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HyUSPRe

Hydrogen **U**nderground **S**torage in **P**orous **Re**servoirs

Review of the window of viability of the different microbial metabolisms relevant for subsurface H_2 storage application

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Disclaimer

Executive summary

The transition to large-scale application of renewable energies (e.g., electricity from wind and solar) requires storing energy in versatile energy carrier molecules, such as hydrogen (H_2). These energy carriers must be stored to buffer fluctuations in electricity production and consumption. Underground H_2 storage, e.g., in depleted natural gas or oil reservoirs, aquifers, and salt caverns, is a potentially more economical and feasible alternative to overground storage installations. However, existing gas storage facilities are designed to harbor natural gas and require adaptation for H_2 storage. One of the risks to consider during H_2 underground storage is that subsurface microorganisms consume the H_2 stored. Apart from the H_2 loss, there are other possible side effects that microbes can cause, such as the contamination of the stored H_2 with, e.g., sulfides and methane, as well as the reduction in H_2 injectivity due to the clogging of the wellbore by the accumulation of bio-based solids (microbial biomass, extracellular polymeric substances (EPS), metal sulfides, etc.). Underground environments have rather inhospitable conditions: limited nutrient availability, high pressure, high temperature, high salinity, etc. Nevertheless, microbial life has been detected under these conditions.

H_2 is an excellent electron donor for several types of microorganisms, including sulfate reducers (producing hydrogen sulfide), methanogens (producing methane), and acetogens (producing acetate). In environments with low partial hydrogen pressure, microbial activity is limited by H_2 due to its low solubility. However, H_2 solubility increases under increasing H_2 partial pressure conditions, becoming more available for microorganisms. Although vast literature is available on the viability of microbial life in underground reservoirs, specific information on H_2-consuming microbial communities in these environments is limited. Also, underground H_2 storage is an emerging practice, with few (pilot) trials over the world, and so far, it is not known how H_2-consuming microorganisms in reservoirs will react to the presence of high H_2 pressures. Finally, it must be noted that microorganisms need a source of carbon as well (e.g., CO_2), so microbial communities in H_2 reservoirs might be limited by carbon availability.

This report provides a literature review of the viability window of the possible H_2-consuming microbial metabolisms encountered under subsurface H_2 storage conditions. Also, we provide preliminary results of incubations with environmental subsurface samples with 80% H_2 and 20% CO_2 and with 100% H_2 at 1.7 bar total pressure, confirming the production of H_2S, CH_4, and acetate at temperatures ranging from 35 to 80 °C.

About HyUSPRe

Hydrogen **U**nderground **S**torage in **P**orous **Re**servoirs

The HyUSPRe project researches the feasibility and potential of implementing large-scale underground geological storage of renewable hydrogen Europe. This includes the identification of suitable porous reservoirs for hydrogen storage, and technical and economic assessments of the feasibility of implementing large-scale storage in these reservoirs to support the European energy transition to net zero emissions by 2050. The project will address specific technical issues and risks regarding storage in porous reservoirs and conduct an economic analysis to facilitate the decision-making process regarding the development of a portfolio of potential field pilots. A techno-economic assessment, accompanied by environmental, social and regulatory perspectives on implementation will allow for the development of a roadmap for widespread hydrogen storage by 2050; indicating the role of large-scale hydrogen storage in achieving a zero-emissions energy system in the EU by 2050.

This project has two specific objectives. Objective 1 concerns the assessment of the technical feasibility, associated risks, and the potential of large-scale underground hydrogen storage in porous reservoirs for Europe. HyUSPRe will establish the important geochemical, microbiological, flow and transport processes in porous reservoirs in the presence of hydrogen via a combination of laboratory-scale experiments and integrated modelling, and establish more accurate cost estimates to identify the potential business case for hydrogen storage in porous reservoirs. Suitable storage sites will be identified and their hydrogen storage potential will be assessed. Objective 2 concerns the development of a roadmap for the deployment of geological hydrogen storage up to 2050. The proximity of storage sites to large renewable energy infrastructure and the amount of renewable energy that can be buffered versus time varying demands will be evaluated. This will form a basis for developing future scenario roadmaps and preparing for demonstrations.

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1 Introduction

'Everything is everywhere, but the environment selects', was stated by the microbiologist Baas Becking (1934). Subsurface porous reservoirs are not an exception and harbor diverse microbial communities able to live under the extreme conditions in these environments (Gniese et al., 2014). Some of the microorganisms in these environments could have a detrimental impact on hydrogen (H_2) underground storage, with the main potentially impacting microbial processes being sulfate (SO_4^{2-}) reduction, methanogenesis and acetogenesis. Their negative impact is linked to the fact that by using H_2 as their energy source, they cause a loss of the injected H_2. Moreover, hydrogen sulfide (H_2S) is generated in the case of sulfate-reducing activity, causing contamination of the H_2 and corrosion of the storage facilities. Microbial activity can also lead to the accumulation of bio-based solids, including microbial biomass, extracellular polymeric substances (EPS), and FeS, in the storage facilities causing well bore clogging and loss of H_2 injectivity. In WP3 of HyUSPRe we aim to assess risks caused by H_2-driven microbial activities, to predict their impact on subsurface H_2 storage conditions in a precise manner.

H_2-utilizing sulfate reducers, methanogens and acetogens have each their specific traits, with certain conditions inhibiting or stimulating their activity. The determination of the "windows of viability" for the different microbial metabolisms will allow us to assess the potential risks from microbial activities for subsurface H_2 storage at a specific site. Recently, this topic has also been broadly covered by the review written by Thaysen et al. (2021), which shall provide the reader with an in-depth overview of this subject. The current report describes the subsurface environment, the main microbial metabolisms impacting subsurface H_2 storage, the influence of the environmental condition on them, the results of the experimental work performed on environmental samples, and finalizes with the future perspectives in this field.

2 Description of the subsurface environment from a microbiological point of view

One could think about the deep subsurface as an extreme environment where life needs to adapt to harsh conditions to persist. Microorganisms in the deep subsurface face extreme temperatures and pressures, salinity levels up to saturation, limited availability of nutrients and energy sources, low water activity (Escudero et al., 2018), and limited pore sizes to pass through or to provide space for colonization(Sharma and McInerney, 1994; Fredrickson et al., 1997). The larger the pore system is, the better it provides space for the microorganisms to colonize it and for nutrient and water accessibility (Sharma and McInerney, 1994; Fredrickson et al., 1997). Despite the extreme conditions, microorganisms were detected at high depth (Zhang et al., 2006; Teske et al., 2013; Soares et al., 2019). The deep biosphere is estimated to account for up to 2–19% of the Earth's total biomass (McMahon and Parnell, 2014). Depending on the source, total microbial cell numbers have been found between 8.65×10^4 to 1.01×10^6 per gram of rock (Dutta et al., 2018). However, the number of microorganisms and their diversity decreases with increasing depth and is, in general, dependent on the physical and chemical constraints of the environment (Escudero et al., 2018). It has also been speculated that microorganisms in the subsurface stay mostly in a dormant state (Wörmer et al., 2019), waiting for conditions such as energy supply to become favorable in order to become active again (Morono et al., 2011). In general, the bacterial representatives are more abundant and diverse than the archaeal. Bacteria detected in the subsurface include Proteobacteria, Actinobacteria, Bacteroidetes, and Firmicutes, whereas for the archaeal representatives, methanogens are commonly retrieved (Dutta et al., 2018; Escudero et al., 2018).

3 Microbiology of Underground Gas Storage (UGS) environments

Multiple recent studies describe the microbiome of Underground Gas Storage (UGS) sites from different site locations, including porous reservoirs (Šmigáň et al., 1990; Ivanova et al., 2007; Buriánková et al., 2022), aquifers (Basso et al., 2009; Gulliver et al., 2019; Ranchou-Peyruse et al., 2019, 2021; Kadnikov et al., 2020), and salt caverns (Schwab et al., 2022) filled with different types of gas (either natural gas, town gas, or CO_2). Cell numbers in the formation water varied per type of UGS, reaching up to 2.1×10^6 cells/ml in a porous reservoir (Ivanova et al., 2007), 2.43×10^4 cells/ml in an aquifer (Ranchou-Peyruse et al., 2021), and 2×10^6 to 7×10^6 cells/ml in the brine of salt cavern (Schwab et al., 2022). In porous reservoirs, it was shown that the microbial composition varies between the groundwater and the surrounding rock (Lehman et al., 2004; Momper et al., 2017), and it is estimated that microorganisms attached to rocks outnumber the ones in the water column by a magnitude of a thousand (McMahon and Parnell, 2014). However, studies tend to focus only on the analysis of microorganisms in groundwater, therefore underestimating the total number of microorganisms in the ecosystem (Escudero et al., 2018). To be able to analyze the full risk of the microbial impact in UGS, further microbial community analyses of potential H_2 storage sites should also include rock material.

The analysis of the whole microbiome of the formation water of the deep aquifer system of the Beynes, in the West of Paris (France), revealed a predominance of sulfate reducers (*Deltaproteobacteria* clade Sva0485, *Thermodesulfovibriona* sp., *Desulfotomaculum* sp., *Desulfomonile* sp., and *Desulfovibrio* sp.), fermenters (*Peptococcaceae* sp. SCADC1_2_3, *Anaerolineae* sp. and *Pelotomaculum* sp.) and homoacetogenic bacteria ('*Candidatus* Acetothermia'). Only a few archaea were detected, which included amongst others *Methanomassiliicoccaceae* sp., *Methanobacteriaceae* sp., and members of the *Bathyarcheia* class (Ranchou-Peyruse et al., 2021). For salt caverns, the microbial community was analyzed in five German natural gas storage caverns. In those caverns, archaeal members of the *Halobacteria* and bacterial representatives of the *Halanaerobiales*, the *Balneolales,* and the *Desulfovermiculus* were detected (Schwab et al., 2022).

Unfortunately, even though several studies of UGS with high partial H_2 pressure have been conducted, microbial community analyses were often not included (Panfilov, 2016), and are, to our knowledge, not even existing for sites filled with pure H_2. However, members of the diverse hydrogenotrophic metabolisms are commonly found in analyzed UGS and subsurface environments (Nealson et al., 2005; Escudero et al., 2018), and it has been shown that once gas containing high partial H_2 pressure is injected into the UGS, the microbial community can utilize it (Šmigáň et al., 1990; Buriánková et al., 2022).

4 Microorganisms impacting subsurface H_2 storage

H_2 is an important energy source in anaerobic ecosystems (Claassens et al., 2018), especially in subsurface environments where electron donors are scarce (Jones et al., 2018). The oxidation of H_2 with its low redox potential offers a rich energy source to fuel life under these oligotrophic and unhostile environments. H_2 can occur naturally in a variety of subsurface environments and can originate from both abiotic and biotic processes (Smith et al., 2005). For instance, abiotic processes include serpentinization[1] or cataclasis[2], and biotic mechanisms comprise nitrogen fixation or fermentation, amongst others (Gregory et al., 2019).

Hydrogenotrophic microorganisms can impact subsurface H_2 storage in various ways. They could cause: (i) loss of the stored H_2, and (ii) formation of H_2-contaminating products such as H_2S and CH_4 (Dopffel et al., 2021). The production of H_2S induces microbial-influenced corrosion (MIC) through the formation of FeS-rich deposits, which enhances the consumption of H_2 generated at the metal's surface. Another microbial reaction causing MIC is the direct uptake of electrons from the metal's surface (Enning and Garrelfs, 2014). Through MIC, microorganisms can impact the durability and integrity of the equipment utilized for either storage, transportation, or use of H_2. Another detrimental effect of microbial activity and growth is the loss of H_2 injectivity of the wellbore as a result of the accumulation of bio-based solids as extracellular polymeric substances or FeS in the near-wellbore area of the reservoir (Dopffel et al., 2021).

Hydrogenotrophic microorganisms use H_2 as an electron donor and couple its oxidation to the reduction of various electron acceptors, such as O_2 (aerobic bacteria), NO_3^- (nitrate reducers), Fe^{3+} (iron reducers), SO_4^{2-} (sulfate reducers) or CO_2 (acetogens, methanogens) (Table 1). However, not all microbial metabolisms exert a relevant risk on subsurface H_2 storage. This risk is evaluated based on the availability of the electron acceptors under subsurface storage conditions, as well as on the ranking of the metabolic process depending on their needed H_2 threshold concentration and their standard Gibbs free energy yield ($\Delta G^{0'}$) (Table 1) (Thaysen et al., 2021). The threshold concentration of H_2 indicates the minimal concentration at which the metabolic process can occur. The standard Gibbs free energy ($\Delta G^{0''}$) is the free-energy change of a chemical reaction catalyzed by a microorganism at pH 7, 25°C, and at reactants/products concentration of 1 M (soluble reactants/products) or pressure of 1 atm (gaseous reactants/products). It indicates the thermodynamic potential of a reaction. In the case of a negative $\Delta G^{0'}$, a reaction is considered exergonic and therefore thermodynamically feasible (Table 1), with larger negative values indicating a greater amount of free energy yielded by the reaction.

Low risk to subsurface H_2 storage exert the reactions of H_2 with the electron acceptors including amongst others O_2, NO_3^-, halogenated compounds, fumarate or MnO_2/MnO_4^{2-} (Table 1). However, aerobic H_2 oxidation, denitrification, and ammonification could become a threat should contamination, for example, by drilling fluid occur in the storage site (Thaysen et al., 2021). The metabolisms that exert an intermediate risk level are ferric iron reduction (Fe^{3+}) and elemental sulfur (S^0) reduction (Thaysen et al., 2021). Ferric iron reduction (also referred to as iron (III) reduction) can be classified as an intermediate risk factor, since its availability is generally limited at sites for potential subsurface H_2 storage. Sulfur reduction, on the other hand, is limited due to the low solubility of sulfur in aqueous systems (Thaysen et al., 2021). However, sulfur can react with solubilized S^{2-} to form soluble polysulfides (S_n^{2-}). Such reaction

[1] A process, in which through the interaction with low-temperature water the primary, high-temperature igneous ferromagnesium minerals are modified to low-temperature, secondary serpentine minerals (Gregory et al., 2019).
[2] A process, in which chemical bonds are broken by hydromechanical reactions and radicals are produced that interact with the groundwater to produce hydrogen (Gregory et al., 2019).

reaches high conversion rates in solutions under alkaline conditions (Rickard and Luther, 2007). A more detailed description of these low and intermediate-risk metabolisms can be found in the review of Thaysen et al. (2021).

The highest risk to impact subsurface H_2 storage is given by hydrogenotrophic SO_4^{2-} reducers, hydrogenotrophic methanogens, and homoacetogens (Figure 1) (Thaysen et al., 2021). Interestingly, these three main hydrogenotrophic microbial metabolisms in the subsurface possess the highest H_2 threshold concentration and simultaneously a low $\Delta G^{0'}$ (Table 1). Thus, these three metabolisms require the highest H_2 concentrations and gain the lowest energy possible for the microorganisms that carry them (Thaysen et al., 2021).

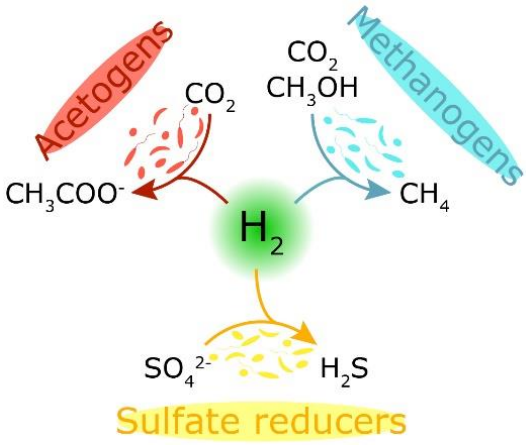

Figure 1. Main hydrogenotrophic microbial metabolisms in subsurface H_2 storage sites.

Should competition for H_2 amongst the metabolic groups happen, the metabolism with the lowest H_2 threshold is expected to outcompete the other functional groups (Lovley and Goodwin, 1988). For example, from the three main metabolic groups believed to have the highest impact on H_2 subsurface storage, based on their higher affinity for H_2 and higher growth rate with H_2, SO_4^{2-} reducers are predicted to generally outcompete methanogens (Robinson and Tiedje, 1984; Oude Elferink et al., 1994) and acetogens (Cord-Ruwisch et al., 1988). However, the predictions can be shifted depending on environmental factors such as temperature (Kotsyurbenko et al., 2001; Fu et al., 2019), the presence of needed substrates (Ranchou-Peyruse et al., 2019), the initial inoculum (Weijma et al., 2002; Braga Nan et al., 2020), or the formation of microniches (Jakobsen, 2007).

Ranchou-Peyruse et al. (2019) studied the microbiome of the aquifer systems of Beynes, in the West of Paris (France), which are used for natural gas storage. In these aquifers, SO_4^{2-} reducers dominated over methanogens except for one aquifer, which had been used 50 years before to store town gas containing at least 50% H_2. Methanogens dominance in this aquifer was correlated with the almost complete depletion of SO_4^{2-} provoked by SO_4^{2-} reducers when town gas was stored. These results indicate the influence of stored gas on the reservoir's microbiome even decades after (Ranchou-Peyruse et al., 2019). An impact on the microbial community composition under high partial H_2 pressure was shown in a biomethanation process, in which, depending on the inoculum, either CH_4, a mix of CH_4 and low levels of volatile fatty acids (VFA), or only VFA (mainly acetate) were produced (Braga Nan et al., 2020). In a gas-lift reactor fed with H_2/CO_2 and SO_4^{2-}, microbial SO_4^{2-} reducers rapidly outcompeted methanogens, allowing acetogens to remain based on the higher settling rate of sulfidogenic-acetogenic sludge compared to that of the methanogenic sludge (Weijma et al., 2002). Finally,

symbiont subsurface microniches can develop, with metabolisms that would otherwise compete with each other, such as SO_4^{2-} reduction and methanogenesis (Jakobsen, 2007).

The ranking of the prevailing metabolic process is based on limiting H_2 concentration. Therefore, under high partial H_2 pressure, found under H_2 storage conditions, no electron donor limitation is imposed, shifting the limitation to the availability of other macro or micronutrients. The influence of high partial H_2 pressure and other environmental factors on the competition, and thus, on the microbial community structures, has received limited attention so far (Ranchou-Peyruse et al., 2019; Braga Nan et al., 2020). Therefore, the impact of high H_2 concentrations on interactions of the metabolic groups and on how the community is shaped needs to be elucidated to better predict community shifts (Thaysen et al., 2021).

Table 1. Microbial H_2 oxidation metabolisms with their respective H_2 threshold, Gibbs free energy yield ($\Delta G^{0'}$) and the H_2 concentrations, which is typical for environments for the respective metabolic reaction. Included in the table are only the relevant metabolic reaction for H_2 storage. NA, Not available. Table was adapted from Thaysen et al. (2021).

H_2- oxidizing process	Reaction		H_2 threshold (nM)	$\Delta G^{0'}$ (KJ mol/H_2)	Typical ambient conc. H_2 (nmol/l)	Relevance for H_2 storage
Chromate reduction	$\frac{1}{2}H_2 + \frac{1}{3}CrO_4^{2-} + \frac{5}{3}H^+ \rightarrow \frac{1}{3}Cr^{3+} + \frac{4}{3}H_2O$	(1)	< 0.1	NA	NA	low
Aerobic hydrogen oxidation (Knallgas)	$H_2 + \frac{1}{2}O_2 \rightarrow H_2O$	(2)	0,051	-237	NA	low
Denitrification	$H_2 + \frac{2}{5}H^+ + \frac{2}{5}NO_3^- \rightarrow \frac{1}{5}N_2 + \frac{6}{5}H_2O$	(3)	< 0.05-0.5	-240,1 -224	< 0.05	low
Halorespiration	$H_2 + halogenated\ compounds \rightarrow dehalogenated\ compounds + HCl$	(4)	0.05-0.27 < 0,3 0.27-2	-230 to -187	NA	low
Iron (III) reduction	$H_2 + ferric(oxy)hydroxides \rightarrow ferrous\ iron + H_2O$	(5)	< 0.11-0.8	-228.3 -182.5 -114	0.2 0.2-1	intermediate
Manganese (IV) reduction	$2\,H_2 + MnO_2 \rightarrow Mn(OH)_2 + 2\,H_2O$	(6)	< 0.05	-163	< 0.05	low
Arsenate reduction	$H_2 + HAsO_4^{2-} + 2\,H^+ \rightarrow H_3AsO_3 + H_2O$	(7)	0.03-0.09	162.4	0.4 - 0.7	low
Ammonification	$4\,H_2 + 2\,H^+ + NO_3^- \rightarrow NH_4^+ + 3\,H_2O$	(8)	0.015-0.025	-150	< 0.05	low
Fumarate reduction	$H_2 + fumarate \rightarrow succinate$	(9)	0.015	-86.2	NA	low
Hydrogenotrophic sulfate reduction	$4\,H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4\,H_2O$	(10)	1-15	-38 -48 -57	1-2	high
Hydrogenotrophic methanogenesis	$4\,H_2 + CO_2 \rightarrow CH_4 + 2\,H_2O$	(11)	0.4-95	-34 -43.9	5-10 7-13	high
Sulfur reduction	$H_2 + S \rightarrow HS^- + H^+$	(12)	2500	-33.1	NA	intermediate
Homoacetogenesis	$4\,H_2 + 2\,CO_2 \rightarrow CH_3COOH + 2\,H_2O$	(13)	328-3640	-26 -36.1	> 100	high

4.1 Hydrogenotrophic sulfur reduction

Sulfur is a ubiquitous element in the environment, of which 94% of the sulfur species are concentrated under the pedosphere, the outermost layer of the lithosphere (Reddy and DeLaune, 2008). Sulfur can go through an eight-electron traverse from SO_4^{2-}, where the sulfur atom is in the [+6] oxidation state, to H_2S, where sulfur oxidation state is [−2]. Given the high relative abundance of sulfur in the environment and the broad range of oxidation states it can have, the metabolization of sulfur compounds is likely one of the most primitive microbial

metabolisms encountered in both Archaea and Bacteria domains (Brimblecombe, 2013). For the subsurface storage of H_2, the extensive microbial sulfur metabolism can be streamlined to the dissimilatory sulfur reduction, where the terminal metabolic product is H_2S, an undesirable and harmful product for the H_2 subsurface storage.

In the metabolism of dissimilatory sulfur reduction, energy is obtained through the organic matter or H_2 oxidation, utilizing SO_4^{2-} and/or S^0 as the terminal electron acceptor(s), resulting in H_2S production. Despite sulfur being the 14th most abundant element on Earth, SO_4^{2-} is the second most abundant anion after chloride (Bharathi, 2008). Consequently, metabolization of SO_4^{2-} is prevalent in natural environments compared to S^0 metabolization. Microbial reduction of SO_4^{2-}, including hydrogenotrophic SO_4^{2-} reduction (Table 1), has been reported in a broad spectrum of conditions. The optimal conditions where SO_4^{2-} reducing microorganisms thrive are comparable to those encountered in depleted or soon-to-be-depleted oil and gas reservoirs targeted for H_2 storage (Thaysen et al., 2021).

Hydrogenotrophic SO_4^{2-} reduction has been reported to take place in a wide range of pH, temperature, salinities, and water pressures, with pH seemingly being the most influential parameter enabling or inhibiting sulfate-reducing microorganisms. Although SO_4^{2-} reduction occurs in extreme low and high pH, the optimal pH for sulfate-reducing microorganisms is reported within a range between 6.5 to 8.0 (Figure 2), with pH ranges lower than 4 or higher than 9 significantly decreasing the rate of SO_4^{2-} reduction (Kaksonen and Puhakka, 2007; Muyzer and Stams, 2008; Bijmans et al., 2010). Likewise, sulfate-reducing microorganisms grow in an extensive range of temperatures, with optimal temperatures ranging from 20 – 80 ˚C, but in the case of spore formation, they can resist temperatures up to 130 ˚C (Rosnes et al., 1991; Isaksen et al., 1994). Sulfate-reducing microorganisms are reported to grow optimally from null salt concentrations until the lower end of moderate halophilic environments (0.5 M NaCl) (Hao et al., 2014). Though SO_4^{2-} reduction has been encountered in hypersaline environments (Minz et al., 1999), salinity concentrations that benefit certain sulfate-reducing microorganisms could inhibit other microbial species capable of SO_4^{2-} reduction (Kaksonen and Puhakka, 2007), and thus its optimal salinity is species dependent. In high-pressure environments such as deep-sea environments, sulfate-reducing microorganisms thrive and are commonly encountered (Kallmeyer and Boetius, 2004; Williamson et al., 2018); however, no data is available in high-pressure gaseous environments.

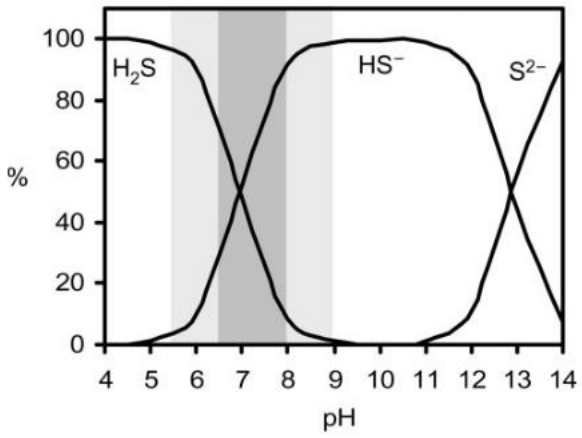

Figure 2. Optimal pH conditions for SO_4^{2-} reducing microorganisms (vertical grey bars) and sulfide speciation across the pH scale at 25 °C (continuous lines). Grayer zones signal the optimum pH of neutrophilic SO_4^{2-} reducers reported (Kaksonen and Puhakka, 2007).

In natural environments, sulfate-reducing microorganisms coexist with other anaerobes, such as methanogens and homoacetogens, resulting in metabolic interactions with neighboring microorganisms. In the presence of SO_4^{2-}, the competitive order benefits the hydrogenotrophic sulfidogenesis through SO_4^{2-} reduction ($\Delta G^{0'}$ -151.9 KJ·mol^{-1}) as it is thermodynamically more favorable than hydrogenotrophic methanogenesis ($\Delta G^{0'}$ -135.6 KJ·mol^{-1}) and homoacetogenesis ($\Delta G^{0'}$ -104.6 KJ·mol^{-1}) (Weijma et al., 2002; Muyzer and Stams, 2008). However, in the absence of SO_4^{2-}, the competitive order shifts towards homoacetogen and methanogen microorganisms, respectively (Weijma et al., 2002). The shift in the competitive order enables sulfate-reducing microbes to grow acetogenically in syntropy with hydrogenotrophic methanogens (Muyzer and Stams, 2008).

Reduction of Sº (Table 1) is less prone to occur than SO_4^{2-} reduction. Though the Sº reduction is also thermodynamically favorable ($\Delta G^{0'}$ -45.64 KJ·mol^{-1}) (Amend and Shock, 2001), Sº reduction is limited by the bioavailability of Sº. The solubility of Sº is low, in the order of 10^{-8} to 10^{-4} M within a temperature range of 0 to 95 ˚C, with higher temperatures increasing the sulfur solubility (Kamyshny, 2009). The Sº solubility is further affected by the salinity concentration, with sea water salinity driving a decrease in the soluble Sº concentration down to 61 ± 13% of the Sº solubility in pure water (Kamyshny, 2009). Nevertheless, the Sº-reducing metabolism is well distributed among extremophiles, which can grow on direct contact with the Sº or by reducing polysulfides (S_n^{2-}) (Florentino et al., 2015), a soluble elongated Sº linear chain with a reduced sulfur atom (S^{2-}) in its tail. Furthermore, at high temperatures (≥65 ˚C), Sº is soluble in compressed gases (10 - 30 MPa) such as nitrogen, CH_4, CO_2, and H_2S, increasing 0.03 mM Sº per degree increase in the centigrade scale (˚C) (Florentino et al., 2016). Thus, likely increasing the bioavailability of Sº under the conditions typically found in potential H_2 storage reservoirs. Consequently, its relevance for subsurface H_2 storage cannot be excluded.

Other sulfur oxyanions (e.g., thiosulfate, sulfite, polythionates) are also viable terminal electron acceptors assimilated by some microorganisms resulting in H_2S production (Zopfi et al., 2004). However, regardless of the pH and redox potential, sulfur oxyanions are never predominant in equilibrium conditions (Steudel, 2020), and therefore its contribution to the microbial H_2S production is likely negligible.

4.2 Hydrogenotrophic methanogenesis

Methanogenesis is an energy-conserving process performed by strictly anaerobic CH_4-producing archaea called methanogens. Methanogens are key players in the Earth's carbon biogeochemical cycle as they generate more than half of all CH_4 found on Earth (Evans et al., 2019). Biogenic CH_4 production can be found in a wide range of anaerobic environments ranging from animals' (Misiukiewicz et al., 2021) or insects' digestive tract (Brune, 2010), over freshwater (Borrel et al., 2011), saline (Kallistova et al., 2020) and haloalkaline (Sorokin et al., 2015a) lake sediments to subsurface environments including oil and gas reservoirs (Buriánková et al., 2022; Molíková et al., 2022). This diversity of habitats is reflected in their adaptation to various conditions as for instance their ability to grow up to elevated temperatures (≤ 122ºC) (Jabłoński et al., 2015) or pressures (> 20 MPa) (Taubner et al., 2015).

Methanogenic metabolisms can be divided into three main pathways, namely: (i) hydrogenotrophic, (ii) methylotrophic, and (iii) acetoclastic methanogenesis. In more detail, these reactions are described as the reduction (i) of CO_2 with H_2 (or with CO, formate, or certain alcohols) (Table 1), (ii) of methyl compounds (as methanol, (di/tri)methylamine, dimethyl sulfide or other methyl sulfides), which may also use H_2 as electron donor, and (iii) of acetotrophic substrates (acetate, pyruvate) (Liu and Whitman, 2008; Schöne and Rother, 2018; Ferry, 2020; Kurth et al., 2020). Of those metabolisms, hydrogenotrophic methanogens are the most widespread (Jabłoński et al., 2015) and phylogenetically diverse (Buan, 2018).

Hydrogenotrophic methanogenesis is a multiple-step pathway that leads from the reduction of CO_2 to the production of CH_4 involving multiple enzymes and cofactors (Figure 3). Methanogenesis pathway starts with the binding of CO_2 to a methanofuran-containing enzyme (MF) and its reduction to formyl by a reduced ferredoxin (Fd_{red}). Thereafter, the formyl is transferred to a methanopterin-containing enzyme (MP), where it is dehydrated by H_2 and consecutively reduced by $F_{240,red}$ to methylene and then to methyl. The final two steps of the pathway focus on energy conservation and the bifurcation of electrons. First, for the energy conserving step, the methyl group is transferred to coenzyme M (CoM-SH), which is enabled by a methyltransferase pumping sodium outside the cell and creating by this an electrochemical gradient. This sodium gradient motive force is used to form ATP (Adenosine 5′-triphosphate) by an ATP synthase. For the electron bifurcation step, the methyl-CoM is reduced to CH_4 by a methyl reductase in parallel to the oxidation of CoM by the coenzyme B (CoB-SH) and the formation of CoM-S-S-CoB. This heterodisulfide is regenerated to CoM and CoB by its reduction with a hydrogenase-heterodisulfide reductase complex, which couples this reaction to the reduction of the ferredoxin using H_2 as an electron donor. The ferredoxin can then again be used in the first step of the methanogenesis to reduce CO_2 (Evans et al., 2019; Madigan et al., 2019).

The production of CH_4 from H_2 and CO_2 liberates less free energy with increasing temperature (-131 kJ/mol H_2 at 25°C to -100 kJ/mole H_2 at 100°C) (Amend and Shock, 2001). Moreover, this reaction does not gain enough free energy to support the growth of methanogens when growing with elevated temperature and low partial H_2 pressure (<10 Pa) (Thauer et al., 2008).

Hydrogenotrophic methanogens are commonly found in subsurface environments, which were not used before for the storage of gas containing H_2 (Fry et al., 1997; Kotelnikova and Pedersen, 1997; Chapelle et al., 2002; Shimizu et al., 2006; Flynn et al., 2013; Kadnikov et al., 2020). Due to their natural presence in many subsurface environments, and their capacity to transform H_2 and CO_2 into CH_4, it has been proposed to use subsurface reservoirs as a "bioreactor" for biomethanation (Buriánková et al., 2022; Molíková et al., 2022). This process was studied in depth by the "Underground Sun Conversion" project with the aim of producing green gas (https://www.underground-sun-conversion.at/en, visited the 22/07/2022). At reactor level, the process of anaerobic digestion (AD) is also used to transform different organic wastes and residues by bio-methanation to increase the CH_4 content in biogas (Angelidaki et al., 2011).

4.3 Acetogenesis

Acetogenesis is a strictly anaerobic process performed by acetogens; the name derives from the main product of this fermentation, acetate. Homoacetogens are taxonomically widely distributed, but in general, are represented only by a few members within a phylum. The phyla harboring acetogens include Spirochaetes, Firmicutes, Chloroflexi, Deltaproteobacteria, and Acidobacteria (Ragsdale and Pierce, 2008). Acetogens can be found in a wide range of environments, similar to methanogens, also including the digestive tracts of animals (Li et al., 2020), and insects (Leadbetter et al., 1999), freshwater (Gwynfryn Jones and Simon, 1985), marine (Hoehler et al., 1999) and soda lake (Sorokin et al., 2015b) sediments, surface soils (Peters and Conrad, 1995; Küsel et al., 1999) and the deep subsurface as oil and gas reservoirs (Maune and Tanner, 2012; Buriánková et al., 2022). Acetogens are able to use next to H_2 also C_1 compounds (CO_2, CO, methanol), methyl groups of many methoxylated aromatic compounds, sugars, organic acids, amino acids, alcohols, and some nitrogen bases. In addition to CO_2 reduction, some acetogens can also perform nitrate (NO_3^-), nitrite (NO_2^-), and thiosulfate ($S_2O_3^{2-}$) reduction (Ragsdale and Pierce, 2008; Madigan et al., 2019).

A main feature of acetogens is the reduction of CO_2 by the Wood-Ljungdahl pathway, also referred to as reductive acetyl-CoA pathway, to produce energy and acetyl-CoA (precursor for biomass production) (Ragsdale and Pierce, 2008). In this pathway, one molecule of CO_2 is

reduced into the methyl group, while a second CO_2 molecule is converted into CO, forming the carboxyl group of acetate (Figure 3). The reduction of the first CO_2 molecule uses one ATP and the coenzyme tetrahydrofolate (THF) in a multistep transformation of CO_2 over formate to methyl-THF. Subsequently, the methyl group is transferred from the THF to a cobalt- and iron-containing corrinoid iron-sulfur protein (CoFeSP) coenzyme. The reduction of the second CO_2 molecule is catalyzed by the carbon monoxide dehydrogenase in combination with the oxidation of ferredoxin (Fd). The methyl- and the carbonyl-group are then combined by the carbon monoxide dehydrogenase and the acetyl-CoA synthase to produce acetyl-CoA. This latter compound is then converted to acetate, and one ATP is formed by substrate-level phosphorylation (Figure 3). Energy through acetogens is gained by the ion motive force of the Rnf complex (or Ech complex), generating either a H^+ or Na^+ motive force by pumping one H^+ or Na^+ per exchanged electron from reduced ferredoxin to NAD^+ (Nicotinamide adenine dinucleotide) outside the cell. The ATP is then synthetized by a H^+ or Na^+-dependent ATP synthase using the H^+ or Na^+-motive force, respectively. In total, only 0.3 ATP are generated per 4 H_2 and 2 CO_2 utilized. To regenerate the oxidized ferredoxins, an electron-bifurcating hydrogenase couples this process to the reduction of NAD^+ (Madigan et al., 2019).

Homoacetogenesis is the process of H_2 oxidation with the lowest $\Delta G^{0'}$ and the highest H_2 threshold concentration (Table 1) (Thaysen et al., 2021). Therefore, the general consensus is that acetogens will be outcompeted by energetically more favorable metabolisms as methanogenesis and SO_4^{2-} reduction. However, co-existence might also be possible as acetogens can take advantage of niche differentiation due to their diverse substrate range and low energetic biosynthesis costs by the utilization of the reductive acetyl CoA pathway. However, environmental conditions have a significant influence as well. Lever et al. (2012) showed that homoacetogenesis with H_2 and CO_2 at low H_2 concentrations (0.1 nM) and high temperature (122 °C) is endergonic, and it only turns energetically favorable at H_2 concentrations of 0.1 µM and lower temperatures. Moreover, reactions that can be performed with or without H_2 are under low H_2 concentrations (0.1 nM) only energetically favorable without H_2, but most become more favorable than without H_2 under high H_2 concentrations (0.1 µM). (Lever, 2012). In general, $\Delta G^{0'}$ of acetogenesis based on H_2 and CO_2 increases with increasing H_2 concentration, increasing pH, and decreasing temperature (Thauer et al., 2008).

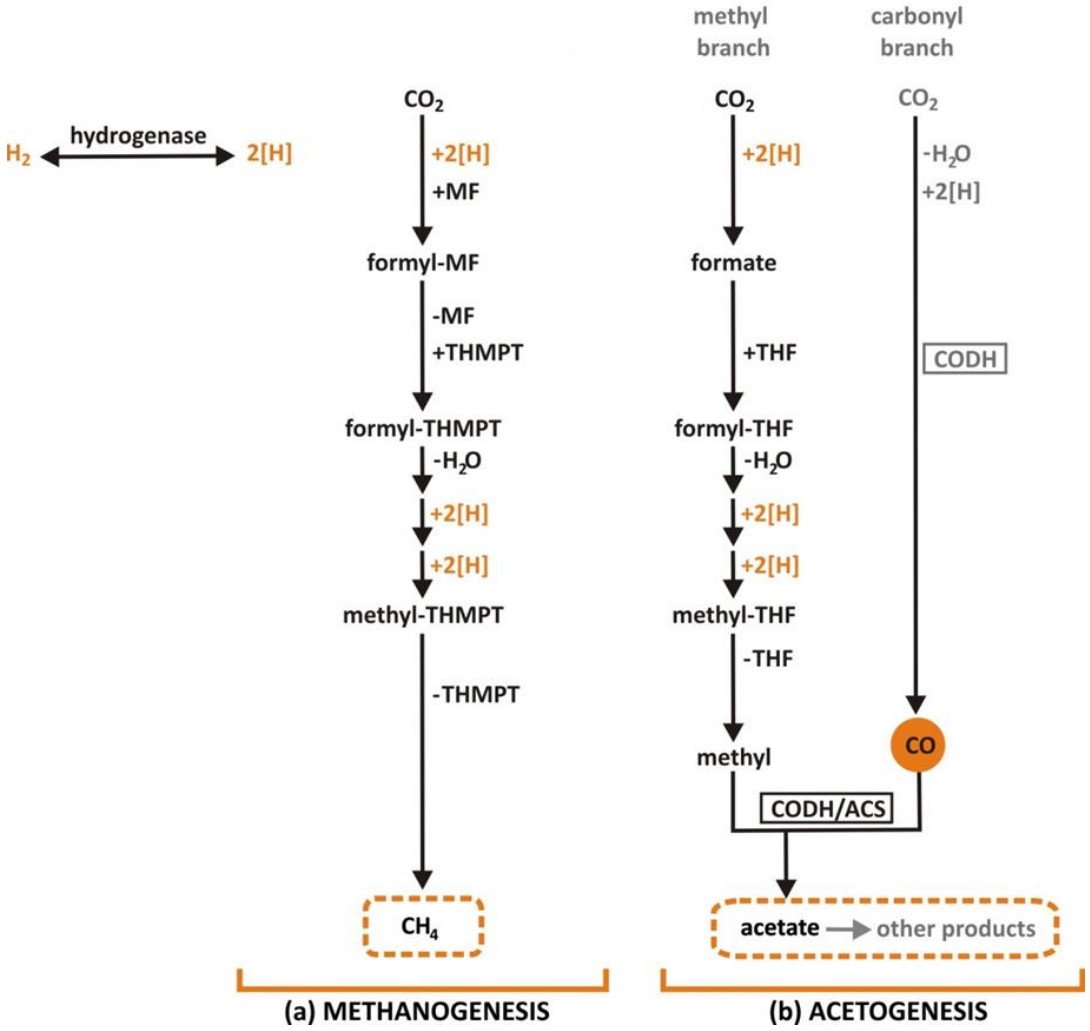

Figure 3. Simplified pathways for CO and CO_2/H_2 conversion by (a) methanogens (CO_2 reduction pathway) and (b) acetogenic microorganisms (Wood-Ljungdahl pathway). Abbreviations. CODH: CO dehydrogenase; CODH/ACS: CO dehydrogenase/acetyl-CoA synthase complex; MF: methanofuran; THMPT: tetrahydromethanopterin; THF: tetrahydrofolate.

5 Impact of environmental conditions on microbial communities in subsurface H_2 storage

The different life forms on Earth have all their specific optimal and limiting growth factors. The subsurface as an extreme environment offers multiple life-constraining parameters. The main conditions having an impact on microorganisms are temperature, pressure, salinity and pH. Below, the impact on microorganisms of these constraining parameters are summarized. For a more detailed review, please refer to Thaysen et al. (2021), in which also additional stress factors are discussed, including limited availability of nutrients, brine complexity, and inhibitors (Thaysen et al., 2021).

5.1 Temperature

Temperature has a tremendous impact on microbial growth and survival. In the subsurface, only high-temperature stress is relevant as temperature increases in average 25°C per km, and after approximately 5 km, it would pass the border of the highest recorded growth temperature of 122°C (Takai et al., 2008; Oger and Jebbar, 2010). This record is performed by the methanogen *Methanopyrus kandleri* in combination with a pressure of 20 MPa (Takai et al., 2008). Therefore, subsurface H_2 storage sites with temperatures higher than 122°C can be considered safe regarding microbial risk (Thaysen et al., 2021). However, previously, temperatures in the range of 20 to 100°C have been proposed for H_2 storage sites (Matos et al., 2019; Shi et al., 2020), which would offer a niche for microbial hydrogenotrophic activity. Moreover, a problem that could arise even at sites with initial high temperatures are cooling effects of sea water injections (Thaysen et al., 2021).

Physiological effects of high temperatures on the cell are the increase in membrane fluidity impacting the electron transport and respiratory chain (Sinensky, 1974), the induction of oxidative stress (Marcén et al., 2017) and the denaturation of DNA, RNA, and proteins (Holden, 2009). On a microbial community level, high temperatures will decrease the diversity of the microbial community (Li et al., 2015). An advantageous physical effect of high temperatures at equal pressure for subsurface H_2 storage is the decrease in solubility of gases in the liquid phase, rendering it less accessible to the microbial community (Thaysen et al., 2021).

Analyzing the optimal and limiting growth conditions of relevant strains, Thaysen et al. (2021) found that so far, known acetogens will not grow at temperatures higher than 72 °C, and the maximal recorded growth temperature for sulfate reducers is 94 °C. However, the maximum growth temperature for known elemental sulfur reducers and methanogens are even higher and reach 113 °C and 122 °C, respectively.

5.2 Pressure

On Earth, hydrostatic pressure ranges from 0.1 MPa at sea level to up to 110 MPa in trenches of the deep ocean (Jebbar et al., 2015). In the subsurface, pressure increases on average 30 MPa per km (Oger and Jebbar, 2010). For potential H_2 storage reservoirs, pressure ranges from 1 to 40 MPa (Matos et al., 2019; NAM, 2021).

Until today no limits for pressure resistance of microbial life could clearly be defined (Dopffel et al., 2021). Mesophilic organisms adapted to atmospheric pressure are generally resistant to up to 30-50 MPa (Abe et al., 1999). The highest recorded pressure resistance was achieved by adapting step-wise a *Shewanella oneidensis* strain to 1.5 GPa (Hazael et al., 2014) and an *Escherichia coli* strain to 2 GPa (Vanlint et al., 2011). Physiological effects of high pressure on microorganisms are caused at the level of the membrane fluidity, affecting the membrane transport and respiratory chain, the microbial metabolism, DNA stability, the ribosomal and protein structure, and motility (Simonato et al., 2006).

Microbial isolations from subsurface reservoir or aquifer environments showed that the encountered pressure ranges do not indicate for environmental selection of obligate piezophiles (Haveman and Pedersen, 2002; Basso et al., 2009; Leandro et al., 2018; Karnachuk et al., 2019). However, Nyyssönen et al. (2014) described a correlation between the pressure and the structure of the microbial community present in deep crystalline rocks.

Pressure also interacts with other physical and chemical parameters of the subsurface environment. For instance, an increase in pressure leads to a higher solubility of gases in the liquid phase, according to Henry's law, raising the availability of H_2 and CO_2 to microorganisms (Follonier et al., 2012; Thaysen et al., 2021). Furthermore, to allow the growth of hyperthermophiles at temperatures higher than 100°C, pressure needs to be high enough to avoid boiling of the liquid phase, with elevated pressures also observed to induce stabilization of multiple enzymes of hyperthermophiles (Holden, 2009). A synergetic effect of high temperature on the growth rate at high pressure has also been observed for *Desulfovibrio indonesiensis* (Fichtel et al., 2015). For strains of *Pyrococcus* sp., *Thermococcus* sp., and *Desulfurococcus* sp., an increase of the maximum growth temperature was noted at increased pressure, and other strains increased their growth rate at high temperatures in combination with high pressure (Holden, 2009). This synergetic effect could be explained by a similar physiological impact of both physical conditions on the microbial cell.

5.3 Salinity

Salinity concentrations in potential H_2 storage sites can range from 0 to 5 M NaCl (Shi et al., 2020; Schwab et al., 2022). However, various microorganisms are known to grow until high salt concentrations (Thaysen et al., 2021). In general, methanogens favor a salinity of up to 0.77 M NaCl, but growth of two representatives was detected until 3.4 M salt. For SO_4^{2-} reducers, optimal salinity concentrations are situated between 0 and 0.4 M NaCl. However, members were also isolated, growing until 4.2 M NaCl. The activity of SO_4^{2-} reducers has been recorded up to 346 g/l of salt in sediment enrichments of Searles Lake (California, USA) (Blum et al., 2009) and up to 475 g/l for Lake Tanatar I (Kulunda steppe, Russia) (Foti et al., 2007). Finally, most acetogens prefer low salt concentrations ranging from 0 to 0.4 M NaCl, but exceptions are recorded with limits up to 4.4 M NaCl (Thaysen et al., 2021). The risk for microbial growth is reduced at extreme salinities, but a salinity limit for microbial growth has not yet been defined (Oren, 2011; Dopffel et al., 2021; Thaysen et al., 2021).

However, it is not the salinity on its own, but the brine's composition, which influences the window of viability of microorganisms (Thaysen et al., 2021). For instance, some ions are stronger electrolytes than others (NaCl vs. $NaHCO_3$/Na_2CO_3) exerting a higher osmotic stress on the cell (Sorokin et al., 2011). Moreover, the effect of chaotrophic ($MgCl_2$, $CaCl_2$ or $FeCl_3$) or kosmotrophic molecules (ex: NaCl, KCl or $NaHCO_3$/Na_2CO_3) (Zhang et al., 2005; Cray et al., 2013), and reduced water activity (a_w) are also important factors defined by the brine impacting microbial life (Thaysen et al., 2021).

Metagenomic analysis of salt caverns showed that five salt caverns with a salt concentration of 4.7 M NaCl harbor a diverse halophilic community in high cell numbers inside each sample, but also across the different samples. The detected community contained members of the phyla *Halobacterota*, *Halanaerobiaeota*, *Desulfobacterota*, *Firmicutes*, *Bacteroidota*, *Actinobacteriota,* and *Proteobacteria*. As the detected abundance and diversity is generally higher than in groundwater systems, the authors propose that the present microbial community originates not only from initial presence in the rock but also from external import during, for example, solution mining or gas charging (Schwab et al., 2022).

High salinity concentration stresses the cell's Na^+ homeostasis and leads to osmotic pressure on the cell. Ion pumps, antiporters, and other transport proteins maintain intracellular Na^+ concentrations low. To hold the osmotic pressure, cells adopt either the salt-in or the salt-out

strategy. In the salt-in strategy, the cell accumulates a high concentration of mainly K^+ inside the cell, a strategy energetically not very costly but allows only growth at low salt concentrations. In comparison, in the salt-out strategy, the cell accumulates high concentrations of compatible solutes, so-called osmolytes. Osmolytes are small zwitterions as for example glycine betaine or ectoine. Their synthesis is, however, energetically costly for the cell but allows growth up to high salinities (Oren, 2012).

5.4 pH

The measured pH of UGS aquifers, reservoirs, and salt caverns is in general around neutrality, but spans from 6.2 to 9.2 (Šmigáň et al., 1990; Ivanova et al., 2007; Ranchou-Peyruse et al., 2019, 2021; Kadnikov et al., 2020; Buriánková et al., 2022; Schwab et al., 2022). Microorganisms can grow on a broad pH range going from a pH of 1 to 10.7. However, most methanogens, SSRM (Sulfur species reducing microorganisms), and acetogens have their pH optimum at 6.5 to 7.5 (Thaysen et al., 2021).

The pH of the environment induces physiological effects on the microorganisms but also influences the redox reactions (Thaysen et al., 2021). Deviation from the circumneutral pH challenges the cell to maintain pH homeostasis and imposes a pH gradient across the membrane as the cytoplasmic pH is maintained at neutral pH ($\Delta pH = pH_{in} - pH_{out}$). The pH gradient influences together with the electric membrane potential ($\Delta\psi$) the difference in transmembrane proton potential (the proton motive force (*pmf*)), impacting by this the cell's bioenergetics (Slonczewski et al., 2009). Furthermore, the microorganisms need to adapt their membrane fluidity and permeability under pH deviations from neutrality (Siliakus et al., 2017).

5.5 Limits of microbial life for subsurface H_2 storage conditions and effect of the combination of stress factors

Collecting optimal and limiting growth parameters of published data of 532 pure strains of acetogens, SSRM, and methanogens, Thaysen et al. (2021) defined parameters limiting microbial activity to help in the selection of potential sites for subsurface H_2 storage. Table 2 summarizes their findings for the key parameters.

Table 2. Summary of optimal and limiting conditions for microbial growth under subsurface H_2 storage conditions. NA, Not available. Values were retrieved from Thaysen et al. (2021).

Parameters	Microbial optimum & limit	Methanogens	Sulfate reducers	Acetogens
Temperature (H_2 storage: 22.5-100°C)	Optimum	15-98°C	10-106°C	20-30°C
	Limits	122°C	113°C	72°C
Pressure (H_2 storage: 1-50 MPa)	Optimum	0-30/50 MPa	0-30/50 MPa	0-30/50 MPa
Salinity (H_2 storage: 0-5 M NaCl)	Optimum	0-0.77 M NaCl	0-0.4 M NaCl	0-0.4 M NaCl
	Limits	3.4 M NaCl	4.2 M NaCl	4.4 M NaCl
pH	Optimum	4-9.5	4-9.5	NA
	Limits	4-10	1-10	3.6-10.7

Thaysen et al. (2021) evaluated that temperature alone or in combination with salinity exerts the most constraining parameters for microbial growth and activity in the subsurface. In general, sites with a temperature higher than 122 °C could be considered sterile. To decrease the probability of S^{2-} formation, sites with temperatures above 80 – 94 °C should be chosen.

Plotting critical temperature and NaCl concentrations, Thaysen et al., (2021) could show the limits of possible growth for methanogens, SSRM, and acetogens in the subsurface environment for these two parameters. They proposed that storage sites with a temperature and salinity higher than 55 °C and 1.7 M should be selected to reduce the probability of the microbial impact (Figure 5). Interestingly, below these borders, a high abundance of cultivated strains was found for a wide range of NaCl concentrations at a critical temperature of 40-50 °C, as well as for a whole panel of temperatures at a salinity below 1 – 1.7 M NaCl (Thaysen et al., 2021). On the other hand, sites with low salinity, low temperature, and a circumneutral pH are the most prone to host substantial microbial growth and activity (Thaysen et al., 2021).

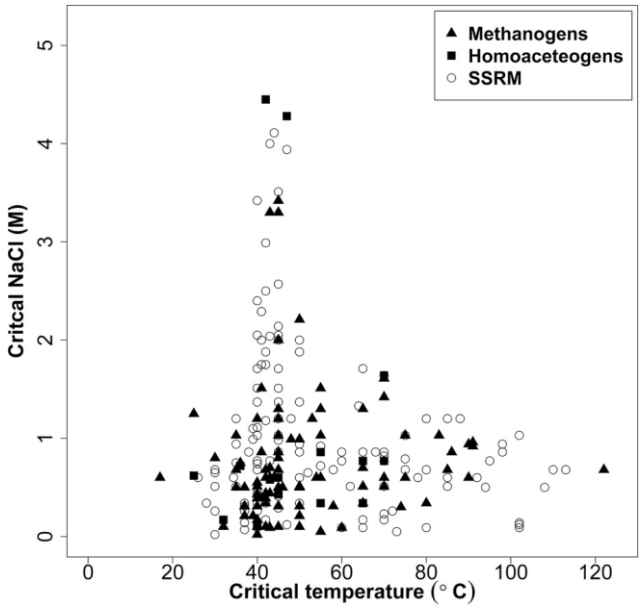

Figure 4. Critical temperature and salinity of 287 methanogens, homoacetogens and sulfur-reducing microorganisms in the absence of the other respective stressor (Thaysen et al., 2021).

An important remark to make is that certain bacteria are able to form spores, which can tolerate, for instance, much higher deviations of temperatures, pressures, and pH than those required for optimal growth (Setlow, 2014). This life form enables them to stay in this dormant state for millions of years until conditions become viable again (Cano and Borucki, 1995). Therefore, special attention needs to be given to possible changes in the limiting growth parameters in the reservoir to ensure the site's sterility or to reduce the impact of microbial activity (Thaysen et al., 2021).

6 H_2 headspace incubation experiments

Within HyUSPRe an experimental setup was designed for the initial assessment of H_2-utilization potential of several underground reservoirs (provided by the industrial partners). The summary of this approach and main results so far are shown in this section.

6.1 Material and methods

6.1.1 Sampling

Reservoir brine samples from different partners were provided: A, five and six runs from two locations, respectively; B, eight (with two runs for three wells), three, five (with five and three runs for two wells), and eight wells for four locations; C, two runs from one location . Once they arrived at Wageningen University, the samples were stored at 15°C until analysis.

Samples from A1 and A2 were taken via a bailor that was sterilized with bleach (10%) and washed with water. The decompression of the sample was not controlled, but slow using a pressure control equipment at the well head. Sub-samples were taken from a tank, flushed with nitrogen and stored at room temperature until shipment.

Samples for three locations from B were acquired by a pipe, from which the sample was taken at the end of the individual tie of a given well before the common gathering point. The device was not sterilized beforehand. The decompression of the samples was not controlled, and the sub-sampling was performed by filling bottles via a ball-valve, for which new sampling bottles were used. The bottles were not flushed, and then stored at room temperature until shipment. For one location, the procedure different by the fact that the sample was taken from a common separator used for several wells. For taking this sample, the production was stopped, and the separator unloaded. Next, the selected well for sampling was put back into production only and the sample was then taken after a reasonable time from the separator.

Samples from C were taken via a bailor, which was beforehand sterilized with 90°C hot water followed by distilled water. Decompression of the samples were performed with 2 bar/min. The samples were then transferred to sterile glass bottles containing atmospheric nitrogen under argon atmosphere and the bottles were flushed with argon. Until shipment, samples were stored at 4°C.

The temperature, pressure, pH and conductivity of the different well samples are given in Annex 1.

6.1.2 Incubations

For A and B, brine samples of the different runs were mixed together per location in equal volumetric proportion, and for C, the samples from the different wells and the different locations were all mixed together in equal proportion due to their high number. Brine samples (2 ml) were incubated in 48 ml of bicarbonate-buffered mineral salt medium (also called CP medium) (Stams et al., 1993; Plugge, 2005). Mineral medium contained per L: 0.53 g $Na_2HPO_4 \times 2H_2O$, 0.41 g KH_2PO_4, 0.3 g NH_4Cl, 0.11 g $CaCl_2 \times 2H_2O$, 0.10 g $MgCl_2 \times 6\ H_2O$, 0.3 g NaCl, 4.0 g $NaHCO_3$, 0.48 g $Na_2S \times 9\ H_2O$. Acid and alkaline trace elements (each, 1 ml/liter) and vitamins (0.2 ml/liter) prepared as described by Stams et al. (1993) were also added. Depending on the incubation, medium was supplemented with additional 0.45 g/l Na_2SO_4 (3 mM), and 25.52 g/l or 112.52 g/l NaCl to reach 0.5 or 2 M Na^+, respectively. The basic medium was boiled and N_2-flushed before being dispatched into 120 ml serum vials, sealed with black (butyl), red (bromobutyl) or blue (butyl) rubber septa, and crimped with aluminum caps. The headspace of the vial was gas exchanged with N_2 up to the final level of 1.5 bar, and the medium was autoclaved. Prior to inoculation, solutions of vitamins and a reducing agent ($Na_2S \times 9\ H_2O$)

were added via filter-sterilization. For environmental samples supplemented with trace elements, 45 ml of brine sample were added to a sterile and N_2-flushed 120 ml serum vial, to which a total of 5.3 ml filter-sterilized concentrated stock solutions in the same concentrations as for the bicarbonate-buffered mineral salt medium were added. After inoculation, the headspace of the cultures was gas exchanged to either 80% H_2/20% CO_2 or 100% H_2 at 1.7 bar, and the cultures were incubated at 35, 50, 65, or 80°C in the dark and without shaking. The mixture of 80% H_2/20% CO_2 was used to allow a better buffering of the medium as well as to provide a sufficient amount of electron acceptor for methanogens and acetogens.

6.1.3 Gas composition (H_2, CH_4) analysis

Concentrations of H_2 and CH_4 were measured by gas chromatography with a thermal conductivity detector (GC-TCD) on a CompactGC4.0 (Interscience, The Netherlands). For the analysis, 0.2 ml of headspace sample was injected into Carboxen GC 1010 PLOT Capillary pre-column (3 m × 0.32 mm, Sigma-Aldrich Co. LLC, USA), followed by a separation on a Molsieve 5Å column (30 m × 0.32 mm, Agilent, USA). The temperatures in the injector, column, and detector were maintained at 100, 140, and 110°C, respectively. Argon gas was used as a carrier gas at a flow rate of 1 ml/min.

6.1.4 Liquid phase composition (H_2S, SO_4^{2-}, acetate)

H_2S concentrations in the liquid phase of the sample were quantified using the methylene blue method (Standard methods for the examination of water and wastewater, 2017). For this, H_2S was fixed in 1 ml of sample with 250 µl of 5% (w/v) zinc acetate ($Zn(CH_3COO)_2$) previously alkalified with 40 µl of 1M NaOH. 50 µl of the fixed S^{2-} sample were added to 4.5 ml demi-water, to which simultaneously 500 µl reagent A (0.2% (w/v) dimethyl-p-fenyldiamine (oxalate salt), 20% (v/v) concentrated H_2SO_4) and 50 µl reagent B (9 % (w/v) $FeCl_3$ × 6 H_2O, 2 % (v/v) concentrated H_2SO_4) were added and vortexed. Sulfide concentrations were read after 10 min with a Spectroquant Multy colorimeter (Merck, Germany).

Aliquotes of liquid samples for SO_4^{2-} and acetate analysis were centrifuged at 15,000 x *g* for 5 min and stored at -20°C until further processing. SO_4^{2-} was measured with the ion exchange chromatography Dionex ICS-2100 (Dionex, USA) equipped with a Dionex IonPac AS17 column (Dionex, USA) operating at 30°C, and a suppressed conductivity detector. For sample preparation, 100 µl of the supernatant were added to 900 µl of MilliQ water in HPLC vials, and vortexed. The mobile phase consisted of KOH with a concentration gradient ranging from 1 to 40 mM at a flow rate of 0.3 ml/min. Concentrations of acetate and other volatile fatty acids were measured with High-Performance Liquid Chromatography (HPLC) on a Shimadzu Prominence LC2030C Plus (Shimadzu Corporation, Japan), equipped with UV/Vis detector. The 0.01 N aqueous solution of sulfuric acid served as mobile phase at flow rate of 1 ml/min, eluting through the Shodex Sugar SH1821 column (8.0 mm × 300 mm × 6 µm, Showa Denko K.K., Japan) guarded with Shodex Sugar SH-G precolumn (6.0 mm × 50 mm × 10 µm, Showa Denko K.K., Japan), both maintained at constant temperature of 45°C.

6.2 Results and discussion

6.2.1 Incubation of environmental samples with 80%H_2 and 20%CO_2 at different temperatures and media compositions

Incubations of the environmental samples with 80%H_2/20%CO_2 at 1.7 bar of total pressure at different temperatures confirmed methanogenic, SO_4^{2-} reducing, and acetogenic activities in the samples of A, B, and C incubated at 80%H_2/20%CO_2 at different temperatures and media (Table 3).

In general, acetogenesis and SO_4^{2-} reduction were only observed at 35 and 50°C, whereas methanogenesis was observed at 35, 50, and 65°C. SO_4^{2-} reduction was only observed when SO_4^{2-} (3 mM) was added to the media. Naturally, the tested environmental samples only contained low concentrations of SO_4^{2-}, which explains the boost in SO_4^{2-} reduction activity once SO_4^{2-} was added. No microbial activity could be obtained at 80°C as well as for the sample A2 incubated with CP trace elements at any temperature conditions. Samples that did show growth from the samples A, B and C were incubated for five, four, and four months, respectively.

Cultures, in which methanogenic activity was observed, showed an increase in pH due to the lack of buffering capacity of the medium. This increase in pH led to a stop in the microbial activity. In future experiments, grounded core material will be added to provide a better buffering capacity.

Table 3. Microbial activities detected in the environmental samples incubated at 80%H_2/20%CO_2 with different incubation temperatures and different media. Yellow: sulfate reduction, blue: methanogenesis; red: acetogenesis. CP: bicarbonate-buffered mineral salt medium.

Sample	Medium	35°C	50°C	65°C	80°C
A1	Sample amended with CP trace elements	Acetogen	Methanogen	Methanogen	
	CP	Methanogen	Methanogen	Methanogen	
	CP with 0.5 M Na^+ + 3mM SO_4^{2-}	SO_4^{2-} reducer	SO_4^{2-}reducer		
A2	Sample amended with CP trace elements				
	CP	Methanogen	Acetogen		
	CP with 0.5 M Na^+ + 3mM SO_4^{2-}	Methanogen + SO_4^{2-} reducer	SO_4^{2-} reducer		
B	Sample amended with CP trace elements	Methanogen + Acetogen	Methanogen	Methanogen	
	CP with 0.5 M Na^+ + 3mM SO_4^{2-}	SO_4^{2-} reducer + Acetogen	Methanogen	Methanogen	
C	Sample amended with CP trace elements	Methanogen	Methanogen		
	CP with 0.5 M Na^+ + 3mM SO_4^{2-}	Methanogen			

6.2.2 Incubation of environmental samples with 100% H_2 at different temperatures

Incubations of pure environmental samples with 100% H_2 at 1.7 bar of total pressure at different temperatures showed methanogenic activities in the samples of A1 at 35, 50, and 65 °C, and of B at all four tested temperatures (Table 4). The samples A2 and C did not show microbial activity after three months of incubation.

The observed increase of pH was even stronger in the culture incubated only with 100% H_2 without supplemented nutrients than in the experiment above as the buffering capacity of the dissolved carbonates and of the added CO_2 in the gas phase was missing. For future

experiments, grounded rock material should be added to provide a better capacity of the medium as well as to better simulate the reservoir system by providing minerals present in the rock material.

Table 4. Microbial activities detected in pure environmental samples incubated at 100%H_2 with different temperatures. Yellow: SO_4^{2-} reduction, blue: methanogenesis; red: acetogenesis.

Sample	35°C	50°C	65°C	80°C
A1	Methanogen	Methanogen	Methanogen	
A2				
B	Methanogen	Methanogen	Methanogen	Methanogen
C				

7 Conclusions and future perspectives

The definition of the 'window of viability' by Thaysen et al. (2021) was based on published data from pure strains. As the growth limits of those cultures were established while other parameters were kept at optimum, this "window of viability" may probably shift to a combination of lower temperatures and salinity concentrations. Moreover, the effect of high partial H_2 pressure might also exert a supplementary stress factor on the microbial community, impacting as well this viability window of life.

For the selection of potential subsurface H_2 storage sites, microbiological and geochemical characteristics need to be analyzed beforehand but also be monitored during the storage period (Dopffel et al., 2021). Even though at the start, the microbial community and/or the geochemical characteristic seem to prevent a detrimental microbial impact on the H_2 storage, changes in the community composition and the geochemical parameters can occur. The storage sites are open systems, and external import of microorganisms during the operation, for example, the injection of the H_2, can occur (Schwab et al., 2022). Moreover, changes in conditions such as cooling effects after long-term injection of cold seawater can transform the site into a hostile environment for microbial growth (Thaysen et al., 2021). Under these circumstances, the subsurface community, which was previously in a dormant state, able to face the unhostile conditions, can become active again (Morono et al., 2011).

For future site-specific case studies, the analysis of the microbial community composition should not be neglected. It does not only evaluate and track the risks of microbial activity but also allows to link the microbial composition to the geochemical parameters of the site. This would as well increase our knowledge of the 'window of viability' for the different functional groups. Therefore, during this project, we will also sequence the microbial community composition of the potential subsurface H_2 storage sites of our partners. To further evaluate the potential microbial risks for the different sites containing environmental conditions within the window of viability, incubations will be performed in high pressure and high temperature (HPHT) reactors to determine the microbial community structure and the microbial kinetics of the relevant hydrogenotrophic communities under the influence of the different parameters. These experiments will also allow to challenge and adjust the borders of the "window of viability" so far defined by pure strains, as well as to evaluate the influence of pressure in combination with temperature and salt concentration. The obtained results shall be used to improve, extend, and validate the open-source simulator for flow and transport processes DuMu^X (Koch *et al.*, 2020) to better evaluate the microbial impact on subsurface H_2 storage depending on given environmental conditions.

8 References

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9 Annex

Annex 1. Table with the temperature, pressure, pH and conductivity (Proxy for salinity) of the different well samples. NK= Not known; ND = Not determined. A, B, C = Partner acronyms, first number indicates a location, second number indicates a well. Pressure for A and C is the pressure at the time of sampling, and for B, the initial pressure of the reservoir.

Samples	Temperature (°C)	Pressure (bar)	pH (Average)	Conductivity (Proxy for salinity) (mS/cm)
A1	51	45	7.72	49,24
A2	51	87	5.95	79,74
B11	83	116	ND	ND
B12	81	111	ND	ND
B13	83	116	ND	ND
B14	81	111	ND	ND
B15	NK	NK	ND	ND
B16	NK	NK	ND	ND
B27	81	111	ND	ND
B28	NK	NK	ND	ND
B21	107	206	ND	ND
B22	107	206	ND	ND
B23	107	206	ND	ND
B31	98	128	5.25	1.52
B32	NK	NK	ND	ND
B33	98-100	128-137	5.25	1.52
B34	NK	NK	ND	ND
B35	NK	NK	ND	ND
B41	72	97	ND	ND
B42	72	97	ND	ND
B43	72	97	ND	ND
B44	72	97	ND	ND
B45	72	97	ND	ND
B46	72	97	ND	ND
B47	72	97	ND	ND
B48	72	97	ND	ND
C	39-41	56	ND	ND