then remained constant. In both variants of the abiotic control tests, the sulfate concentration remained constant at around 750 and 600 mg/respectively, over the course of the test.

The acetate concentration in the experiments was initially around 80 – 90 mg/L. While no clear change in the acetate concentration was detectable in the 1.25 L batches (Figure 31, low pressure A), acetate in one of the two 125 ml bottles was completely converted within 20 days. Acetate concentration in abiotic test remained constant throughout the experiment.

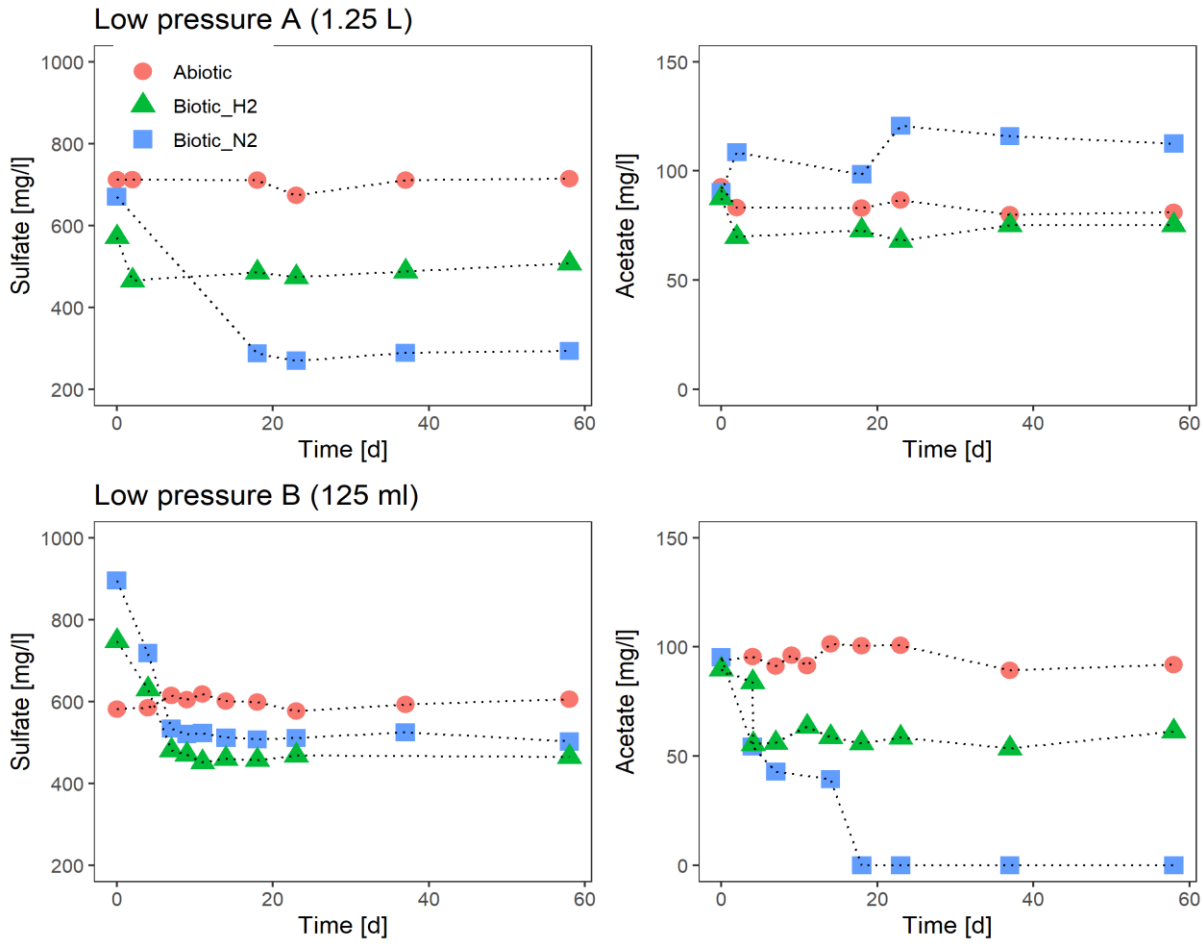

Figure 31: Sulfate and acetate concentrations during hydrogen consumption tests
Low pressure A: *small volume of gas/liquid*
Low pressure B: *larger volume of gas/liquid*
Biotic_H2: *Biotic assays with 100 % H_2*
Biotic_N2: *Biotic assays with initial 100 % H_2 and addition of N_2 to maintain over pressure*

3.5.2. Hydrogen consumption at high pressure

To investigate the influence of increased hydrogen pressure on microbial hydrogen consumption, incubation experiments were carried out at 30 bar with the addition of core material as a carbon source. Pressure and temperature (50 °C) in the high-pressure bioreactor were monitored during the experimental period and are shown in Figure 32. The temporary drop in temperature in the abiotic reactor on day 5 and in both bioreactors on day 47 of the experiment was due to a technical malfunction during operation.

In both high-pressure reactors, a gradual decrease in overpressure can be observed, which is due to technically induced diffusion as well as gas extraction for the analyses. In the reactor inoculated with microorganisms, the formation of methane could be detected by gas chromatography, while only hydrogen was detected in the abiotic control during the test period (Figure 33).

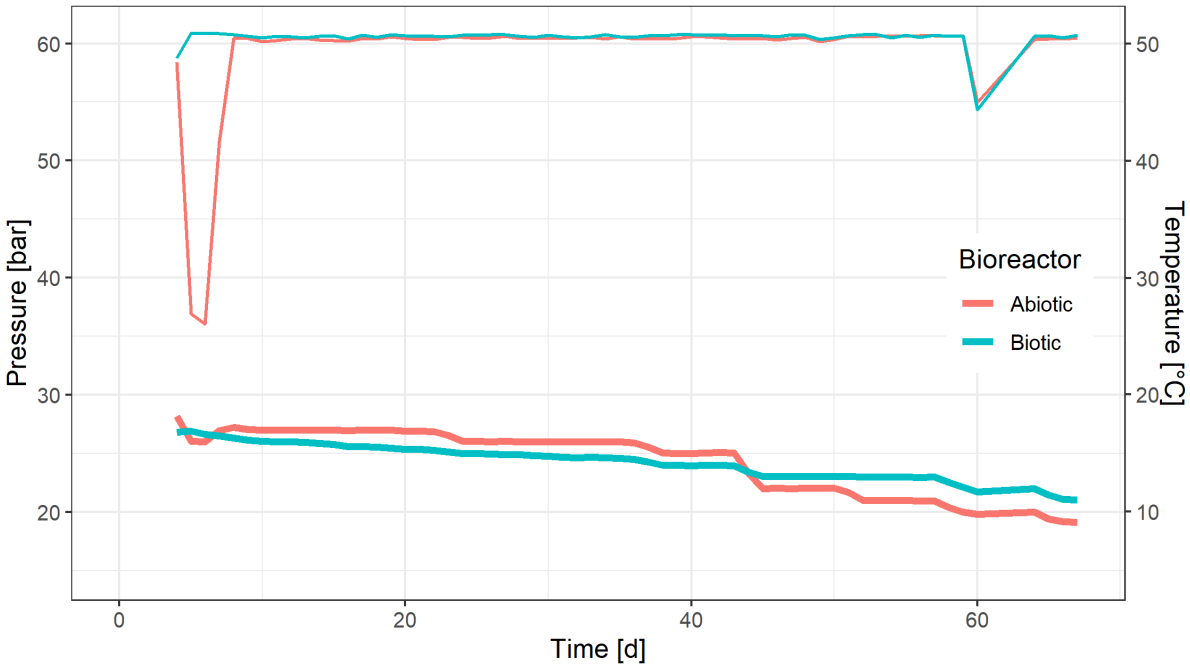

Figure 32: Pressure (thick lines) and temperature (thin lines) monitored during hydrogen consumption test under high pressure condition.

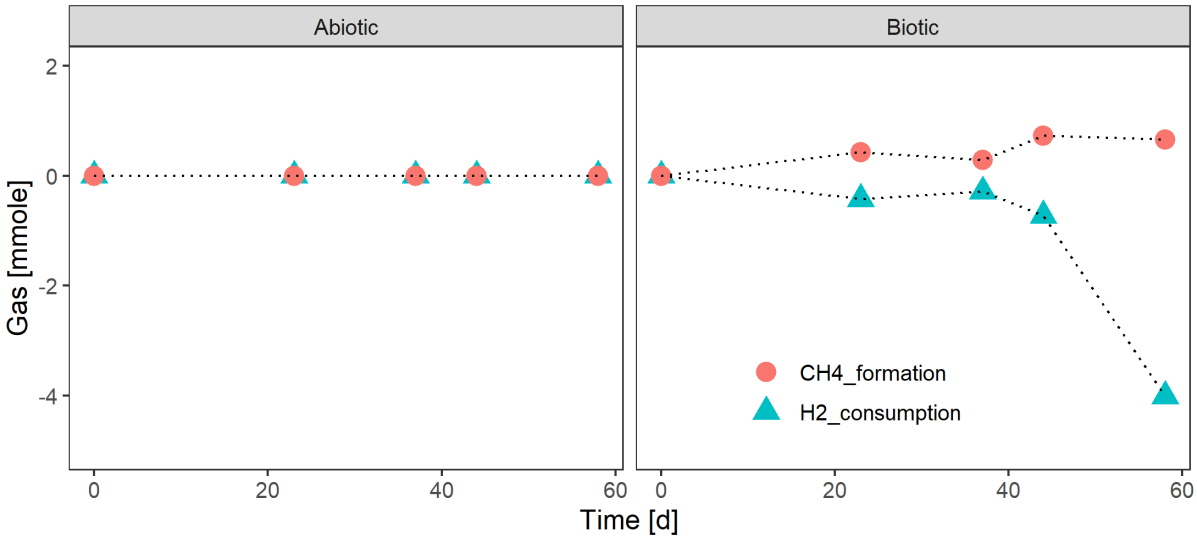

Figure 33: Hydrogen consumption and methane formation due to microbial activities in high hydrogen pressure test (30 bar), temperature 50°C, salinity 1.5 %.

The change in sulfate and acetate concentration, pH and total cell count are shown in Table 19. As shown, sulfate and acetate were present in the liquid phases of both the abiotic and biotic tests. In the abiotic test, the sulfate concentration at the end of the experiment was lower than the concentration measured at the beginning of the experiment. In contrast, the sulfate concentration in the biotic test increased slightly. Furthermore, an increase in pH was observed in both the biotic and abiotic control.

Table 19: Hydrogen consumption test with core materials in the presence of hydrogen at high pressure (30 bar): changes in concentration of sulfate, acetate, pH, and cell numbers.

Reactor	Sulfate (mg/l)		Acetate (mg/l)		pH		Cell numbers (cells/ml)	
	Day 0	Day 70	Day 0	Day 70	Day 0	Day 70	Day 0	Day 70
1 (Abiotic)	859.82	304.60	75.73	83.23	7.48	8.51	n.d	n.d
2 (Biotic)	580.87	644.1	86.22	89.03	7.47	8.39	1.25e+07	1.73e+07

n.d: not detected

3.5.3. Microbial composition

The composition of the hydrogen-consuming microbial groups (acetogens, methanogens, SRA and SRB) at the beginning (inoculum) and at the end of the hydrogen consumption test at high and low pressure was investigated by molecular biological analyses and is shown in Figure 34. For the hydrogen consumption test, the enrichment cultures consisting mainly of methanogens and acetogens were used as inoculum.

Molecular analysis confirmed that methanogens were the dominant hydrogen consuming groups in the inoculum with 3.6 x 10^3 copies/ml, followed by acetogens with only 84 copies/ml. Sulfate-reducing microorganisms were detected at less than 10 copies/ml. At the end of the test, methanogens remained the dominant group in all experimental variants. However, it was found that the microbial counts of the sulfate-reducing microorganisms increased significantly during the test period and exceeded those of the acetogens at the end.
At low pressure and high gas volume, it can be observed that the bacterial content of the methanogens increased at the end of the test, with 6.9 x 10^4 copies/ml and 4.2 x 10^4 copies/ml quantified. The number of sulfate-reducing bacteria changed only insignificantly (> 50 copies/ml).

Similar to the low pressure with high gas volume, methanogens also developed in the low pressure with lower gas volume (low pressure B), with 1.4 x 10^4 copies/ml and 7.2 x 10^4 copies/ml detected in the low-pressure test B with and without N_2 addition during the experiment, respectively. In the low-pressure test B without N_2 addition, an increase in sulfate-reducing bacteria was also observed.

In the high-pressure test, the number of gene copies for methanogens at the end of the experiment was 8.9 x 10^4 copies/ml, which is an increase of more than tenfold compared to the initial methanogens in the inoculum. In this set of experiments, there was also a slight increase in sulfate-reducing prokaryotes (80 gene copies/ml).

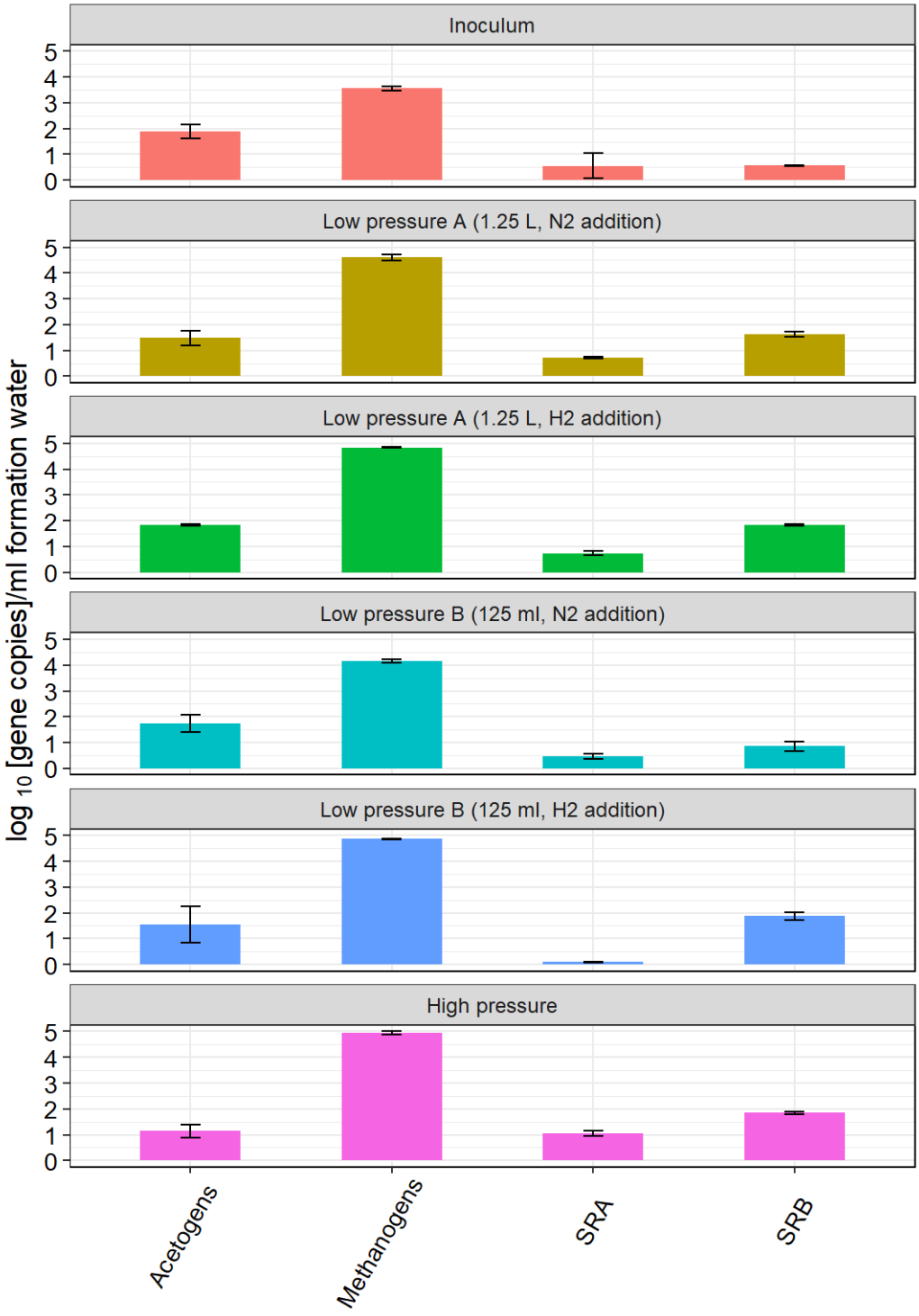

Figure 34: Quantification of hydrogen-consuming microbial groups at beginning (inoculum, same for tests at high pressure and low pressure) and the end of hydrogen consumption tests (low pressure A with large gas volume, low pressure B with low gas volume and high-pressure test). Error bars show standard deviation of duplicate qPCR measurements.

3.5.4. Discussion

The results that for this studied site, methanogenesis was the dominant microbial process consuming hydrogen. The molecular biological analysis showed that methanogens were the dominant group of the inoculum and methanogens also dominated the acetogens and sulfate reducers by orders of magnitude at the end of the experiment.

Under low-pressure conditions, methanogens consumed hydrogen rapidly, regardless the gas volume used for the experiments. The maximum consumption rate remained only within the first 7 days. However, the maximum conversion rate was only reached within the first 7 days in the experiments. Hydrogen is a primary energy source for methanogenesis, which means that its availability can regulate the physiology of the methanogens and energy uptake. As clearly demonstrated in the low-pressure experiments with 125 ml where hydrogen was continuously supplied (Figure 29, low Pressure B), hydrogen was not the limiting factor for microbial activity in the experiments. Moreover, H_2 solubility at a hyperbaric pressure of 50 bar is expected to increase substrate availability to the microorganisms by 25-fold compared to cultivation at 2 bar. Methanogenesis could also take place effectively at a H_2 threshold of only 4 nM (Liu 2012).

Therefore, it can be assumed that during the experimental period H_2 was present in an excess concentration required for methanogenesis. Other factors must be responsible for the decrease in methanogenic activities. Since our experiments were not conducted in continuous systems, the nutrients and carbon source could become limited over time. Hydrogenotrophic methanogenic microorganisms use CO_2/HCO_3^- as a carbon source. In the experiment, the microorganisms obtained their carbon source mainly from the artificial mineral mix. It is possible that the process of methanogenesis is inhibited by a limiting carbon concentration. Studies have shown a decrease in methanogenic activities to 50 % of the maximum when dissolved inorganic carbon was below 59 mM (Garcia-Robledo 2016), and no methane production was detected at less than 44.4 µM dissolved inorganic carbon (Chen 2019). Furthermore, a biological uptake of CO_2/HCO_3^- leads to a decrease in proton H^+, which results in a concomitant increase in pH (Figure 30), with pH values above 8.2 described as inhibiting the methanation process (Luo 2011; Garcia-Robledo 2016; Agneessens 2017; Angelidaki 2018). Therefore, in addition to carbon limitation, high pH value can significantly inhibit the methanogenesis process.

In addition to the methane formation process, heterotrophic sulfate-reducing microorganisms were evidently also present in the low-pressure test B (125 ml experiments), which metabolized acetate for their growth. This would explain the reduction in the acetate concentration over the course of the experiment. However, conversion rates were very low since both sulfate and

acetate were still available after 20 days of the run. A significant sulfate conversion was not detected. Sulfate was therefore not the limiting factor for sulfate-reducing microorganisms in this experiment. The cell content of the sulfate-reducing microorganisms was probably too low to convert sulfate in measurable quantities in the time available.

This case study, which demonstrated the feasibility of methanogenesis process at a temperature of 50 °C and pressure of 50 bar, suggest the likelihood of microbial risks when H_2 is injected into the storage facility and available for active microorganisms. Moreover, in this storage facility, not only methanogens but also other microbial groups were detected (data not shown). Due to the complexity of microbial interaction and the environmental conditions (nutrient availabilities, pH) in the subsurface, more intense microbial activities and secondary microbial process associated with hydrogen could occur, causing severe technical impacts on hydrogen storage system. Therefore, it is highly recommended to identify microbial risks before the injection of hydrogen as well as monitor microbial activities during hydrogen storages to avoid negative impacts of microorganisms on hydrogen underground storage.

3.6. Analysis of storage #6

For the 6th case study on microbial hydrogen turnover, a formation water sample was taken from a reservoir with a salinity of 2.8 % NaCl (w/v), a pH value of 6.54, and a temperature of 40 °C. The formation water was used as an inoculum for the hydrogen consumption experiments.

A culture of methanogenic microorganisms enriched from the formation water served as inoculum for the hydrogen consumption experiments. The other enrichment cultures showed no clear hydrogen consumption in the preliminary experiments and were therefore not used. The temperature for the cultivation of the microorganisms as well as for the hydrogen consumption test was 40 °C. The carbon source was either core material from the horizon of the deposit (40 % w/v), the addition of $NaHCO_3$ (200 mM) or a combination of both carbon sources. Pressure and carbon source for each test are summarized in Table 20

Table 20: Hydrogen consumption tests at low and high-pressure with hydrogen and different carbon sources

Test	Carbon source	Pressure	Volume Gas/Liquid	Liquid phase	Gas phase
Low pressure test (LP1) (Core material and Carbonate addition)	40% (w/v) core, 200 mM $NaHCO_3$	1,500 - 2,000 mbar	65 ml/60 ml	formation water + enrichment cultures	100 % H_2
Low pressure test (LP2) (Core material)	40 % (w/v) core	1,500 - 2,000 mbar	65 ml/60 ml		
High pressure test (HP) (Core material and carbonate addition)	40 % (w/v) core, 200 mM $NaHCO_3$	35 bar	50 ml/200 ml		
High pressure test (HP2) (Core material)	40 % (w/v) core,	35 bar	50 ml/200 ml		
High pressure test (HP3) (Carbonate addition)	200 mM $NaHCO_3$	35 bar	50 ml/200 ml		

3.6.1. Hydrogen consumption at low pressure

In a first step, the microbial hydrogen consumption was investigated at low pressure in two variants with core material and core/carbonate addition (Figure 35). During the 47-day incubation period with 100 % hydrogen in the gas phase, 11.8 - 14.1 mmol of hydrogen were consumed and 1.3 - 3.9 mmol of methane were produced. The hydrogen consumption rate was between 4.6 and 5.7 mmol/m³/day and the methane production rate between 0.8 and 2.1 mmol/m³/day. (Table 21) With a consumption of ~12 mmol hydrogen and a formation of ~3 mmol methane the conversion corresponds to the theoretical H_2 : CH_4 ratio (4 : 1). In the tests with core material, this is valid for one set of tests, while the second set shows a significantly lower rate.

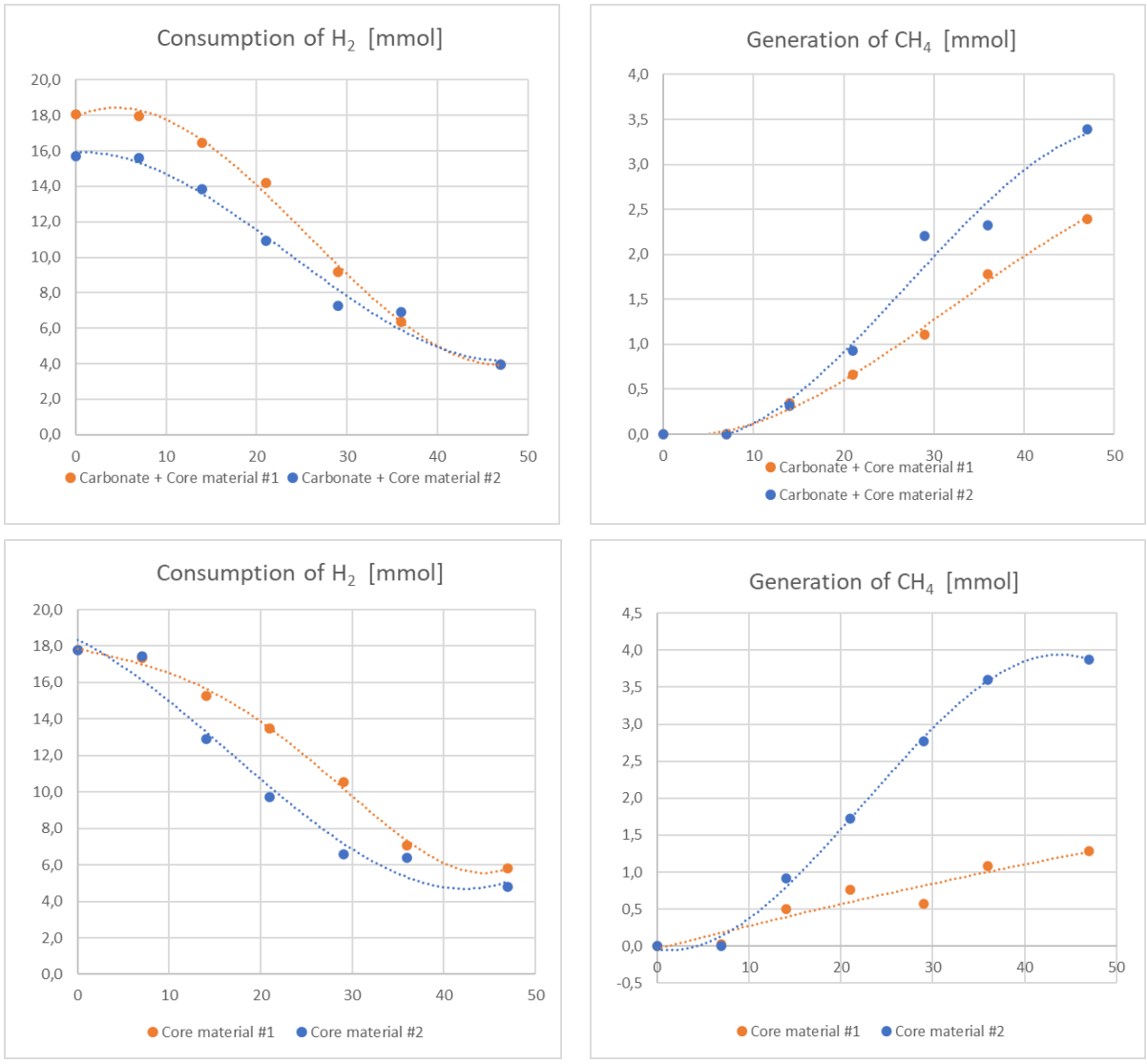

Figure 35: Hydrogen consumption and methane formation with different carbon sources at low hydrogen pressure (2 bar)

Table 21: Consumption rates of low-pressure tests

Test	Max. hydrogen rate *mol/m³/day	Max. methane rate *mol/m³/day
LP1	5.7	0.8
	4.6	1.1
LP2	4.7	0.9
	5.2	2.1

The concentrations of sulfate and acetate, as well as pH and total cell count, are shown in Table 22. In all experimental variants, acetate was detected at the end of the experiment with concentrations between 857 and 929 mg/L. The pH value increased during the test period up

to a pH value of 9.5. The microscopically determined cell count remained almost constant at about 5-6 x 10^7 – 1 x 10^8 cells/mL.

Sulfate was detected at the end of the experiment in all but one batch in the range of 448 to 520 mg/L. In experiment LP2.2, the concentration was significantly lower at 141 mg/L of sulfate.

Table 22: Sulfate and acetate concentration, pH and cell number of low-pressure tests.

Bioreactor	Sulfate (mg/l)		Acetate (mg/l)		pH		Cell numbers (cells/ml)	
	Day 1	Day 47	Day 1	Day 47	Day 1	Day 47	Day 0	Day 47
LP1.1	NA	520,09	NA	928,66	7,0	9.0	1.0e+08	6.0e+07
LP1.2	NA	478,12	NA	928,16	7,0	9.2	1.0e+08	1.0e+08
LP2.1	NA	448,09	NA	928,24	7,0	9.2	1.0e+08	6.0e+07
LP2.2	NA	140.62	NA	857,47	7,0	9.5	1.0e+08	5.0e+07

3.6.2. Hydrogen consumption at high pressure

The real reservoir pressure at the bottom of the borehole is 35 - 78 bar. To investigate the influence of pressure on microbial metabolism in the available test time, high-pressure tests were carried out at 35 bar. Pressure and temperature in the bioreactors were monitored during the test period and are shown in Figure 36.

A decrease in pressure was observed in all three reactors during the experimental period. In the reactor with 200 mM $NaHCO_3$ addition, the pressure remains almost constant after a drop in the first 10 days. In reactors with core and a combination of core and carbonate, the pressure decreased throughout the experimental period. The largest pressure drop during the experimental period was found for the reactor with carbonate and core (35 bar to 13 bar in 47 days).

Hydrogen was consumed and methane was generated simultaneously in all three experimental variants. The maximum hydrogen consumption and methane production rates were observed in the first 14 days of the experiment and amounted to a maximum of 9 mol/m^3 culture and day. The highest methane production rate was 0.5 mol/m^3/day and was observed for reactor 1 with carbonate and core material as carbon sources Table 24.

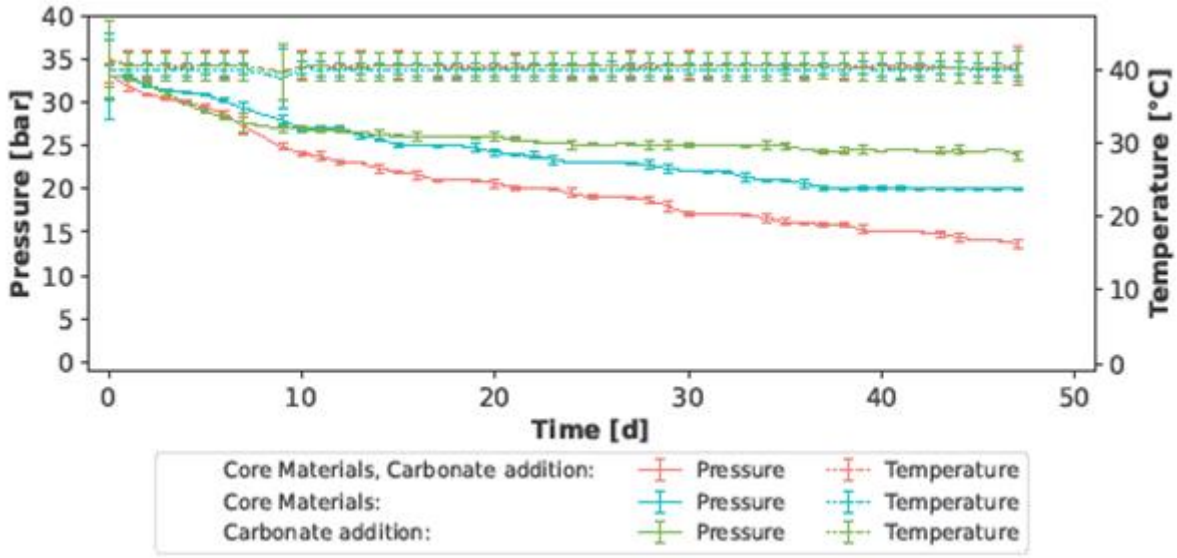

Figure 36: Pressure (thick lines) and temperature (dashed lines) during hydrogen consumption test under high pressure condition with three different carbon sources

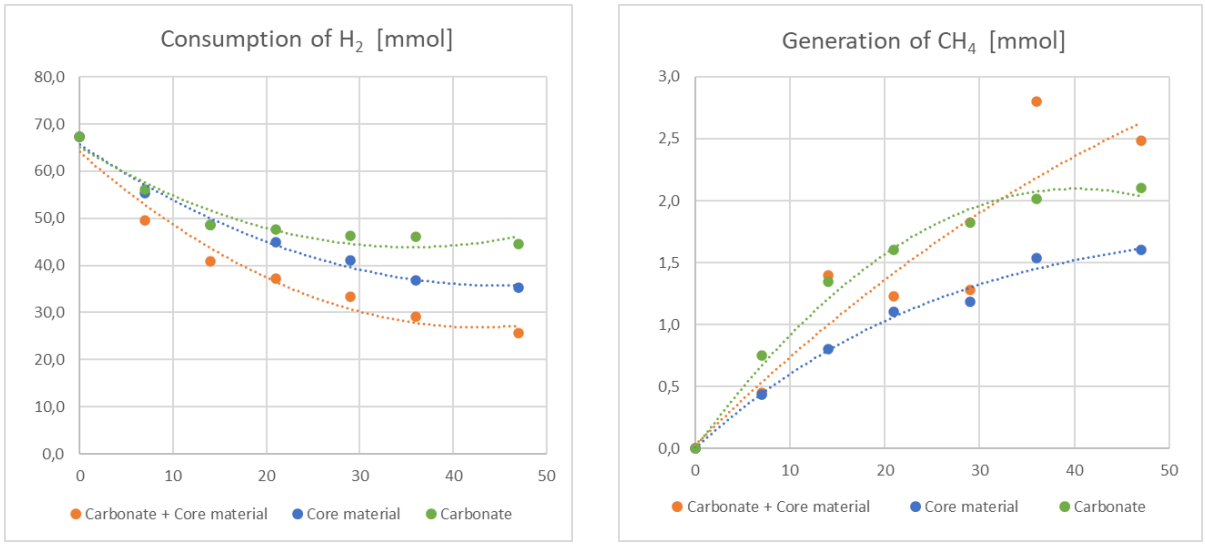

Figure 37: Hydrogen consumption and methane production at high pressure with different carbon sources

Table 23: Maximum hydrogen consumption rates and methane formation rates during low- and high-pressure tests

Test	Max. hydrogen rate *mol/m³/day	Max. methane rate *mol/m³/day
HP1	9.4	0.5
HP2	6.6	0.5
HP3	6.7	0.5

The concentrations of sulfate and acetate, as well as the pH value and total cell count are shown in Table 24. In reactor 1 and 2 (with core material), an increase in cell number and acetate formation was observed during the experiment. In reactor 2, the acetate concentration was higher than in reactor 1 and the cell number was lower. In reactor 3 with carbonate as a carbon source, the acetate production was 5 to 10 times lower than in reactors 1 and 2. Furthermore, the cell number did not increase in this reactor. In all reactors, the pH value increased over time from 7.0 and 7.3 to 8.9 and 9.5.

Table 24: Sulfate and acetate concentration, pH, and cell numbers in hydrogen consumption tests at low and high pressure

Bioreactor	Sulfate (mg/l)		Acetate (mg/l)		pH		Cell numbers (cells/ml)	
	Day 1	Day 47	Day 1	Day 47	Day 1	Day 47	Day 0	Day 47
HP1	NA	509.12	NA	391.61	7.0	8.9	1.0e+08	4.0e+08
HP2	NA	714.54	NA	664.60	7.0	8.9	1.0e+08	2.0e+08
HP3	NA	< 15	NA	62.76	7.3	9.5	7.0e+07	9.0e+07

3.6.3. Microbial composition

The composition of the hydrogen consuming microbial groups (acetogens, methanogens, SRA and SRB) at the beginning (inoculum) and at the end of the high- and low-pressure hydrogen consuming test was investigated using molecular biological methods. The results are shown in Figure 38. The enrichment cultures for methanogens/acetogens were used as the inoculum for the hydrogen consumption tests. The analyses show that methanogens at 5.6 x 10^8 copies/mL were the predominant hydrogen consuming groups in the inoculum, followed by acetogens with 2.1 x 10^6 copies/mL and sulfate-reducing bacteria with 1.2 x 10^6 copies/ml. Sulfate-reducing archaea were the least represented group with 1.0 x 10^4 copies/ml. At the end of the test, methanogens remained the dominant group under all conditions examined.

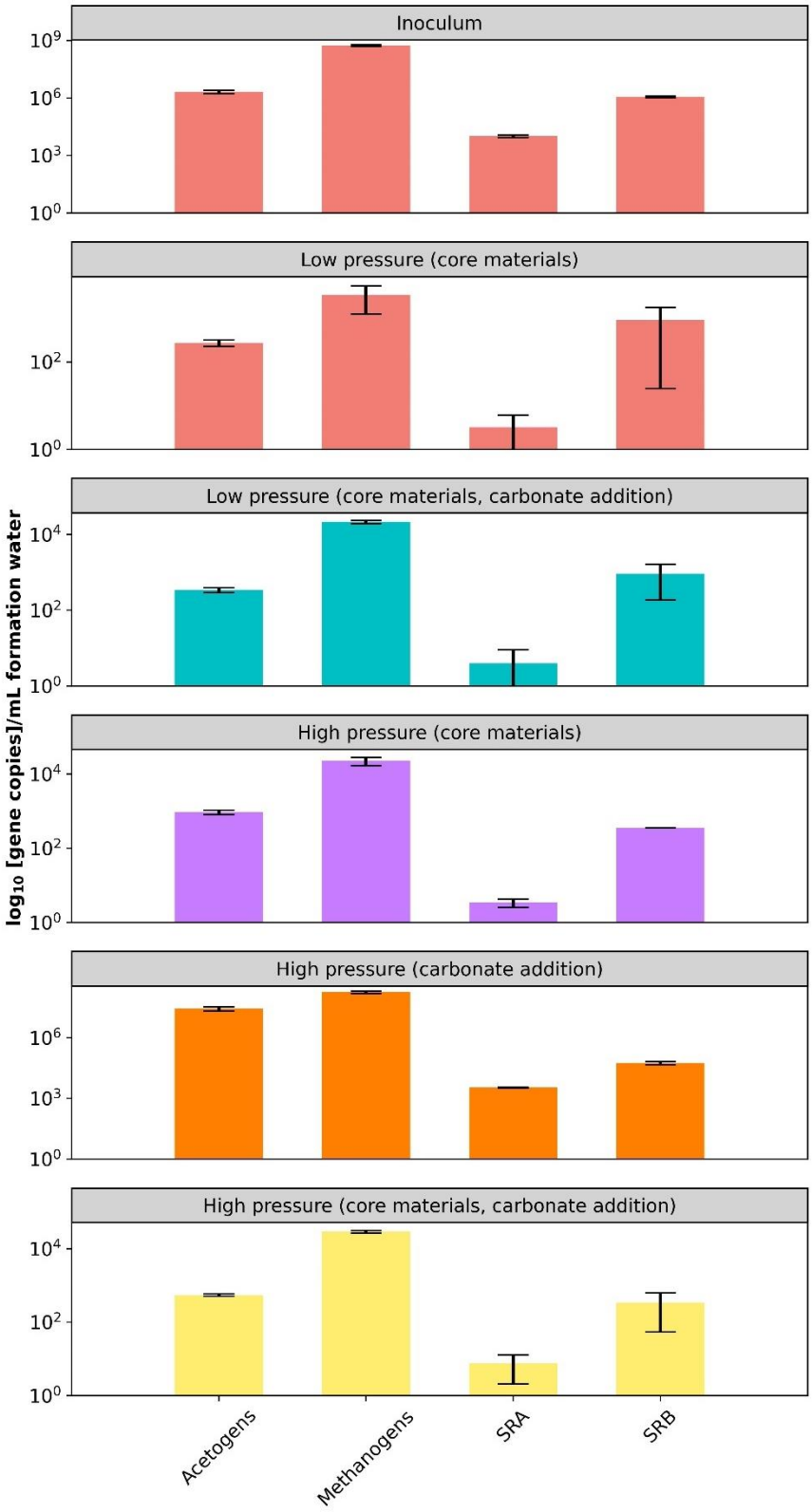

Figure 38: Quantification of hydrogen-consuming microbial groups at beginning and the end of hydrogen consumption tests. Standard deviation of duplicate experiments (low pressure) or single experiments (high pressure) and duplicate qPCR analyses

However, the cell numbers are very different for the individual experiments. In the high-pressure reactors "core materials" and "core material, carbonate addition", the cell count of all microbial groups examined decreased over the course of the experiment. At the end of the test, methanogens with 2.3 x 10^4 copies/ml and acetogens with 5.5 x 10^2 copies/ml were detectable. In contrast, in the high-pressure reactor "carbonate addition", the cell number of methanogens increased to 1.8 x 10^8 copies/ml and that of the acetogens to 2.7 x 10^7 copies/ml. The copy numbers of the sulfate reducers reduced in this experimental approach to 3.4 – 3,6 10^6 copies/ml.

With the same carbon sources at low pressure "core materials" or "core materials, carbonate addition", the cell numbers of methanogens, acetogens and sulfate-reducing archaea are in the same range (methanogens: 4.3 x 10^3 - 2.2 x 10^4, acetogens: 2.7 - 3,4 x 10^2, SRA: 1.3 - 2.2 x 10^0). The gene copies of SRB are higher than those of the high-pressure tests at 1.2 x 10^3 copies/ml.

It is interesting to note that the ratio of the individual groups changes differently depending on the available carbon source. Figure 39 shows the ratios at the start of the test and after the end of the test for the low-pressure tests and high-pressure tests. The strongest change is seen in the low-pressure test with core material, where the number of methanogens is drastically reduced.

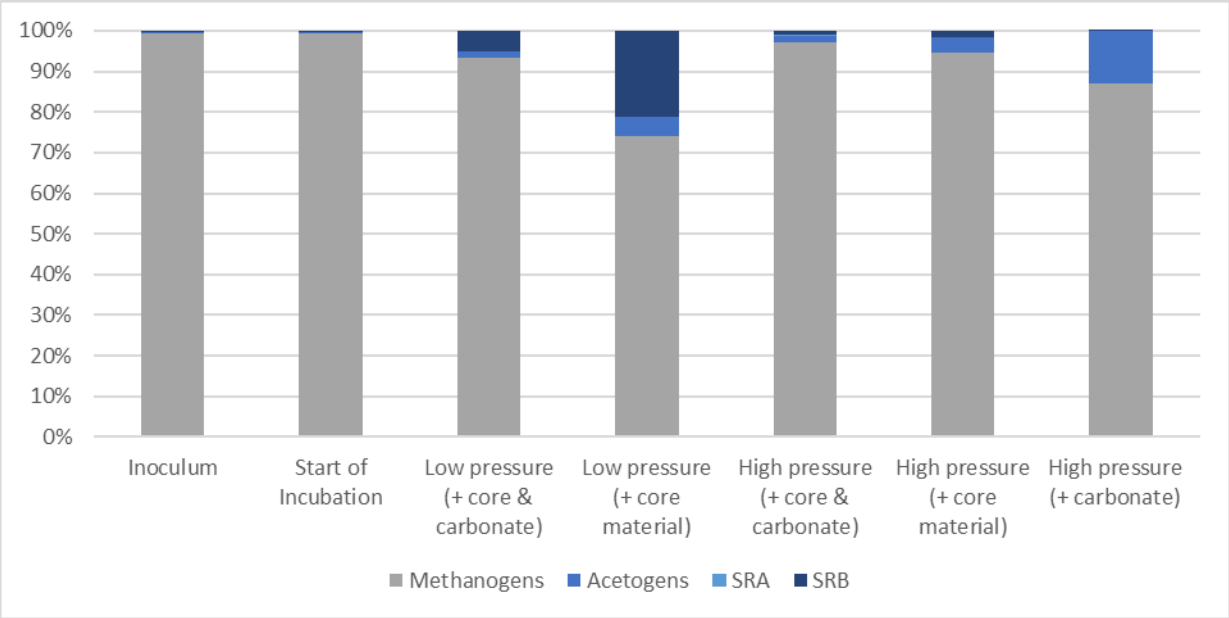

Figure 39: Ratios of the relevant microbial groups before and after the low- and high-pressure tests

3.6.4. Discussion

Under storage-relevant conditions (original formation water and core material), hydrogen consumption with the enrichment cultures from this deposit was observed in the course of the experiment. Biogenic methane production from hydrogen has been demonstrated both at a storage pressure of 35 bar and at only a slight overpressure. Since no other carbon sources were available, it can be assumed that the microorganisms use both the carbonate contained in the core material from the reservoir and artificially added carbonate as a carbon source to produce methane and acetate. The main activity was observed during the first 14 days, after which hydrogen consumption in the bioreactor with carbonate as sole carbon source stopped, while hydrogen consumption continued in the other reactors with core material. Since hydrogen was still available, another factor must have limited hydrogen consumption in this bioreactor. The most probable reason for this is the pH value, which increased drastically from 7.3 to 9.5 as a result of the microbial changes, so that further microbial processes are inhibited. It is known from the literature and from our own experiments that methanogens are inhibited by a high pH above 8.2 (Luo 2011; Garcia-Robledo 2016; Agneessens 2017; Angelidaki 2018).

Although no significant sulfate reduction activities were detected in the precultures, the inoculum included all potentially relevant groups of microorganisms for hydrogen consumption including SRB/SRA (Figure 38). However, the methanogenic microorganisms were the dominant group under all test conditions. Under all conditions except high pressure with carbonate, there was a decrease in cell number in all groups compared to the inoculum. The pure carbonate test is the only heat with no core material from the deposit. Therefore, a connection between the core used and the reduction in cell number could be assumed. In addition, an increase in the pH value to 9.1 was observed in the mixtures of formation water with core material before the start of the test compared to the formation water with a pH of 6.5. This pH effect was corrected by adjusting the pH to pH 7.0 at the beginning of the experiment. However, the unexpectedly sharp rise in pH also indicates that the core could have an impact on the test conditions.

Hydrogen consumption associated with methane production was observed under all conditions. Methane formation rates are nearly stoichiometric in relation to hydrogen consumption, with a ratio of 4:1 expected at atmospheric pressure. During the high-pressure tests, the hydrogen consumption rate dropped to 20-25% of the theoretical rate. It is assumed that in addition to methanogenesis, other metabolic processes also take place under high pressure, so that the stoichiometrically possible rate is not reached.

At the end of all simulation experiments at low and high pressure, acetate was detected in the liquid phase. At low pressure, the concentrations are almost similar at 857.5 to 929 mg/L. In high-pressure tests, the amounts formed were lower overall than in low-pressure tests. The

lowest acetate concentration (63 mg/L) was determined in the test without core material. It is known that many methanogens require acetate for growth (Fardeau 2019). Methanogens within the order Methanosarcinales can grow on CO_2/H_2, acetate or methyl compounds (Kendall and Boone 2006, Thauer 2008). Therefore, it is possible that this approach uses the acetate produced from hydrogenotrophic acetogens as a carbon source when carbonate is no longer available.

According to the experimental results, hydrogen consumption and methane production in situ are possible under these reservoir conditions of 40 °C and 35 bar and a salinity of 2.8 %. In addition, other biological processes cannot be ruled out for this reservoir as molecular biological results showed the presence of sulfate reducers and acetogens before and after low- and high-pressure tests. Active acetogenesis in the reservoir is also demonstrably possible. Since in the experiments under a 100 % hydrogen phase the hydrogen consumption was considerable in the presence of only core material from the reservoir, it can be assumed that the hydrogen fed into the reservoir is also metabolised with a high degree of probability. Hydrogen storage in this reservoir can therefore be classified as risky if no precautions are taken.

3.7. Analysis of storage #7

As part of the Hystories research project, a 7th case study on microbial hydrogen turnover was carried out. For this purpose, a mixture of three formation water samples from a reservoir was prepared in order to have sufficient sample material for the experiments. The mixed sample had a salinity of 4.9 % NaCl (w/v) and a pH of 6.7.

A culture of thiosulfate-reducing microorganisms enriched from the formation water served as inoculum for the hydrogen consumption experiments. This enrichment culture developed extraordinarily slowly and took several months to reach the required minimum microbial content. The other enrichment cultures showed no discernible growth in the preliminary experiments and could therefore not be used to determine the conversion rates. The temperature for the hydrogen consumption test was 45 °C. A mix of five different core material samples from the horizon of the deposit at 1,416 m served as carbon source. The tests were performed with 40 % w/v core material, or the artificial carbon source $NaHCO_3$ (200 mM) or a combination of both.

3.7.1. Hydrogen consumption at low pressure

The microbial hydrogen turnover was investigated at low pressure (1,500 - 2,000 mbar) in duplicates with two variants: with carbonate addition (Biotic + $NaHCO_3$ _1 + _2) or with core material (Biotic + Core _3 + _4) in comparison to uninoculated blind tests (Abiotic + $NaHCO_3$ _5 + _6). Pressure and carbon source for the tests are summarized in Table 25.

Table 25: Hydrogen consumption tests at low pressure with hydrogen and different carbon sources

Test	Carbon source	Pressure [mbar]	Volume Gas/Liquid	Liquid phase	Gas phase
Biotic_1 +$NaHCO_3$	200 mM $NaHCO_3$	1,500 - 2,000	65 ml/60 ml	formation water + enrichment cultures	100 % H_2
Biotic_2 + $NaHCO_3$	200 mM $NaHCO_3$	1,500 - 2,000	65 ml/60 ml		
Biotic_3 + Core	24 g core 40 % (w/v)	1,500 - 2,000	65 ml/60 ml		
Biotic_4 + Core	24 g core 40 % (w/v)	1,500 - 2,000	65 ml/60 ml		
Abiotic_5 + $NaHCO_3$	200 mM $NaHCO_3$	1,500 - 2,000	65 ml/60 ml	Formation water	
Abiotic_6 + $NaHCO_3$	200 mM $NaHCO_3$	1,500 - 2,000	65 ml/60 ml		

The hydrogen conversion in the experimental approaches was generally very low. During the 51-day incubation period with 100 % hydrogen in the gas phase, the carbonate batches consumed on average about 3,4 mmol of hydrogen and produced about 1.4 mmol of methane. The conversion rate of 2.4 corresponds to only part of the theoretical ratio for hydrogenotrophic methanation of 4 mol H_2 : 1 mol CH_4. In the case of the core material variants, the Core_3 trial with 1.6 is still far below this conversion ratio. In the parallel Core_4 experiment, however, a relatively large amount of methane was formed. However, this did not occur with hydrogen. The hydrogen consumption rate was between 113 and 451 mmol/m³/day and the methane production rate was between 360 and 425 mmol/m³/day (Table 26), whereby Core_4 experiment is not taken into account.

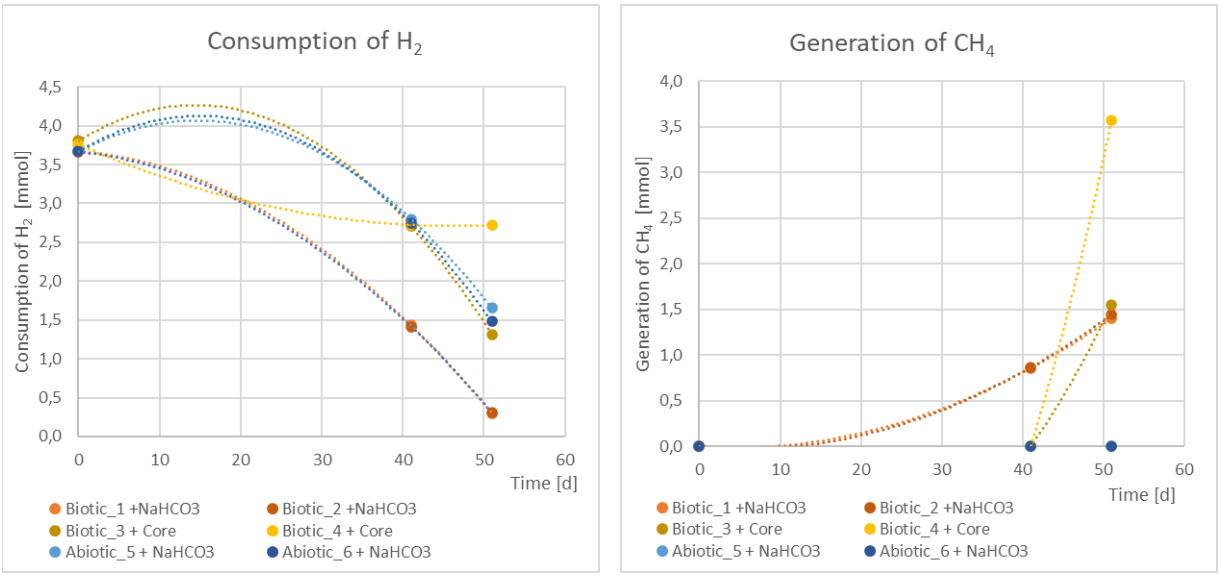

Figure 40: Hydrogen consumption and methane generation with different carbon sources at low hydrogen pressure (2 bar)

Table 26: Consumption rates of low-pressure tests (normalised to abiotic test _5 and _6).

Test	Max. hydrogen consumption rate *mol/m³/day	Max. methane generation rate *mol/m³/day
Biotic_1 +$NaHCO_3$	451.3	425.8
Biotic_2 + $NaHCO_3$	448.1	436.8
Biotic_3 + Core	112.9	359.2
Biotic_4 + Core	-174.2	827.0

The changes in the concentrations of sulfate and acetate as well as the pH value and the total cell count are shown in Table 27. The acetate concentrations remain relatively constant except

for the approach with carbonate (day 51: 2.5 mg/L). Obviously, the core material contains sulfate, as the concentration in these approaches is almost three times higher than in the approaches with carbonate. The pH values increase slightly to pH 8.5 during the experimental period. The microscopically determined cell count decreased from 3.1×10^8 to $2.4 - 5.7 \times 10^7$ cells/mL.

Table 27: Sulfate and acetate concentration, pH and cell number of low-pressure tests.

Bioreactor	Sulfate (mg/l)		Acetate (mg/l)		pH		Cell numbers (cells/ml)	
	Day 1	Day 51	Day 1	Day 51	Day 1	Day 51	Day 1	Day 51
Biotic_1 +$NaHCO_3$	40.94	48.80	131.43	2.47	7.77	8.55	3.1e+08	2.4e+07
Biotic_2 + $NaHCO_3$	40.37	35.73	136.05	122.99	7.66	8.51	3.1e+08	2.7e+07
Biotic_3 + Core	106.81	75.38	158.36	177.43	7.33	7.79	3.1×10^8	5.7×10^7
Biotic_4 + Core	115.20	98.91	155.2	159.06	7.32	7.82	3.1×10^8	3.5×10^7
Abiotic_5 + $NaHCO_3$	NA	NA	50.82	60.56	7.94	8.03	NA	NA
Abiotic_6 + $NaHCO_3$	NA	NA	59.07	59.04	7.80	8.02	NA	NA

3.7.2. Hydrogen consumption at high pressure

The reservoir featured a pressure between 38 and 68 bar. In order to be able to register minimal changes in the time available, the high-pressure consumption tests were carried out in autoclave units at 35 bar and 45 °C. Pressure and temperature in the bioreactors were kept constant during the test period. The tests were carried out in three variants: Inoculated + core material, Inoculated with $NaHCO_3$ and Abiotic with $NaHCO_3$. (Table 28)

Table 28: Hydrogen consumption tests at high pressure with hydrogen and different carbon sources

Test	Carbon source	Pressure [bar]	Volume Gas/Liquid	Liquid phase	Gas phase
HP_1_core material	64 g core 40 % (w/v)	35	76 ml/174 ml	formation water + enrichment cultures	100 % H_2
HP_2_$NaHCO_3$	200 mM $NaHCO_3$	35	110 ml/140 ml		
HP_3_Abiotic + $NaHCO_3$	200 mM $NaHCO_3$	35	90 ml/160 ml	formation water	

For technical reasons, a pressure drop was observed in all three reactors during the test period. Compared to the abiotic test batch, the pressure drop in the core material batch was lower than in the carbonate addition batch.

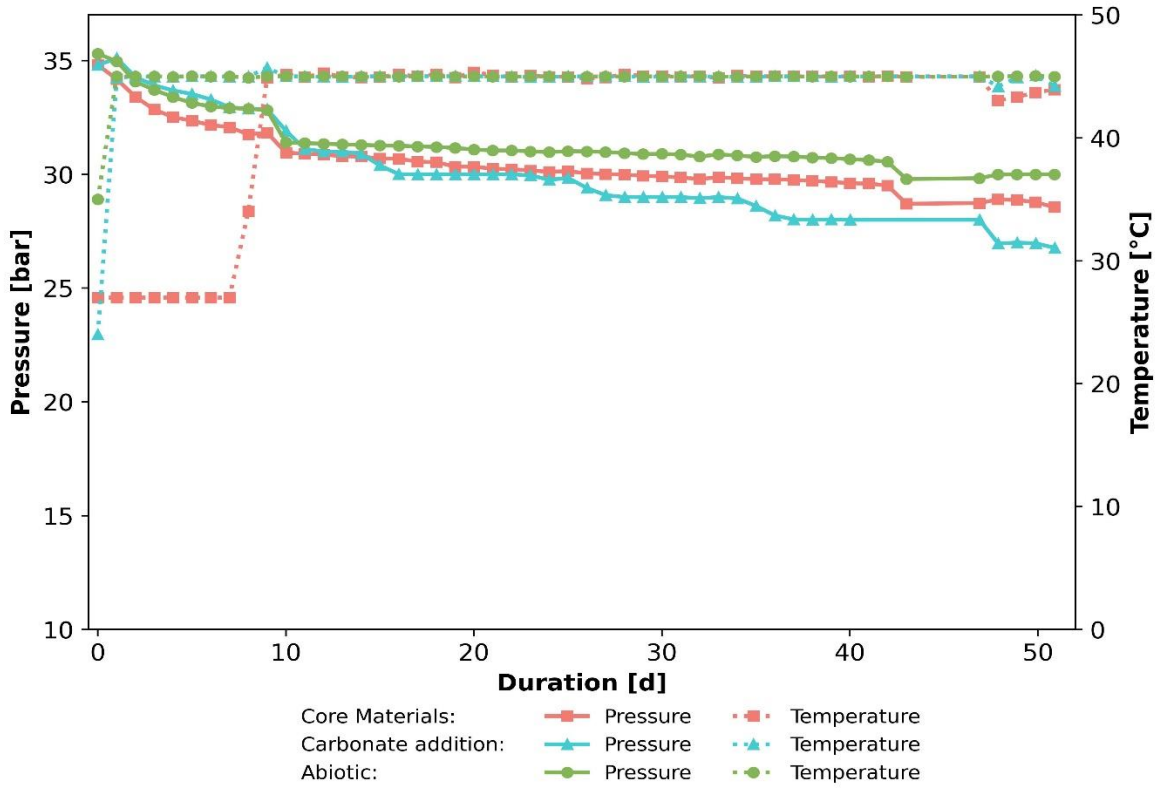

Figure 41: Pressure (thick lines) and temperature (dashed lines) during hydrogen consumption test under high pressure condition with three different carbon sources

The small differences in metabolism are also shown by the hydrogen consumption and methane formation (Figure 42). Normalised with the abiotic control, the hydrogen consumption was 34.98 mmol in the carbonate experiment and 15.71 mmol in the approach with core material. 13.93 mmol were formed in the carbonate experiment and 12.5 mmol in the core experiment. Thus, the methane formation was clearly below the theoretical H2:CH4 ratio. As shown in Table 30, a proportionate acetate formation took place in both approaches. The pH values hardly changed in the core approach. In the carbonate approach, the pH value increased to 8.3, similar to the abiotic control approach.

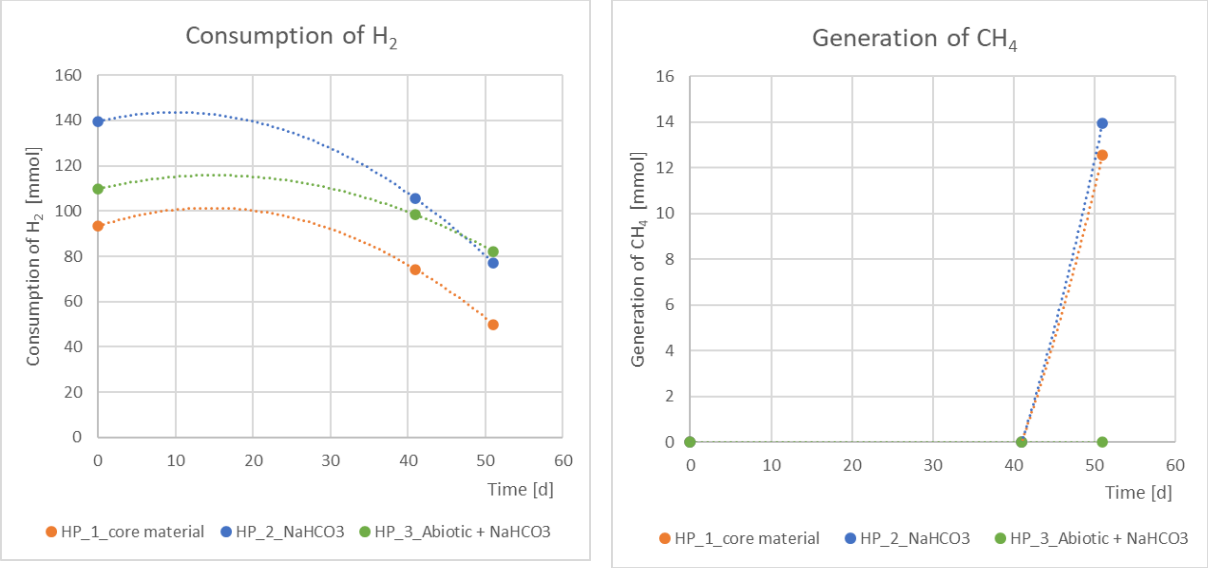

Figure 42: Hydrogen consumption and methane generation at high pressure with different carbon sources

Table 29: Maximum hydrogen consumption rates and methane formation rates during high-pressure tests (normalised to abiotic test)

Test	Max. hydrogen rate *mol/m³/day	Max. methane rate *mol/m³/day
HP_1_core material	4,528.9	2,909.1
HP_2_$NaHCO_3$	10,781.7	3,231.1

Table 30: Sulfate and acetate concentration, pH and cell number of high-pressure tests

Bioreactor	Sulfate (mg/l)		Acetate (mg/l)		pH		Cell numbers (cells/ml)	
	Day 1	Day 51	Day 1	Day 51	Day 1	Day 51	Day 1	Day 51
HP_1 core material	NA	NA	58.86	294.13	7.29	7.34	2.7e+08	5.9e+07
HP_2 $NaHCO_3$	NA	44.69	60.66	153.25	7.22	8.34	2.7e+08	4.5e+07
HP_3 Abiotic+ $NaHCO_3$	NA	NA	52.72	49.78	7.23	8.17	NA	NA

3.7.3. Microbial composition

The microbial composition of the fluid samples was analysed before and at the end of the high- and low-pressure test with respect to hydrogen consuming microbial groups (acetogens, methanogens, SRA and SRB). The results are shown in Figure XXX. The corresponding enrichment culture for methanogens/acetogens from the deposit was used as inoculum for the hydrogen consumption tests. The analyses show that the inoculum was mainly formed by

methanogens (5 x 10^5) and sulfate-reducing bacteria (3 x 10^5). Acetogens (3 x 10^3) and sulfate-reducing archaea (4 x 10^2) were also represented. Analysis at the end of the low-pressure test showed an almost unchanged result for the carbonate approach. In the core material test, significantly fewer cells were detected, although no microscopic differences in microbial content were found. There was little change in the ratio of microbial groups compared to the inoculum. Methanogenic and sulphate-reducing bacteria remained dominant. The analysis after completion of the high-pressure test showed virtually no changes in the microbial content and in the numerical ratio of the microorganism groups examined.

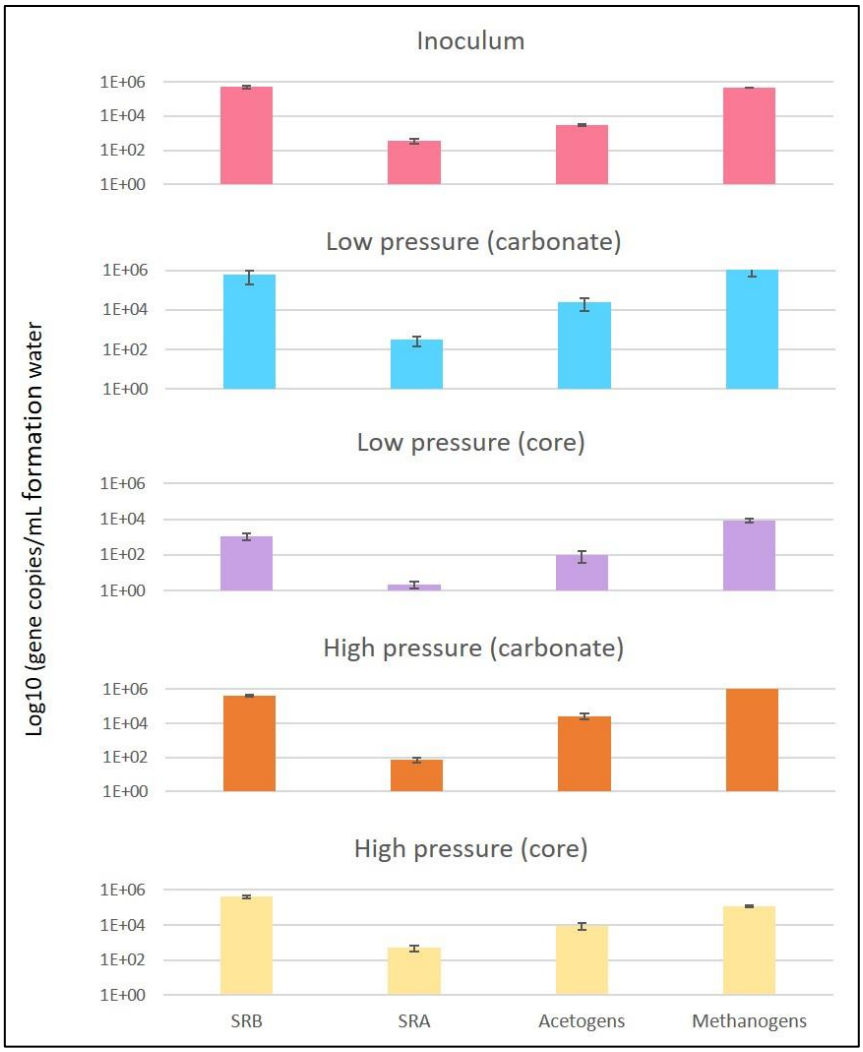

Figure 43: Quantification of hydrogen-consuming microbial groups at beginning (inoculum) and the end of hydrogen consumption tests under low pressure and high pressure and addition of care material or carbonate as a carbon source.

3.7.4. Discussion

Under storage-relevant conditions and with the original formation water sample, hydrogen turnover was investigated at 2,000 mbar and 35 bar, respectively with original core material and with artificial $NaHCO_3$. Significant microbial hydrogen turnover was observed in all tested variants, partly accompanied by methanogenesis. However, there are also clear indications of other parallel processes such as sulfate reduction (e.g. Biotic_3 + core), acetate turnover (Biotic_1 + $NaHCO_3$) or acetogenesis (HP_1_core material and HP_2_$NaHCO_3$). The maximum hydrogen turnover rates are accordingly very different and range between 113 and 10,782 mol/m³/day.

The two experimental variants with core material and artificial carbonate ($NaHCO_3$) show that carbonates in different forms can be used as carbon sources by the microorganisms present in the reservoir. In some cases, slightly higher conversion rates can be shown for the carbonate variant, which is probably due to better solubility and thus easier bioavailability. For the deposit, this means that the carbonate components present in the rock matrix are not expected to limit microbial hydrogen consumption. In addition, the sulfate analyses of the core approaches (Table 30) show that the rock samples contain some soluble sulfate components, which means that sulfidogenic processes in the deposit cannot be excluded.

In all test series, the initial microbial content of 3×10^8 decreased by about one order of magnitude. Molecular biological analyses show a lower cell content both at the beginning and at the end of the experiments of a maximum of 10^6 cells per ml. Except for the test with core material, the cell contents also remained approximately at this level. There was no proliferation of microorganisms during the 51-day test period. The pH values increased in all test series, including the abiotic controls, from approximately 7.3 - 7.8 to 7.8 - 8.5.

Although the hydrogen turnover was relatively low in the available experimental time, the metabolic processes taking place can be clearly identified. The microbially induced hydrogen consumption with accompanying methanogenesis, as well as partial acetogenesis and sulfate reduction, could be clearly demonstrated under the selected experimental conditions and with the microorganisms enriched from the deposit. For a detailed prediction of the conversion rates, further experiments with longer cultivation times and subsequent hydrogen dosing are required.

4. Discussion and conclusion

The extensive simulation tests carried out within the framework of this project with representative depth samples from 7 different reservoirs clearly demonstrate that under real storage conditions, microbial utilisation of hydrogen as an energy source for anaerobic microorganisms can indeed take place. Requirements for a corresponding microbial activity are conditions that basically allow microbial growth as well as the presence of a carbon source in the form of carbon dioxide or carbonates. The essential parameters that influence the microbial activity of hydrogen-utilising microorganisms are among others temperature, salinity, pH-value, mineral composition of the water (carbonate and sulfate source) as well as organic and toxic compounds. Of course, the degree of colonisation and the composition of the microbial community are decisive for the extent and direction of metabolic processes. Environmental conditions and microbial risks are discussed in detail in Deliverables D3.4 and D3.5 of the Hystories project.

The simulation tests were carried out with enrichments from the original downhole samples of the reservoirs in order to achieve measurable hydrogen conversions in the available test time. Due to the unfavourable gas : liquid ratio, this applies in particular to the high-pressure tests, where low substance conversions are otherwise not measurable. In this respect, the determined conversion rates represent rather a worst-case scenario, which can only be expected under real conditions with a correspondingly high cell content and optimal conditions.

Since the hydrogen turnover rates strongly depend on the respective conditions and the composition of the microbial population, it is almost impossible to make generalised statements about this. In addition, microbial populations in geological structures - as in nature in general - do not occur as pure cultures, and very likely different metabolic pathways run simultaneously. Nevertheless, in our experiments we determined maximum turnover rates of up to 500 mM/h for the enriched cultures of sulfate-reducing microorganisms and up to 27 mM/h for methanogens. Another aspect that must be taken into account when interpreting the results is the ratio between liquid volume and rock matrix, which was used in the experiments for technical reasons. In a porous reservoir with an average porosity of 20 %, this ratio is the other way round. (Sedlacek 1999)

Nevertheless, a biogenic conversion of hydrogen was detected both at low overpressure and at real storage pressures. Depending on the composition of the inoculum and the respective experimental conditions (e.g. pH value, availability of SO_4^{2-}), different microbial processes (methanogenesis, acetogenesis, sulfate reduction) dominated. Through the experiments it became clear that the microorganisms can use both the carbonate contained in the core

material of the reservoir and artificially supplied carbonate as a carbon source to produce methane or acetate (Figure 35, Figure 37). Often, the main activity was observed only during the first 14 days of the experiment. In some of these experiments, a very significant increase in pH was observed, which apparently rose up to pH 9.5 due to microbial activities, so that further microbial processes are inhibited as a result. It is known from the literature and from own experiments that the pH value can rise sharply as a result of hydrogen utilisation by sulfate-reducing microorganisms, leading to an inhibition of further microbial processes. (Liamleam 2007; Dopffel 2023) The extent to which this effect occurs under real conditions in a porous reservoir must be clarified by further investigations.

In summary, it can be concluded that through the simulation tests with enriched reservoir samples and under practical conditions, it has been shown that microbial hydrogen depletion is highly likely to occur in some reservoirs and that there is thus a real risk for certain reservoir configurations. A precise prediction about the extent and direction of these processes cannot be made in general, as this depends on numerous factors. Case-specific investigations are required for a corresponding risk analysis.

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