Hydrogenotrophic metabolisms in the subsurface and their implications for underground hydrogen storage and natural hydrogen prospecting

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Abstract

Hydrogen is a fundamental electron donor in diverse microbial metabolisms and it is considered the energetic currency exchanged within microbial communities in anaerobic environments. Hydrogen is also the major actor in the transition to alternative low-carbon energy sources, primarily due to its dual role as energy source and energy carrier and to the production of water as a byproduct of its combustion. The geological storage of hydrogen gas produced from diverse sources in stable terrestrial reservoirs, known also as Underground Hydrogen Storage (UHS), is a key prerequisite to decouple production from utilization. UHS targets include depleted porous natural gas reservoirs, salt caverns and deep aquifers. Studies carried out in the past 30 years have unveiled a large subsurface ecosystem able to interact with the geochemical cycles and volatiles present in Earth's crust. When hydrogen is stored underground, the microbial communities present *in situ* can interact with it, consuming it as electron donor, potentially producing undesired metabolic byproducts capable of affecting the success of UHS operations. Additionally, subsurface microbial communities might impact the geological production, migration and accumulation of hydrogen in natural reservoirs. Here, we review the current state of knowledge in

hydrogenotrophic metabolisms capable of affecting UHS operations and natural hydrogen prospecting, and discuss how the microbiology of natural hydrogen-rich springs can be used as analog to model the state space of hydrogen operations. We discuss our current knowledge of the limits of life in the context of hydrogen economy, and the complex trophic network that hydrogen might sustain in the subsurface. While energy demands increase globally, the ability to effectively operate geological hydrogen storage and identify natural hydrogen deposits will become a key prerequisite to reduce the global carbon footprint. Understanding the potential for microbes to interact with hydrogen in the subsurface is therefore at the forefront of the ecological transition.

Introduction

As of writing, carbon dioxide levels in the atmosphere have reached 425 ppm (Keeling and Keeling, 2017); https://keelingcurve.ucsd.edu/, accessed 25 December 2024) as a result of increased fossil fuel consumption since the industrial revolution. We are currently beyond the point of containing the global climate warming effects below 1.5 °C (2023 IPCC Report on Climate Change, (Calvin et al., 2023). A radical shift toward low carbon energy sources is required in order to contain global warming to below 2 °C and avoid a number of ecological and climatic cascading effects already taking place (Armstrong McKay et al., 2022). Renewable energy sources, such as wind, solar, tidal and geothermal energy produce electricity limiting the emission of greenhouse gases. However, among the many challenges related to their large-scale adoption as a viable substitute of fossil fuels, the mismatch between energy generation and demand, and the ability to create strategic long term energy reserves need to be addressed (Thiyagarajan et al., 2022). Storage of the electricity surplus from low-carbon energy resources might come in diverse solutions, such as the use of accumulators, conversion into gravitational potential energy or chemical storage (Sayed et al., 2023; Jafarizadeh et al., 2024). Among chemical storage, the conversion of the energetic surplus into hydrogen, known as green hydrogen, is especially appealing, due to the conceptually simple conversion process involving water electrolysis and the production of water vapor as the sole byproduct of hydrogen combustion (Ishaq et al., 2022). Hydrogen produced from renewable energy sources can be safely stored in specific geological underground formations for later use (Zivar et al., 2021a; Takach et al., 2022).

Medium/long-term storage solutions for hydrogen include geological storage in depleted natural gas reservoirs, salt caverns and saline aquifers (Heinemann et al., 2021), collectively known as "Underground

Hydrogen Storage" (UHS) solutions. The three possible storage reservoir types offer each a number of advantages and disadvantages. Compared to salt caverns, geological porous formations, such as deep saline aquifers and depleted oil and gas reservoirs, offer reasonably larger hydrogen storage capacities (several orders of magnitude) (Hassanpouryouzband et al., 2021; Heinemann et al., 2021; Zivar et al., 2021b). Additionally, previous experiences in gas storage operations in saline aquifers and gas/oil reservoirs provide significant hydrological and geological information required to evaluate the potential for UHS (Michael et al., 2009, 2010). This strategy makes hydrogen usable with the times and methods already developed for natural gas, ensuring high safety and versatility in energy production. On the other side, salt caverns storage are suggested to be more suitable for short-to medium-term hydrogen storage (Heinemann et al., 2021), mainly because of their lower working gas volume capacity, which is the amount of gas that can be withdrawn or injected with installed subsurface and surface facilities (Ozarslan, 2012). These environments have been widely used for various industrial applications such as natural gas storage, crude oil storage, and waste disposal (Firme et al., 2019).

Irrespective of the storage reservoir type, when hydrogen is stored underground, microbial communities present *in situ* interact with it, consuming it as electron donor and potentially producing undesired metabolic byproducts capable of affecting the success of UHS operations. The last 30 years of research have revealed the presence of a ubiquitous subsurface ecosystem harbouring the largest diversity of prokaryotes and nearly 14 % of the living biomass of our planet (Kallmeyer et al., 2012; Bar-On et al., 2018; Magnabosco et al., 2018a). Research carried out in diverse continental and oceanic tectonic settings have revealed the presence of abundant (between 10^2 and 10^6 cell g^{-1}) and active microbial communities down to several km depths, with theoretical studies suggesting that under favourable conditions it might reach 15-20 km depths (Power et al., 2018). Subsurface microbial communities have been shown to interact with biogeochemical cycles (D'Hondt et al., 2019; Magnabosco et al., 2019; Robador, 2024) and geological volatile cycling (Hinrichs and Boetius, 2003; Barry et al., 2019; Fullerton et al., 2021; Giovannelli et al., 2021; Rogers et al., 2022b) and are well known to impact fossil fuel reservoirs (Mcinerney et al., 2007; Hagar et al., 2022; Mu and Nazina, 2022). The theoretical maximum depth at which deep subsurface microbes can survive is related to the temperature gradient and available pore space. The current maximum temperature at which life can thrive in laboratory conditions is 122 °C (Takai et al., 2008) however in the environment maximum temperatures for life might reach 135-150 °C (Merino et al., 2019). Similarly, the range of parameters that defines the limits of life in the crust is quite large, and subsurface microbes pose a significant challenge to geological gas storage (Dopffel et al., 2021a; Thaysen et al., 2021a, 2023).

The ability of subsurface microorganisms to interact with hydrogen is relevant also to the natural hydrogen search as an energy source (Gregory et al., 2019b; Zgonnik, 2020a). Geological manifestations of hydrogen have been acknowledged for millennia (Zhao et al., 2023). While hydrogen (H_2) can be found as part of gas mixtures in a number of geological settings globally (see the MAGA gas database; Cardellini et al., 2020), the fundamental questions revolve around its existence as a volumetrically significant, discoverable, predictable, and exploitable energy resource. Preliminary investigations indicate that the characterization of potential hydrogen sources, the depths at which they are likely to be generated, the mechanisms governing their migration, and the factors influencing their retention or loss concerning the microbial utilization of this gas, could be an interesting and life-changing future challenge. While there have been an increasing number of reports of hydrogen-containing seeps (Larin et al., 2015; Prinzhofer et al., 2018; Frery et al., 2021; Milkov, 2022; Etiope, 2023), consensus over the diversity play connected with natural hydrogen exploitation is still lacking. Subsurface microorganisms might play a pivotal role in natural hydrogen research, as they might impact natural hydrogen migration and accumulation in the crust. Therefore, research into the ability of microorganisms to use hydrogen in the subsurface has important implications both in the Underground Hydrogen Storage sector as well as in the search for natural hydrogen.

This review will delve into the diversity of hydrogenotrophic metabolism in the subsurface, discussing their potential role in Underground Hydrogen Storage in diverse reservoirs. We start by discussing the diversity of enzymes used by microorganisms to interact with molecular hydrogen. These enzymes, called hydrogenases, are incredibly diverse and widespread among microorganisms and research suggests that hydrogen oxidation might be an ancestral metabolism that possibly appeared at the origin of life (Weiss et al., 2016; Martin and Thauer, 2017). We then present a list of hydrogenotrophic metabolisms relevant for subsurface hydrogen and discuss their relative importance in diverse UHS reservoirs and possible role in natural hydrogen. We conclude discussing the use of deeply-sourced springs as a model to understand the possible role of microorganism in subsurface hydrogen cyclings.

Hydrogen as a key resource for energy transition

Several countries have prepared or ratified joint strategies to achieve climate neutrality in the near future, presenting a vision to transition to an economy with net-zero greenhouse gas (GHG) emissions by mid-century (United Nations, 2016; Hale et al., 2022). The increase of the use of low-carbon electricity will play a critical role in decarbonizing the energy landscape, necessitating an increase in its share of

final energy consumption from the current 20 % to approximately 50 % by the latter half of the century (International Energy Agency, 2023). Crucially, electricity must be sourced from carbon-neutral systems. While nuclear energy and gas generation coupled with carbon capture technologies may contribute, additional technological domains are indispensable. Hydrogen (H_2) is considered a key compound for the energy transition due to its non-toxic nature, its high energy density and the production of water as a sole combustion product. Compared to methane (CH_4), hydrogen has lower molecular weight, lower viscosity and water solubility, a lower boiling point (−252.8 °C at atmospheric pressure) and a higher energy density per mass (120 kJ g^{-1}) but has a very low volumetric energy density of 8 MJ/L for liquid hydrogen at atmospheric pressure which further motivate the underground large storage space (Tarkowski and Uliasz-Misiak, 2021; Thiyagarajan et al., 2022).

Hydrogen production can be achieved through a variety of processes, e.g., electrolysis, steam methane reforming, gasification, thermochemical, photochemical, biochemical, and biological processes, that result in varying degrees of environmental sustainability (Miocic et al., 2023). At the same time, diverse energy sources can be used to produce electrical energy necessary for the water electrolysis process, with no involvement of fossil fuels, such as renewable energy sources or nuclear energy. Currently, the majority of hydrogen is produced from fossil fuels (Afanasev et al., 2024), mainly from steam methane reforming (SMR) without Carbon Capture Storage (CCS) technologies, referred to as "Gray hydrogen". The remaining is mainly obtained from coal or lignite usage (so-called "Black" and "Brown hydrogen", respectively). On the other hand of the spectrum, hydrogen produced from electrolysis from renewable energy ("Green hydrogen"), nuclear energy ("Pink hydrogen") or from direct stimulation of water rock interactions ("Orange hydrogen") aligning seamlessly with the global agenda to reduce greenhouse gas emissions (Incer-Valverde et al., 2023). Hydrogen produced from water electrolysis using electricity from renewable energy sources has been investigated in the last decade as one of the most promising technologies capable of near-zero carbon emissions (Incer-Valverde et al., 2023). Several challenges prevent the large-scale adoption of this technology, including the low efficiency of electrolyzers and challenges associated with the hydrogen transport and storage (Tarkowski and Uliasz-Misiak, 2022). Despite this, green hydrogen remains one of the most promising technologies for the rapid transition to a net-zero carbon society.

On the transitional front, a number of other hybrid hydrogen producing technologies have been developed associating hydrogen production to CCS technologies to offset the CO_2 produced. These approaches have expanded the rainbow of hydrogen (Incer-Valverde et al., 2023), and created hydrogen technologies "superfamilies", distinguishing low-carbon emission hydrogen technologies from traditional production processes. Among the most promising transitional technologies, the coupling of steam methane reforming

with CCS ("Blue hydrogen") is gaining momentum. While not entirely emission-free, this technology minimizes the environmental impact compared to conventional methods, and might be more easily implemented in current production. Another emerging technology, yet to be demonstrated on large-scale, is the generation of hydrogen through methane pyrolysis, a process that produces hydrogen and solid carbon ("Turquoise hydrogen")(Incer-Valverde et al., 2023). This hydrogen type has the potential to be a low-emission hydrogen source, provided the thermal process is powered by renewable energy and the solid carbon byproduct is permanently stored or repurposed.

Hydrogen is continuously produced by a variety of geological processes, including water-rock interactions, water radiolysis and the thermal and biological decay of deeply buried organic matter (Figure 1) (Wong et al., 2019; Wong et al., 2025). Hydrogen produced through these processes (known as geological hydrogen, natural hydrogen or "White hydrogen") can accumulate in crustal reservoirs and can be exploited as a clean energy source (Parnell and Blamey, 2017; I. Epelle et al., 2022; Milkov, 2022). While natural hydrogen production is currently represented by a single field (Bourabougou, Mali) (Prinzhofer et al., 2018), evidences for natural molecular hydrogen seepages are growing fast in different areas of the planet, such as Russia, USA and Brazil (Larin et al., 2015; Zgonnik et al., 2015; Prinzhofer et al., 2019). Natural hydrogen prospecting is a fast growing sector with an estimated global market value of 3.4 Billion Euro in 2023 with an anticipated CAGR of 9.2 % through 2029. Subsurface microbial communities might play a key role also in the natural hydrogen economy as they can alter or consume hydrogen in its geological reservoir significantly impacting the hydrogen migration and accumulation processes. For these reasons, a better understanding of the diverse hydrogenotrophic metabolisms is a key prerequisite to improve current natural hydrogen prospecting efforts. The knowledge presented in this review is equally relevant to Underground Hydrogen Storage and Natural hydrogen prospecting.

Besides the generation processes, the storage of the gas and its delivery issues expose the need for a comprehensive, end-to-end assessment of the whole pipeline: from the production of hydrogen gas to its final transformation into usable energy (Ishaq et al., 2022). Irrespective of the origin, the storage of large quantities of hydrogen may require UHS technologies to be developed, putting our understanding of the role of subsurface microbial communities with regards to hydrogen at the forefront of the energetic transition (Dopffel et al., 2021a; Zivar et al., 2021c; Miocic et al., 2023; Thaysen et al., 2023).

Subsurface geological storage is increasingly considered one of the more suitable spaces for storing hydrogen to be used as a carrier of energy (Figure 1) (Muhammed et al., 2022). Underground gas storage (UGS) facilities have many advantages over other surface solution, including: i) increased safety, as they are less vulnerable to fire, terrorist attacks, or military actions compared to surface storage; ii) space

efficiency as they require significantly less surface area than traditional surface storage allowing for easier integration with existing infrastructures; iii) increased cost-effectiveness since constructing UGS facilities is orders of magnitude more economical than building surface-based storage of comparable capacity. Available geological formations for UGS are widespread, making them appealing for decentralized storage, limiting the necessity for long-range transport (Plaat, 2009). Underground hydrogen storage facilities may be created at sites with suitable geological conditions with features allowing for suitable operations, such as the depth of the geological structure, formation thickness, tightness, reservoir pressure, porosity and permeability, geomechanical properties and proper characteristics of the insulating caprock (Tarkowski, 2019). The presence of spaces, either in rock pores or in large cavities, together with an impermeable caprock, is essential for an efficient hydrogen storage and maintenance in place (Tarkowski, 2019). The most accredited potential hydrogen storage types are porous rocks reservoirs, that include depleted natural gas and oil deposits and deep aquifers, and artificial underground spaces, such as salt caverns and decommissioned mines. Each storage type requires specific conditions to facilitate effective hydrogen storage.

Deposits of natural gas and petroleum occur in so-called geological traps (Jia, 2012; Bjørlykke, 2015; de Jager, 2020). These usually consist of a reservoir (the accumulation of hydrocarbons in the pore space of rocks such as sandstones or carbonates), its seal and an underlying aquifer. The rocks that seal the trap (low permeable and non-fractured) keep the hydrocarbons in the reservoir and do not allow them to migrate beyond its limits. The most common type of underground gas storage sites are depleted gas fields. Deposits of this type are usually equipped with necessary surface and subsurface installations, which may be repurposed for hydrogen storage (Muhammed et al., 2023; Perera, 2023; Sadkhan and Al-Mudhafar, 2024). Their adaptation to the needs of underground hydrogen storage allows costs to be reduced. Some geological criteria have to be met for a depleted hydrocarbon deposit to be used as an underground hydrogen storage site, where the safety of storage is the key issue (Jackson et al. 2024). Depleted oil deposits are not frequently converted to underground gas storage facilities because of concerns that large amounts of hydrogen may participate in chemical reactions with residual oil and formation fluids, become converted (for example, into CH_4), dissolve in the oil and become irreversibly lost (Sadkhan and Al-Mudhafar, 2024).

Hydrogen storage in deep aquifers is similar to that in depleted oil and gas deposits. Aquifers are porous and permeable rock formations which have the pore space occupied by brackish or saline waters (Jafari Raad et al., 2022; Amirthan and Perera, 2023). They are common in all sedimentary basins all over the world and they may present an alternative for underground hydrogen storage in those areas where depleted hydrocarbon fields or salt caverns are not available. Many of them are situated close to major

energy consumers or large cities and urban agglomerations. They have been safely used as natural gas storage sites for decades (Schultz and Evans, 2020; Jafari Raad et al., 2022). The reduced geological knowledge in comparison with depleted hydrocarbon fields, means there is greater uncertainty for storage integrity and potential hazards include possible migration of hydrogen along undetected rock fractures or poorly defined sealing caprocks, biochemical reactions or reactions of hydrogen with minerals in the reservoir rock.

Salt caverns (artificial chambers created through solution mining in halite deposits) are also suitable for storing gases under high pressures (Ozarslan, 2012; Caglayan et al., 2020; Li et al., 2024). UGS in salt formation is appealing due to the specific geological conditions that make the deposits tight, the favorable elastic behaviour of salt and its inertness to chemical reactions with most of the stored substances. Salt deposits of suitable thickness, purity and extent, enables the construction of underground storage facilities with multiple caverns hosting significant capacity. These specific properties of salt guarantee long-term stability and the tightness of storage. Hydrogen has been already successfully stored in several salt caverns in the UK and the US (Evans and Holloway, 2009; Caglayan et al., 2020; Jahanbakhsh et al., 2024). The presence of contaminations of non-salt rocks in the salt beds, as well as by highly soluble potassium-magnesium salts, may provide a migration path for the stored gas and might compromise the salt storage tightness (Minougou et al., 2023; Tarkowski et al., 2024).

Over the past century, the number and capacity of UGS facilities have consistently increased, particularly in the Northern Hemisphere. Referring to the IGU Storage Committee Database portal (http://ugs.igu.org/index.php/ugs_list/get_list) as of 2021, there are 690 operational UGS facilities worldwide, primarily located in gas fields (472), followed by salt caverns (101), aquifers (72), oil fields (42), rock caverns (3). The East and Midwest regions of the United States have the highest concentration of facilities, followed by the North and South Central region, CIS (Commonwealth of Independent States) countries, Europe, Asia (mainly China), Oceania and several other areas worldwide. According to more recent information by CEDIGAZ, the International Association for Natural Gas Information (Carnot-Gandolphe, 2016), by the end of 2023, the global working gas capacity of underground gas storage (UGS) reached 437 billion cubic meters (bcm), reflecting a 2 % year-on-year increase since 2015. This expansion was driven largely by significant capacity additions in China, with further contributions from Europe, Kazakhstan, and Canada. Globally, the number of operational UGS facilities reached 681, including five newly commissioned sites in China and the inauguration of Saudi Arabia's first facility. Additionally, the global peak withdrawal rate rose by 1.6 % to 7,516 million cubic meters per day (mcm/d). Finally, the underground gas storage (UGS) market is concentrated among a few key players, with the top five countries—the United States, Russia, Ukraine, Canada, and China—accounting for 68 %

of global capacity. China's ascent to fifth place, surpassing Germany, underscores the rapid growth of its gas market and signals a broader shift toward expanding markets in China and the Middle East. While depleted fields comprise 81 % of global working gas capacity, salt caverns, representing just 8 % of capacity, contribute 25 % of global gas delivered, playing a critical role in addressing short-term demand fluctuations. More detailed data is presented in "Underground Gas Storage in the World - 2024 Status", a reference report that builds on the CEDIGAZ Underground Gas Storage Database (Carnot-Gandolphe, 2016). Overall, the global landscape of underground gas storage is characterized by ongoing expansion and strategic investments.

Hydrogen as key energy source in microorganisms

Biology derives energy for its sustenance and growth from thermodynamically favourable chemical redox reactions (Hay Mele et al., 2023). Irrespective of the metabolism employed by the single organisms (not limited to microorganisms, but including animal and plant life), the oxidation of an electron donor is coupled to the reduction of an electron acceptor to derive reducing equivalents (i.e. electrons) and energy to be used for downstream metabolic needs. Life is able to control redox chemistry through a series of complex enzymes called oxidoreductases. The diversity of redox couples that life can use is astonishing (Jelen et al., 2016; Hay Mele et al., 2023), and include a large number of elements in the periodic table (Bastoni et al., 2024). While animal life relies on sugar and organic carbon as a source of electrons, and plants rely on high energy photons to extract electrons from water, microorganisms are capable of obtaining electrons from a large number of reduced compounds (Hay Mele et al., 2023). Molecular hydrogen (H_2) is a key electron donor for microbial redox reactions due to its negative redox potential ($E°' = -0.42$ V) making it easy to couple with a vast array of oxidants (called in biology "electron acceptors"). Additionally, it easily diffuses across biological membranes and it is widespread in diverse environments, generated through biological and geological processes.

Microorganisms are capable of interacting with H_2 through specialized oxidoreductases known as *hydrogenases* (Schwartz et al., 2013), which catalyze the reversible conversion between H_2 and protons, making them essential players in the biogeochemical cycle of hydrogen (Greening and Boyd, 2020). Microorganisms can oxidize H_2 in diverse ecosystems, ranging from hypoxic and anoxic H_2-enriched environments, like animal guts and subsurface environments, to oxygenated soils and waters where H_2 is present only in trace amounts (Greening et al., 2016). Globally, H_2 oxidation is coupled to a number of biogeochemically important metabolisms. For example, hydrogen produced by microbial fermentation in marine sediments is coupled to sulfate reduction and iron (III) reduction (Jørgensen et al., 2019).

Hydrogen oxidation is also responsible for the production of 20-50 % of the biogenic methane globally (Rosentreter et al., 2021). In several environments, the concentrations of free hydrogen are kept at the nanomolar level by the fast consumption of a large number of microbial groups (Chen et al., 2015; Adhikari et al., 2016). Hydrogen is also considered the ancestral electron donor, has been often cited as present in the Last Universal Common Ancestors and might have played a key role in controlling the emergence of life (Weiss et al., 2016). All this highlights the importance and versatility of hydrogen as electron donor for microorganisms, so much so that hydrogen is considered the energetic currency exchanged within the microbial world.

Hydrogen utilization is widespread across the Bacteria and Archaea domains. Besides the classic microorganisms requiring hydrogen as sole electron donor for their metabolisms (known as obligate hydrogen oxidizers), recent studies have highlighted the utilization of hydrogen as supplementary electron donor in aerobic microorganisms present in soil and surface ocean waters (Greening et al., 2022). These microorganisms, represented by members of the Actinobacteria and Acidobacteria phyla, are capable of scavenging H_2 present in trace amounts in the atmosphere (~530 ppbv), challenging the conventional notion that hydrogen metabolism is limited to environments with low O_2 and high H_2 concentrations (Greening and Cook, 2014). These investigations have provided biochemical insights into mechanisms that enable certain hydrogenases to function in the presence of oxygen, which was traditionally considered an inhibitor of their active sites (Shomura et al., 2011; Schwartz et al., 2013; Horch et al., 2015). Minimalistic hydrogenase-containing respiratory chains have been identified in anaerobic systems, demonstrating their involvement in efficient energy generation within oligotrophic environments (Kim et al., 2010; Lim et al., 2014).

Hydrogenases are classified into three types based on their metal cofactors as [NiFe]-hydrogenases, [FeFe]-hydrogenases and [Fe]-only hydrogenases (Figure 2) (Vignais et al., 2001). These three types in turn can be classified in 39 different groups based on their sequence similarity, structure and function (Table 1) compiled from the literature and available sources (Greening et al., 2016, 2024; Søndergaard et al., 2016). Understanding the diversity, functions and distribution of these enzymes is crucial for unraveling ecological processes in natural and engineered environments and their implications for UHS. Hydrogenases catalyze the reversible oxidation of H_2 to H^+ and electrons according to the following reaction:

$$H_2 \leftrightarrow 2H^+ + 2e^-$$

The electrons are transferred one by one to an external electron carrier (such as membrane quinones) via iron-sulfur clusters present within the enzyme (Figure 3) or utilized directly within a multimeric complex

to reduce other molecules such as NAD^+ or ferredoxin. As such, hydrogenase groups came in a variety of shapes and tertiary and quaternary structures, being part of relatively simple enzymes or large multimeric and multifunctional complexes (Figure 3 and Table 1). The different groups of hydrogenases are characterized by diverse functions, that include hydrogen utilization as electron donor, hydrogen sensing and uptake, and hydrogen production (Table 1). [NiFe]-hydrogenases are capable of all known functions and are among the most diverse types present in all domains of life (Bacteria, Archaea and Eukarya). Crystal structures of [NiFe]-hydrogenases (Figures 2 and 3) show that the iron ion in the dinuclear centers is coordinated by sulfur (from a cysteine amino acid residue), carbon monoxide (CO), and cyanide (CN) ligands while the nickel is coordinated by 4 cysteines; 3 and a modified seleno-cysteine in the case of [NiFeSe] (Volbeda et al., 1995, 2005). [FeFe]-hydrogenase are involved in hydrogen production and sensing in Bacteria and Eukaryotes (Figure 2 and 3) (Peters et al., 1998; Nicolet et al., 1999, 2001; Bennett et al., 2000). Both iron atoms in the dinuclear center are coordinated by thiol groups from the dithiolate ligand, as well as CO and CN ligands. The nature of the bridge in the dithiolate ligand was unknown, thought to be either CH_2, NH or O (Grigoropoulos and Szilagyi, 2010), but it was later shown that NH is likely the naturally occurring group (Berggren et al. 2013, Birrel et al. 2021). [Fe]-only hydrogenases instead, are present exclusively in methanogens (Archaea) and catalyze hydrogen uptake coupled to methenyl-tetrahydromethanopterin (methenyl-H_4MPT+) reduction (Schwörer et al., 1993; Hartmann et al., 1996; Lyon et al., 2004), a key step in the methane-generation pathway that characterizes these microorganisms. [Fe]-only hydrogenase active site contains a labile light-sensitive cofactor with a mononuclear low-spin iron that binds two CO ligands, and lacks the iron-sulfur clusters cubic centers (Figures 2 and 3) (Korbas et al., 2006).

All hydrogenases could be classified into eight previously described major lineages or supergroups (Greening et al. 2016; Vignais and Billoud, 2007; Calusinska et al., 2010): supergroups 1 to 4 for [NiFe]-hydrogenases, supergroups A to C for the [FeFe]-hydrogenases and a single supergroup for [Fe]-hydrogenases (Figure 4 and Table 1). Despite the similarities in structure and functions, phylogenetic analysis suggests that the three hydrogenase types have originated independently (Figure 4) (Vignais et al., 2001; Peters et al., 2015), and that the major supergroups within each hydrogenase type represent phylogenetically distinct evolutionary radiations. Among the different hydrogenases, [NiFe]-hydrogenases are the most widespread and well-studied. Each supergroup exhibits unique structural and functional characteristics. Supergroup 1 and 2 [NiFe]-hydrogenases are typically found in aerobic bacteria and some anaerobes, while supergroup 3 is prevalent in anaerobes. Supergroup 4 [NiFe]-hydrogenase enzymes are predicted to be the most abundant supergroup of hydrogenase on the planet, detected in both bacteria and archaea, which preferentially generates hydrogen exploiting various

partner enzymes or ferredoxin (Finney and Sargent, 2019). [FeFe]-hydrogenases, supergroups A to C, primarily found in numerous anaerobic microorganisms, are also present in all domains of life (Peters et al., 2015; Greening et al., 2016, 2024), exhibiting exceptional catalytic efficiency and playing a crucial role in the microbial production of H_2 anoxic environments. The process of H_2 evolution efficiently releases excess reducing agents as a diffusible gas during microbial fermentation and photobiological processes (Schwartz et al., 2013). [Fe]-hydrogenases are numerically less diverse and are exclusively found in methanogenic Archaea microorganisms. They often contain multiple types of hydrogenases simultaneously, allowing them to sense and interact with H_2 under diverse environmental and physiological conditions.

Despite significant progress, many aspects of microbial H_2 metabolism remain unexplored at the enzymatic, cellular and ecological levels. Detailed phylogenetic and biochemical informations, and atomic-resolution structures are available for only a subset of hydrogenases (Table 2) (Volbeda et al., 1995; Peters et al., 1998; Shima et al., 2008; Fritsch et al., 2011; Mills et al., 2013). For example, recent advancements in sequencing technologies have expanded genome and metagenome sequence data have provided important insights into the phylogeny and diversity of hydrogenases (Vignais and Billoud, 2007; Peters et al., 2015; Greening et al., 2016). The recent analysis of publicly available genome and metagenome resources have revealed that H_2 metabolism is more diverse and widespread at both taxonomic and community levels than previously recognized (Greening et al., 2016). Specifically, [NiFe]-hydrogenases exhibited the broadest distribution, being consistently found in all classes of Proteobacteria, as well as in Firmicutes, Cyanobacteria, Aquificae, Euryarchaeota, and Crenarchaeota. [NiFe]-hydrogenases were also found in phyla lacking previous hydrogenase descriptions, including Bacteroidetes, Chlorobi, Chloroflexi, Planctomycetes, and Verrucomicrobia. Putative group A1 [FeFe]-hydrogenases were detected in anaerobic bacteria, unicellular eukaryotes, and a number of archaea, mainly from uncultured lineages (Greening et al., 2024). Other [FeFe]-hydrogenase types, encompassing bifurcating, ancestral, and sensory variants, were exclusive to anaerobic bacteria such as member of the phyla Firmicutes, Bacteroidetes, Spirochaetes, Thermotogae, and Fusobacteria. In contrast, genes encoding the functionally restricted [Fe]-hydrogenases were exclusively found within methanogens genomes (Greening et al., 2016). The phylogenetic distribution of hydrogenases seems to be strongly correlated with oxygen preference: mid-branching heterotetrameric hydrogenases (groups 1c, 1e, 1g) show broader taxonomic dispersion, supporting roles in various metabolic processes across diverse taxa. Conversely, more recent branching lineages (groups 1d, 1h, and 2a) appear to be oxygen-tolerant enzymes involved in respiration in aerobes and facultative anaerobes. These subgroups were predominant in aerated samples, suggesting independent development of O_2 tolerance mechanisms after the emergence of

oxygenic photosynthesis. This data suggests that the O_2-sensitive deepest-branching forms (groups 1a, 1b) are involved in anaerobic respiration in strictly anaerobes, prevalent in hypoxic soils and subsurface. Metagenomic analyses on the hydrogenase distribution in the cold desert soils of continental Antarctica, showed that the most abundant and prevalent microorganisms are metabolically versatile aerobes that use atmospheric hydrogen to support aerobic respiration, through a phylogenetically and structurally distinct enzyme, the latest new group 1l [NiFe]-hydrogenase, encoded by nine bacterial phyla (Ortiz et al., 2021). Although the importance of H_2 metabolism in certain environments is well recognized, the precise function of hydrogenases in overall soil and aquatic ecosystems remains largely unclear (Barz et al., 2010; Constant et al., 2011; Beimgraben et al., 2014; Greening et al., 2015). Recent evidence suggests that H_2 serves as a crucial energy source for marine communities (Lappan et al., 2023). Through a combination of biogeochemical, metagenomic, and thermodynamic modeling analyses, they demonstrated that a diverse yet limited subset of community members oxidize H_2 at rates conducive to lithotrophic growth. Specifically, groups 1d, 1l and 2a [NiFe]-hydrogenases, enabling cells to uptake electrons from H_2 into the aerobic respiratory chain (Podzuweit et al., 1986; Søndergaard et al., 2016; Islam et al., 2020; Ortiz et al., 2021), are by far the most abundant among the H_2-oxidizing enzymes detected in surface seawater samples (Lappan et al., 2023).

The complexity of the molecular machinery utilized by microorganisms to interact with H_2 highlights the key role that these molecules play in diverse ecosystems. Investigating the role that microbial communities have in the global hydrogen biogeochemical cycling is fundamental to achieve a deeper knowledge about our planet functioning and to access important biotechnological applications and innovative strategies in energy research. For example, biologically produced hydrogen from diverse biomasses through fermentation and biophotolysis might constitute an important source to add to the portfolio of low-carbon hydrogen production processes (Akhlaghi and Najafpour-Darzi, 2020; Baeyens et al., 2020; Hitam and Jalil, 2023). At the same time, the ability of microorganisms to efficiently utilize H_2 in trace amounts in the atmosphere might provide a solution to the potential threats generated by the leak of H_2 as greenhouse gas into the atmosphere as a result of increased societal usage (Conrad, 1995; Greening et al., 2015; Greening and Grinter, 2022). Current models assume that about 70 % of the hydrogen leaked into the atmosphere will be "uptaken by soils", that is, consumed by soil microorganisms (Constant et al., 2009; Bertagni et al., 2022; Ocko and Hamburg, 2022; Sand et al., 2023). However data on the actual utilization of hydrogen by soil microorganisms, by ocean and subsurface microorganisms as well, is severely limited, and the actual fate of hydrogen leaked into the atmosphere as a result of the transition toward an hydrogen economy remains to be investigated.

The limits of life in the subsurface and their relevance for an hydrogen economy

The limits of life are defined as the range of parameters beyond which life cannot sustain itself (Merino et al. 2019). This is a rather complex concept for a number of reason: i) the limits of life are a moving target, i.e. the actual absolute values at which life ceases to exist have been updated over the years as new knowledge about microorganisms is gained; ii) while we tend to think about the limit of life in term of major physico-chemical parameters, life boundary conditions are defined by a long list of necessary elements and conditions, each of which acts in combination with the other; and iii) the limits of life can be a relative concept depending on the boundary we are defining. For example, the current absolute limits of life are very different to the limit of phototrophic life and might be—and most probably will be—different for any other metabolic or taxonomic group. Let's explore them individually before delving in the major parameters controlling microbial life in the subsurface.

Our knowledge of life on Earth is constantly expanding, and microorganisms —especially those inhabiting the subsurface (Lloyd et al., 2018)— are the main reservoir of unknown genetic and physiological diversity. Given that our current approaches only allow us to directly culture a severely limited number of environmental microorganisms (Lloyd et al., 2018), it is possible that life might thrive in conditions beyond the current recognized limits of life. Take for example the control that temperature imposes on the limits of life. The current highest temperature at which life can be sustained in the lab is 122 °C, the temperature at which the hyperthermophilic and piezophilic methanogen *Methanopyrus kandleri* strain 116 can be cultivated (Takai et al., 2008). Before this discovery, the known temperature limits of life have changed rapidly in the span of 50 years, moving from ~70 °C (Pasteur, 1880) believed to be the limits until ~1960 (Brock, 1967). While theoretical reason have been set forth to support the known temperature limits at the time (Blöchl et al., 1997), subsequent discoveries and the isolation of new microorganisms from extreme environments has pushed the boundary of temperature from ~70 °C (Pasteur, 1880), 85 °C (Brock et al., 1972), 89 °C (Huber et al., 1998), 97 °C (Stetter et al., 1981), 110 °C (Stetter, 1982), 113 °C (Blöchl et al., 1997) and 122 (Takai et al., 2008) in the span of 50 years. Environmental observation always preceded the isolation of new record holder microorganisms, with yellowstone observation of the presence of bacteria at 89 °C in 1903 (Setchell, 1903), bacteria living in boiling pools in Yellowstone (Brock, 1967) and of microorganisms at temperatures above 100 °C at deep sea hydrothermal vents in 1981 (Baross et al., 1982). While the temperature limit for life in natural environments is debated, observations about the hyperthermophilic archaea *Geogemma barossii* strain 121 show that this microorganism is viable but not growing at temperature of 130 °C (Blöchl et al., 1997),

environmental studies suggest that the limit in the subsurface is well above 120 °C (Heuer et al., 2020). Theoretical work suggests that 135-150 °C might provide a biophysical limit to the stability of essential molecules (e.g. ATP) (Daniel* and Cowan, 2000; Cowan, 2004; Merino et al., 2019), however future discoveries might raise this limit further, as it has already happened in the past. A similar progressive expansion of the limits of life in the last 50-70 years can be drawn for every physico-chemical parameter.

While the classic limits of life are defined in terms of fundamental physico-chemical factors such as temperature, pressure, pH and salinity, life requires a diverse set of conditions and a long list of elements and molecules to thrive, each of which might impose limits on its existence. For example the availability of suitable carbon sources, either organic or inorganic, can severely limit the existence of life in certain conditions. Similarly, the presence of specific types of salts, such as chaotropic salts, might be a stronger limiting parameter rather than the absolute salinity (Yakimov et al., 2015; Belilla et al., 2019). Related to the subsurface, the availability of living space, in the form of pore space and fractures, and connectivity between spaces to avoid single organism isolations might be a prerequisite for life existence. Additionally it is worth noting that parameters interact in further expanding or constraining the ecological niche under which life might thrive. Classic examples are the ability of life to survive above 100 °C in the presence of high pressure preventing water from boiling and the ability to survive at temperatures below 0 °C in the presence of high salt concentrations preventing water from freezing (Merino et al., 2019). Similarly, high temperatures and high concentrations of salts, as well as their chemical composition, might impose controls limiting life presence to a narrower range of conditions than if confronted in isolation (Belilla et al., 2019). The contraction and expansion of absolute and specific niches due to the combinations of different parameters is difficult to predict, and might pose a challenge to modelling efforts required to assess the potential effect of microbiology on UHS and natural hydrogen accumulation.

In addition, the limits of life are both absolute, in the way they pertain to all life as we know it, and a relative concept when used in the context of diverse definitions of "living" and "thriving" as well as when applied to diverse metabolic or taxonomic groups. A well known example is the temperature limits of life that move from 122 °C in the lab, when considering all known life to 72–74 °C, when considering phototrophic organisms (Ferrera and Reysenbach, 2007), a limit connected with the thermal stability of the required photosynthetic pigments. Similar changes in the limits might be present—and most probably are present—for many taxonomic and metabolic groups. Understanding where these relative limits lie is fundamental in understanding the effect that microbial life might have on UHS (Thaysen et al., 2021a) as well as natural hydrogen prospecting (Zhao et al., 2023). Defining these relative limits is however challenging, especially in an environmental context where surviving and thriving under certain conditions might take different meanings. Under laboratory conditions the most commonly used parameter to

investigate microbial fitness is microbial growth, measured through the duplication of the population. However, microorganisms in natural ecosystems have been shown to thrive without the need of entering an exponential growth phase, challenging the notion that growth is the main parameter to be considered when investigating microbial fitness under selected conditions (Zhao et al., 2023; Boyd et al., 2024). Similarly, microorganisms might be present in the environment in resting stages that might allow them to survive much harsher conditions. For example sporulation, a resting strategy common in numerous environmentally relevant microorganisms in the Firmicutes phylum, allows these mesophilic and moderately thermophilic bacteria to survive for extended periods of time to temperatures up to 131 °C (Wells-Bennik et al., 2016), and after dehydration some spores have been reported to withstand temperatures as high as 420 °C (Beladjal et al., 2018).

Considering all of the above, the limits of life remain an elusive concept that has important implications for UHS and natural hydrogen prospecting and exploitation. When considered together in the context of geological hydrogen storage (Table 2), culture based and environmental based limits of life far exceed the average reservoir conditions found in the literature (Figure 5), suggesting that the identification of the limits of life for specific metabolic groups of interest might be more promising in yielding usable information during UHS site selection or natural hydrogen prospecting. While conditions falling outside the known limits are present in the crust, their reliability regarding the absence of microbial effects needs to be balanced against operational costs to represent viable sites for UHS (Dopffel et al., 2021a). The current limits of life derived from culture-dependent approaches for the classic physico-chemical parameters (namely -15–122 °C, 0–12.4 pH, 1–125 MPa and up to saturation limits for NaCl concentrations) define large boundary conditions for life existence (Figure 5A). In the subsurface, a few additional parameters might contribute to determine the limits of life and the extent to which life might be relevant for UHS and natural hydrogen operations.

Life in the subsurface has been demonstrated to occur in every known lithology, with cell concentrations between 10^2 and 10^6 cell g^{-1} of rocks down to several km depths. The depth to which subsurface microbial communities extend to is directly related to the space and time they have available to interact with geological volatiles (Giovannelli and Lloyd, 2024), and can directly influence the interaction of subsurface microorganisms with stored or accumulated hydrogen in the crust. Generally speaking, the depth of the subsurface biosphere will be limited primarily by the depth of the isotherms, considering a conservative upper temperature for life of 135 °C. This corresponds to a depth of approximately 4 km considering a common geothermal gradient of 30 °C km^{-1} (Nash et al., 2022), which puts this limit beyond sustainable operations unless placed in a context with much higher geothermal gradients (>80 °C km^{-1} to have the 135 °C isotherm at depth of less than 1.5 km). In addition to temperature, available living space

and its connectivity has been proved to be fundamental for the existence of a subsurface biosphere. Highly porous lithologies, or lithologies highly fractured, have been shown to contain higher abundances of cells (Gold, 1992; Männik et al., 2009; Nielsen and Fisk, 2010). By contrast unfractured crystalline rocks are generally considered sterile. Unfortunately, the presence of high porosity and permeability is a prerequisite for UHS operations, overlapping with the requirements for life in the subsurface. Beyond these two physical parameters (i.e., temperature and available space), life can withstand virtually all the conditions found in the shallow crust, often surviving well beyond, for example when surviving GPa pressure exposure (Merino et al., 2019). The availability of suitable electron donors and acceptors for metabolic redox reactions might impose a strong control on the presence and survival of subsurface microorganisms, together with the availability of essential trace metals required by life to interact with specific redox couples (Giovannelli, 2023; Bastoni et al., 2024).

Storage temperatures for hydrogen in depleted oil/gas fields and saline aquifers and caverns range from about 20 to 100 °C, within a depth range of 500 to 2000 m (Matos et al., 2019; Hassanpouryouzband et al., 2020; Shi et al., 2020). At elevated temperatures, thermophiles and hyperthermophiles face challenges associated with increased reaction rates. The rapid abiotic reactions may negate any benefits to microorganisms catalyzing the reactions, prompting high-temperature-adapted microorganisms to produce enzymes with faster reaction rates (Steinweg et al., 2013). Hydrogenotrophic methanogens span the entire range of temperatures, from mesophilic conditions to 122 °C (Liu and Whitman, 2008; Hagen et al., 2016; Maus et al., 2016; Bu et al., 2018; Holden and Sistu, 2023); (Kurr et al., 1991; Takai et al., 2008). Homoacetogens show optimum growth temperatures from 20 to 70–72 °C (Leigh et al., 1981; Basen and Müller, 2017; Basen et al., 2018). Cultivated sulfur species-reducing microorganisms exhibit optimum growth temperatures from 20–30 °C to 113 °C (*Pyrolobus fumarii*).

On the other hand, a net upper salinity limit to microbial activity has not been established (Yakimov et al., 2015; Merino et al., 2019). It appears to be the brine composition, rather than the salinity alone, that puts a hard limit on microbial growth (Singh, 2016; Payler et al., 2019). During the water evaporation process, small parts of the brine become trapped in the salt and end up as fluid inclusions. Any halophilic microorganisms present in the fluid will be trapped as well and subsequently freed during the process of solution mining. For example, the sulfate minerals in Permian salts are potential electron acceptors for microbial energy generation, while the presence of carbonate rock may provide an inorganic carbon source for anabolism and cell growth (Vreeland et al., 2000). The typical salt concentration in hydrogen storage sites, such as depleted gas/oil reservoirs and deep aquifers, ranges from 0 to 3 M NaCl (Buttinelli et al., 2011; Shi et al., 2020; Delshad et al., 2023), at which highly diverse microbial communities can live (Rothschild and Mancinelli, 2001; Telesh et al., 2013; Merino et al., 2019). Hydrogenotrophic

methanogens cultivated in these conditions generally prefer salt concentrations up to 0.77 M NaCl, very close to the salinity of seawater. However, few strains demonstrate resilience under more halophilic conditions, of which two correspond to extremely halophilic mesophilic hydrogenotrophic methanogens, tolerating salt concentrations of approximately 3.3–3.4 M (Ollivier et al., 1998; Zhilina et al., 2013). The majority of cultivated single-strain sulfate-reducing mesophiles (SSRM) thrive optimally at low salinities between 0 and 0.4 M NaCl. Nonetheless, some SSRM strains, including thirty-four mesophiles, exhibit the capability to grow at NaCl concentrations up to 1.7 M (OLLIVIER et al., 1991; Krekeler et al., 1997; Jakobsen et al., 2006). Studies on the salt tolerance of homoacetogens are limited, with cultivated strains showing low optimum salinities of 0–0.4 M NaCl and upper growth temperatures of 42 and 47 °C, respectively (Zhilina and Zavarzin, 1990; Zhilina et al., 1996). The pH of the brine determines the *in situ* environmental redox conditions, with an important outcome on the microbial growth and metabolic activity. Most methanogens and homoacetogens are adapted to a pH range of 6.5–7.5, with the inability to grow outside the pH range of 4–9.5 for most of them (Bu et al., 2018). Only seven known methanogens can withstand a critical pH of 10, and only nine known methanogens can withstand acidic conditions of pH <5 (Takai et al., 2002; Bräuer et al., 2011; Ganzert et al., 2014; Stewart et al., 2015). SSRM are considered more widely adapted to different pH conditions, ranging from 1 to 10.7 values of pH (Thaysen et al., 2021a). Generally, pH values below 7 favor the growth of methanogens over sulfate reducers, whereas above pH 7.5, sulfate reducers tend to outcompete methanogens (O'Flaherty et al., 1998).

Reported pressure conditions for H_2 storage range from 5–20 MPa (Matos et al., 2019) to 1–50 MPa (Shi et al., 2020). On Earth's surface, pressure spans from 0.1 to 125 MPa, with elevated pressures documented in subsurface settings, where it has been estimated that microbial life could thrive at pressures around 340 MPa (Merino et al., 2019). While an upper pressure limit for H_2 storage microbial life has not been established (Merino et al., 2019; Dopffel et al., 2021b), studies indicate that high pressures in crustal pore spaces are generally less inhibitory to microbial cellular activity than high temperatures (Yernazarova et al., 2016). Growth of various mesophilic microorganisms adapted to atmospheric pressure is inhibited at 30–50 MPa, whereas pressure effects are favorable for hyperthermophiles. Microorganisms thriving optimally at 10 MPa or above are obligate and facultative piezophiles (Rothschild and Mancinelli, 2001; Merino et al., 2019). Most mesophiles and thermophiles from habitats with pressures <50 MPa can grow in enrichment cultures at atmospheric pressure (Payler et al., 2019). The majority of identified cultivated piezophiles are psychrophiles, with limited relevance to our study. Only four mesophilic strains, three of them hydrogenotrophic sulfate reducers, were reported, growing optimally at 10–40 MPa. Thermophiles such as the hydrogenotrophic methanogen *Methanococcus thermolithotrophicus*, were identified, with optimal growth at 50 MPa (Bernhardt et al., 1988). The hyperthermophilic group includes

hydrogenotrophic *Methanopyrus kandleri* and *Methanocaldococcus jannaschii*, growing optimally at 20–75 MPa, respectively (Salwan and Sharma, 2020). A temperature-dependent pressure response was reported for the SSRM *Desulfovibrio indonesiensis*, exhibiting similar growth rates at high and ambient pressure (45 °C) but reducing growth rate at 20 °C and 30 MPa compared to 0.1 MPa (Fichtel et al., 2015). Pressures above 1 bar of carbon dioxide can be toxic for some microorganisms, including SSRM and methanogens (Dupraz et al., 2013). Oxygen is also toxic for many anaerobic methanogens and homoacetogens (such as members belonging to the genus *Clostridium*) (Lu and Imlay, 2021).

The microbial diversity of potential UHS sites

The assessment of the risks associated with hydrogen loss requires the knowledge of biotic and abiotic processes occurring in storage sites, in order to estimate hydrogen production and consumption rates (Gregory et al., 2019a). When hydrogen is stored underground, the microbial communities present *in situ* can use it as an electron donor for anaerobic metabolisms, consuming important fractions of stored hydrogen (Hagemann et al., 2016). Research carried out in the past 30 years has unveiled a large subsurface ecosystem (Magnabosco et al., 2018b) capable of interacting with the deep carbon cycle and volatiles present or recycled in Earth's crust (Fullerton et al., 2021). When there are suitable electron acceptors, hydrogen concentration can control the speed of microbial metabolic reactions occurring: this suggests that the high amount of hydrogen injected in a storage site could accelerate the metabolic reactions (Zivar et al., 2021b).

Very diverse microorganisms have been isolated and/or detected from oil fields, such as aerobic, microaerophilic and anaerobic microorganisms. Among the aerobic bacteria, *Pseudomonas* and *Acinetobacter* are known to be oil degraders (van Beilen et al., 1994; Bach et al., 2003). Bacteria related to the genera *Xanthobacter polyaromaticivorans* and *Stenotrophomonas sp.* are also detected in such environments and are known to be capable of growing in presence of molecular oxygen (Hirano et al., 2004; Yoshida et al., 2005). Another microbe that can grow using oxygen as an electron acceptor is *Shewanella putrefaciens*, isolated from an oil field site (Yusupova et al., 2020). Members of this species are defined as facultative anaerobic because of their additional sulfur- and iron-reducing properties (Nazina et al., 2007). Microaerophilic microorganisms belonging to the genera *Campylobacter*, *Oceanospirillum*, *Thiomicrospira* and *Geobacillus* are also known to inhabit petroleum reservoirs (Magot et al., 2000; Singh and Choudhary, 2021). In particular *Geobacillus* is a microaerophilic thermophile able to degrade alkanes under strict oxic conditions, but there are some strains known as nitrate reducers under

anoxic conditions. The great majority of the oil field microbial community is dominated by strictly anaerobic microorganisms such as *Methanolobus*, *Archaeoglobus*, *Desulfosarcina*, *Deferribacter*, *Oceanospirillum* and *Thiomicrospira* (Voordouw et al., 1996; Greene et al., 1997; Hubert et al., 2003; Li et al., 2017).

Although we know that these microbial groups mainly include fermentative bacteria, sulfate reducing bacteria (SRB), iron reducing bacteria, syntrophic bacteria and methanogens (Beeder et al., 1994; Orphan et al., 2001; Dopffel et al., 2021b), detailed reports about their *in situ* metabolic activities are currently missing. Microbial activity can cause the formation of biofilms, which may affect the porosity and permeability of the storage formation. Mineral precipitation or dissolution caused both by abiotic and biotic processes may have the same effect (Truche et al., 2010; Etiope et al., 2011; Zivar et al., 2021b). The possible negative implications are hydrogen flow path blockage, pores clogging, corrosion of the infrastructures by H_2S, losing caprock integrity, and the subsequent hydrogen leakage from the caprock (Dopffel et al., 2021b; Heinemann et al., 2021; Thaysen et al., 2021b; Zivar et al., 2021b). Despite many studies have reported alterations in gas composition, microbial diversity, and biotic/abiotic clogging affecting injection wells, very few have presented quantitative data on microbial growth or permeability modifications over time (Shafahi and Vafai, 2009; G. Haddad et al., 2022).

Additionally, the lack of standardized and well described sampling strategies makes it impossible to be sure that the detected microbiome, often sampled at the wellhead or in proximity of the separator for the formation waters, is representative of the in situ subsurface community rather than representing a subset or being the result of near-surface and handling contaminations. There is also the probability that microorganisms could have been introduced into the reservoir by waterflooding or other operations during production (Matz et al., 1992). To date, available data suggest that especially the strict anaerobes could be considered as indigenous of oil reservoirs since the analysis of the genome of some isolates confirms their adaptation to those peculiar conditions (Bernard and Connan, 1992; Magot, 2005). In addition, the detection of taxonomically close microorganisms in similar oil fields (Grassia et al. 1996; Magot 1996) supports the existence of a widespread anaerobic microbial community in such environments. To confirm this data, a significant amount of similar studies need to be carried out.

Aquifers are considered the second-most economically-attractive option for geological hydrogen storage after depleted oil and gas reservoirs (Jafari Raad et al., 2022). Aquifer storage operations do not involve freshwater injection nor the disposal of brines if compared to depleted reservoirs, and the decennal experience gained in the storage of town hall gas makes them an attractive alternative. On the other hand, subsurface aquifers, even deep ones (i.e., with depth below 800 meters) harbor diverse microbial

communities that might impact stored hydrogen. Depending on storage formation and physico-chemical properties such as temperature, pressure, pH, and salinity, different groups of microorganisms in common with the natural gas reservoirs (e.g., methanogens, sulfate-reducers, homo-acetogens, and iron-reducing bacteria), could be stimulated during UHS operations (Heinemann et al., 2021; Thaysen et al., 2021a; G. Haddad et al., 2022).

Salt caverns are thought to be the best solution for short and medium-term hydrogen storage (Böttcher et al., 2017; Heinemann et al., 2021). Salt crystals are formed when saline water evaporates and since microorganisms can be included in these geological formations (Vreeland et al., 1998; Park et al., 2009), it is relatively easy to detect them during the process of solution mining. The sulfate minerals in Permian salts are potential electron acceptors for microbial energy generation, while the presence of carbonate rock may provide an inorganic carbon source for anabolism and cell growth. This allows the assumption that autotrophic microbial life is feasible under certain circumstances, given the presence of a potent electron donor such as the H_2. Microbial groups such as *Halobacteria*, *Halanaerobiales*, *Balneolales, Halanaerobiaeota, Proteobacteria*, *Halobacterota*, *Bacteroidota*, *Firmicutes*, *Actinobacteriota*, and *Desulfobacterota* have been detected in these environments (Schwab et al., 2022). They are able to live at high salt concentration conditions (Oren, 2014) and/or saturated solutions (Fendrihan et al., 2006). Some of them are homo-acetogenic microorganisms and strict anaerobes able to grow autotrophically on H_2/CO_2 or CO, and heterotrophically on a wide range of sugars, alcohols, methoxylated aromatic compounds and one carbon compounds, yielding acetate as their sole metabolic end-product. Their presence in the salt caverns might promote H_2 oxidation by halophilic sulfate reducers. Hydrogenotrophic methanogens may also compete for H_2, since to date they have been detected in hypersaline environments such as solar salterns, soda and salt lakes (Foti et al., 2007; Mcgenity and Sorokin, 2018; Pal et al., 2020) and deep-sea salt-saturated formation (Yakimov et al., 2013). This suggests that, even at salinities approaching to saturation, the reduction of sulfur species is possible, and anaerobic microorganisms, including methanogens and also sulfate reducers (Foti et al., 2007), can be enriched from such environments (Sorokin, 2011; Blum et al., 2012; Sorokin et al., 2017).

Coherently with these studies, a 250 million-year-old halotolerant bacterium has been isolated for the first time from a salt mine constructed 650 meters below the ground surface in the geologically stable Permian age Salado salt formation (Vreeland et al., 2000). These observations suggest that some microorganisms may be active over geologic time scales (Lloyd, 2021) and might be stimulated during UHS operations.

Hydrogenotrophic metabolisms in the subsurface

Microorganisms are known to couple the oxidation of hydrogen to the reduction of a large number of oxidized elements and molecules (Table 3 and Supplementary Table 3). The diversity of possible electron acceptors that can be coupled to hydrogen oxidation is astonishing, and goes well beyond the classically investigated examples in UHS environments such as CO_2, SO_4^{2-} and Fe^{3+} (Rathnayaka and Ranjith, 2024; Wu et al., 2024). For this review we have limited ourselves to the most common molecules of carbon, nitrogen, oxygen, sulfur, manganese and iron, in the form of some of the most common minerals (Table 3 and Supplementary Table S3). However, it must be clear that microorganisms can use hundred of molecules and tens of elements as electron acceptors, including but not limited to fumarate, acetate, tellurate (TeO_4^{2-}), selenate (SeO_4^{2-}), arsenate (AsO_4^{3-}), chromate (CrO_4^{2-}), uranate (UO_4^{2-}) and dozens of minerals (Heim, 2011). These electron acceptors are deemed to be of secondary relevance to UHS and natural hydrogen operations unless locally abundant due to the specific geological settings. Additionally, several known intermediates of sulfur metabolism can be used as electron acceptors by diverse microorganisms. These include polysulfide, tetrathionate, sulfite and dimethylsulfide and many others that are not explicitly included in this review.

Hydrogen holds a pivotal role in the energy metabolism of underground life forms (Colman et al., 2017). Numerous recent reviews have pointed out the relevant role of microbial activity in geological H_2 storage sites (Gregory et al., 2019a; Ranchou-Peyruse et al., 2019; Strobel et al., 2020; Dopffel et al., 2021b; Aftab et al., 2022). The majority of available reviews about hydrogen storage in engineered environments focuses on porous media (Ranchou-Peyruse et al., 2019; Strobel et al., 2020; Heinemann et al., 2021; Perera, 2023; Vasile, 2024), including depleted porous reservoirs and deep aquifers. Recent attention is posed on hydrogen storage in salt caverns and the potential microbiology affecting the technology (Haratian et al., 2022; Schwab et al., 2022; Dopffel et al., 2024). On the other hand, the subsurface environments have gained much more attention from the scientific community (Gregory et al. 2019, Rachel C Beaver, Josh D Neufeld 2024, Francis H. Chapelle et al. 2002) and the natural hydrogen seeps as well, considered as analogues for engineered environments where hydrogen is artificially injected (McMahon et al., 2023).

The oxidation of hydrogen using a variety of electron acceptors yields different energy depending on the redox couple and the environmental conditions (Table 3 and Supplementary Table S3). Oxygen respiration is the most energetic metabolism in reservoir conditions (ΔG° -227.71 kJ/mol at 60 °C and 30 MPa), while the reduction of pyrite provides the least amount of energy (ΔG° -2.88 kJ/mol at 60 °C and 30 MPa). Despite its high energy yield, oxygen respiration is expected to be of minor significance in UHS

reservoirs, and mainly linked either to contaminations during operations or due to contaminations of the reservoir with recent surface waters (Table 3). Analyzing the availability and origin of the diverse electron acceptors in subsurface environments and their relevance to different UHS reservoirs (Table 3 and Supplementary Table S3), it appears evident that sulfate reduction, iron reduction and hydrogenotrophic methanogenesis play a potential key role in the consumption of stored hydrogen.

Sulfate reducing bacteria and archaea are capable of reducing a large number of compounds in addition to sulfate, sharing substrates with elemental sulfur and thiosulfate reducing microorganisms (Porter et al., 2007). For these reasons they are collectively defined as sulfur-species reducing microorganisms (SSRM). SSRM are of particular interest for UHS operations, since the final product, hydrogen sulfide (H_2S) is toxic and corrosive for the infrastructures. SSRM dominate in anoxic environments rich in oxidized sulfur species, and often outcompete methanogens and homoacetogens for hydrogen utilization. Only in certain low temperature and/or low salinity environments, homoacetogens outcompete SSRM (Lackner et al., 2020; Thaysen et al., 2021b). Elemental sulfur respiration is constrained by low solubility of the substrate, requiring cell attachment to sulfur particles (Hedderich et al., 1998) or the utilization of polysulfides and sulfur nanoparticles under certain conditions (Rickard and Luther, 2007; Jelen et al., 2018).

Given the high abundance of iron in the crust, iron (III) reduction is among the most important metabolism for UHS sites. Iron reduction is performed by heterotroph iron reducing bacteria, and relies on iron oxides and minerals such as iron ferrihydrite, hematite, smectite and chlorite, as well as on the presence of organic carbon (Hernsdorf et al., 2017). In natural environments rich in Fe oxides and organic carbon, iron reducing bacteria may have a great advantage over SSRM, methanogens and homoacetogens, due to a very high affinity for H_2 (Stefanie et al., 1994; Colleran et al., 1995; Chen et al., 2008; Paulo et al., 2015; Lackner et al., 2020). Contrary to many sediments and aquifers, in carbon-rich oil fields iron oxides are poorly present and dissimilatory iron reduction may be less relevant (Wiegel et al., 2003).

Hydrogenotrophic methanogens reduce carbon dioxide to methane through H_2 oxidation, and are key player in several subsurface ecosystems (Chapelle et al., 2002; Gregory et al., 2019a; Milkov, 2022; Beaver and Neufeld, 2024) and in UHS reservoirs (Pannekens et al., 2019; Dopffel et al., 2021b). They are known to consume large amounts of hydrogen in diverse conditions, and global estimates suggest that between 10 and 50 % of the biogenic methane is derived from hydrogen oxidation (Wong et al 2025). By contrast, the role of homoacetogens, bacteria capable of coupling the oxidation of hydrogen to carbon dioxide reduction to acetate, is less clear (Lever, 2012). The same three groups of microorganisms are additionally involved in subsurface corrosion (Loto, 2017; Skovhus et al., 2017).

The utilization of H_2 by deep microbial metabolisms in specific UHS sites such as salt caverns, gas and oil reservoirs, and deep aquifers remains largely unexplored (Zgonnik, 2020b). In oil and gas depleted reservoirs, the most likely electron acceptors are sulfate (SO_4^{2-}), thiosulfate ($S_2O_3^{2-}$), pyrite, carbon dioxide (CO_2), ferric iron (Fe^{3+}) and iron-oxide minerals (Table 3 and Supplementary Table S3). The presence of these compounds is generally linked with the specific geological settings of the reservoir. For example, sulfate might be abundant in the formation waters especially in reservoirs of marine origin or situated offshore, or might be linked to the presence of sulfate mineral deposits in the bedding. Thiosulfate and other intermediate sulfur species can be present as the result of *in situ* microbial sulfur metabolism. Iron and iron-oxide minerals are common in sedimentary rocks, and their availability in the reservoir largely depends on the type of rock formation being investigated (Pasquier et al., 2022). Carbon dioxide, on the other hand, can be present in the subsurface generated by the dissolution of carbonate rocks, by the thermal and biological degradation of organic matter and oil, or directly injected into the reservoir as cushion gas during UHS operations (Head et al., 2003; Baines and Worden, 2004; Cardoso and Andres, 2014; Muhammed et al., 2023; Mao et al., 2024).

In deep aquifers, the most likely electron acceptors are oxygen (O_2), pyrite, carbon dioxide (CO_2) and ferric iron (Fe^{3+}) and iron-oxide minerals (Table 3 and Supplementary Table S3). Oxygen traces are often co-injected in the gas network and can be stored in UGS during the process of desulfurization, causing unclear consequences (Haddad et al., 2022). At the same time dissolved O_2 can be present in deep aquifers due to events of reaeration or infiltration through fractures and migration paths from the upper layers of the reservoirs (Foulquier et al., 2010). Pyrite is generally found among the major electron acceptors in deep aquifers, both as sedimentary or hydrothermal pyrite or might be microbially or abiotically precipitated. Some H_2-induced pyrite reduction and re-precipitation of pyrrhotite may be crucial in some sites at low temperatures (~ 25 °C) (Reitenbach et al., 2015). CO_2 is often used as cushion gas before H_2 storage and can also be produced by subsurface microbial heterotrophic activity, from carbonate dissolution and/or mixing with meteoric waters. Fe^{3+} and iron-oxide minerals can be easily detected even if their concentrations generally decrease with depth (Li et al., 2020; Sun et al., 2021) and are generally sourced from the surrounding rocks.

In salt caverns, the most probable electron acceptors are thiosulfate ($S_2O_3^{2-}$), sulfate (SO_4^{2-}) and carbon dioxide (CO_2) (Table 3 and Supplementary Table S3). Thiosulfate could be found in these reservoirs if a sulfur cycle is established either starting from sulfate reduction or from the oxidation of reduced sulfur species. Sulfate might be present as sulfate-minerals (e.g. gypsum) in the evaporite bedding or as minor phase in the evaporite units. Carbon dioxide is often injected as cushion gas before the H_2 storage also in

this context and might be derived from subsurface heterotrophic microbial activity which, in hypersaline environments, might be lower if compared to other settings.

Powerful oxidants, such as oxygen, nitrate and other oxidized nitrogen species (for example NO and N_2O) are available in the subsurface mainly as the result of mixing with surface waters (possibly agriculturally contaminated waters for nitrogen species), either during drilling operations (Gittel et al., 2009; Zettlitzer et al., 2010) or from other episodic events. Recently, new pathways of biological oxygen production in the subsurface independent from oxygenic photosynthesis have been described (Ruff et al., 2023), as well as the presence of abiotic production of oxygen in subsurface ecosystems (Stone et al., 2022; Sweetman et al., 2024). The relevance of these new sources of oxidants to UHS operation has yet to be evaluated. Additional acceptors like halogenated compounds and other oxyanions (e.g., CrO_4^{2-}, SeO_4^{2-}, UO_4^{2-}) might be present in deep aquifers but their concentrations are generally very low. All other possible electron acceptors are either improbable in UHS reservoir conditions due to the trace content of these compounds in the environment, or due to the low energy yields (or in some cases positive Gibbs free energies) at *in situ* conditions.

Necessary nutrients and elements to sustain microbial communities in subsurface storage sites might be directly released by biotically induced mineral weathering (Berthelin, 1988). For example, a group sulfur-oxidizing bacteria known as *Thiobacilli*, widely present in potential UHS sites like petroleum reservoirs (Tian et al., 2017; Al-Khazaali and Ataei, 2023), plays a significant role in the production of sulfuric acid through the oxidation of various sulfur compounds, including sulfide, sulfur, sulfite, and thiosulfate (Ehrlich, 1981). The resulting acidity is recognized as a key factor in the solubilization of carbonates in soils (Dommergues and Mangenot, 1970; Duchaufour, 1982), in the weathering of limestone (Kauffmann, 1960; Jaton, 1972) and of serpentinized ultrabasic rocks (Lebedeva et al., 1978). In soils, over 50 % of the isolated microbial strains are capable of mineral weathering, as demonstrated by their ability to mineralize biotite (Huang et al., 2014). It is also noteworthy to highlight that the abiotic interactions of hydrogen with the rock minerals available in a reservoir's rock and caprock can cause mineral dissolution (e.g., carbonate, sulfate, feldspars, clay) and precipitation (e.g., illite, sulfide, pyrrhotite) (Perera, 2023), further providing substrates to subsurface microbial communities. Mineral dissolution can also determine changes in the rock structure, activating faults and causing a mechanical weakening of the reservoir rocks. For example, pyrite dissolution, caused by the thermodynamically unstable nature of pyrite in the presence of hydrogen (Ebrahimiyekta, 2017), is considered the major abiotic mineral dissolution process during hydrogen storage.

All the hydrogenotrophic metabolisms discussed above generate a number of chemical by-products, some of which are of particular relevance for UHS operations: i) methane (CH_4); ii) hydrogen sulfide (H_2S), iii) organic acids. Microbial production of methane could be either considered a desirable process enhancing the reservoir storage capacity or an unwanted by-product. This biogenic gas potentially serves as an additional energy resource, contributing to the overall energy output from storage reservoirs, especially in the presence of simultaneous UHS and CCS. On the contrary, hydrogen sulfide, a toxic gas produced by SSRM, is highly toxic and corrodes steel infrastructures in a process called Microbial Induced Corrosion (MIC) causing economic damages (Hemme and van Berk, 2017; Dopffel et al., 2021b). Ferrous-ferric oxide (also called "magnetite") produced in the iron-reduction carried out by iron-reducing bacteria (IRB), can react with hydrogen sulfide leading to precipitation of iron sulfide (FeS) which causes clogging events affecting permeability and fluid flow. Moreover, if magnetite formation occurs near wellbores or within infrastructure, it may pose challenges related to wellbore stability, casing integrity, and the following loss of H_2 reservoir injectivity (Enning and Garrelfs, 2014). The production and accumulation of organic acids might increase microbial-induced rock weathering leading to structural instabilities and the availability of additional substrates and acid-producing bacteria have been described to be mainly responsible for the corrosion problems (Thaysen et al., 2021b). The major organic acid produced in fuel-oil/aqueous systems is acetic acid (Loto, 2017). Additionally, organic acids might be used as substrates by a number of other microorganisms, either as carbon source or for respiration. The relevance of these metabolisms for UHS operation remains unknown.

The accumulation of hydrogenotrophic products also might inhibit microbial communities either through thermodynamic inhibition or through direct toxicity. The accumulation of products slows down the forward reaction favouring the back reaction and making the metabolism energetically unfavoured. On the toxicity side, several byproducts might play a key role either directly or indirectly through changes in key environmental parameters (like pH). For example, exposure to H_2S, and its bisulfide ion, HS^-, induces damage to microbial proteins and coenzymes (Chen et al., 2008; Ntagia et al., 2020). At 5.0–6.3 mM H_2S, SSRM growth is completely inhibited, although metabolic activity persists under these conditions (Choi and Rim, 1991; Kushkevych et al., 2019). For methanogens and homoacetogens 3.8–7.5 mM H_2S and total sulfide concentrations of 3.3 mM, respectively, stop the growth (Choi and Rim, 1991; Ntagia et al., 2020). In systems characterized by circumneutral pH and ferric ion concentrations surpassing 1 mM, the precipitation of H_2S in mackinawite is predicted to maintain concentrations below toxic levels (Rickard and Luther, 2007). Ammonifiers, such as the hydrogenotrophic nitrate reducers to ammonia (DNRA) and sulfide, sulfur and thiosulfate oxidizers are known to alkalize or acidify their media, respectively,

releasing by-products (such as NO_2^- and NO by denitrifiers and H_2S by sulfate reducing bacteria) to levels that sometimes become toxic for the organism (Reis et al., 1992; Albina et al., 2019).

Hydrogen might also directly inhibit microbial metabolism when present at high concentrations. The potential physiologic response of subsurface microorganisms to various H_2 concentrations, both in engineered and in natural subsurface environments remains uncertain (Osman et al., 2022; Liu et al., 2023). A range of studies investigated the metabolism of methanogens at excess H_2 concentrations and ambient pressure, with contrasting results. Conrad et al. (Conrad et al., 1987) demonstrated that excess H_2 stimulated methanogenesis and growth rates in anoxic paddy soil. On the contrary, results by Topçuoğlu et al. (Topçuoğlu et al., 2019) and Stewart et al. (Stewart et al., 2019) suggest an inhibitory effect of high partial pressures of H_2. Similar observations were made for *Methanothermobacter thermoautotrophicus* using comparative proteomic analysis (Enoki et al., 2011). Members of the order Thermotogales have often been detected both in potential hydrogen storage sites such as aquifers and depleted gas reservoirs (Ben Hania et al., 2013; Bassani et al., 2023) and in natural gas field (Dohrmann and Krüger, 2023). Several studies demonstrate that Thermotogales tolerance to H_2 differ greatly between species depending on H_2 partial pressure and hydrogen excess can inhibit the growth of most of them, with few exceptions such as *Mesotoga spp*. (Huber and Stetter, 2001; Dohrmann and Krüger, 2023). The majority of available data on microbial H_2 turnover rates derive from batch cultures under selected growth conditions (de Poorter et al., 2007; Esquivel-Elizondo et al., 2017), where environmental variables affecting the *in situ* microbial metabolisms, may strongly differ from the subsurface (Hoehler and Jørgensen, 2013). Additionally, many microorganisms in the deep subsurface are not culturable and subject to study only through metagenomics (Long et al., 2016; Xie et al., 2021; Soares et al., 2023), therefore the response to variable *in situ* hydrogen concentrations remains unknown.

While inventories of microbial metabolisms occurring in subsurface environments and relevant to UHS have been attempted before (Dopffel et al., 2021b, 2024; Thaysen et al., 2021b; Boyd et al., 2024; Wu et al., 2024), they often fail to convey the complexity of the trophic interactions and cascading effects that each of these individual metabolisms alone might generate. For example, considering the metabolism presented in Table 3, it is possible to draw a complex reaction network map highlighting the high number of cross reactions focusing on H_2 as electron donor (Figure 6). Additional reactions are possible within the presented network (not drawn for clarity), such as the oxidation of sulfide at the expenses of a number of electron acceptors (e.g. Fe^{3+}, NO_3^-, NO_2^-), the disproportionation of thiosulfate (Finster, 2008) or the oxidation of methane at the expense of nitrate, nitrite or sulfate (Zhao et al., 2024). Microorganisms carrying out these reactions might be both autotrophic, heterotrophic or mixotrophic, further contributing to complex trophic interactions. These three lifestyles coexist in subsurface ecosystems, and communities

might be sustained by allochthonous organic carbon —that is carbon derived from ex situ processes, such as transported by aquifers or the presence of diagenetic organic carbon (gas, oil, complex sapropels and coal layers)— or by autochthonous organic carbon produced *in situ* by chemolithoautotrophs, for example in SLiME communities (Kieft, 2021; Al-Yaseri et al., 2023; Aslannezhad et al., 2023; Ranchou-Peyruse, 2024). Additionally, syntrophic interactions, involving the tight cooperative relationship between different microbial groups are common in the subsurface and UHS reservoirs. For instance, SSRM and homoacetogens collaboratively participate in microbial-induced corrosion of steel, where SSRM grow on acetate produced by homoacetogenesis (Usher et al., 2015). Syntrophy explains the co-occurrence of sulfate-reducing *Desulfovibrio* and homoacetogens, like *Acetobacterium*, in environments such as petroleum and subsurface CO_2 reservoirs (Grabowski et al., 2005; Freedman et al., 2017). Similarly, H_2-producing heterotrophs coexist with methanogens in petroleum reservoirs, where the former facilitate H_2 transfer to the latter (Topçuoğlu et al., 2019). Special habitats for syntrophic growth of methane-producing Archaea are found in depleted oil reservoirs during oil biodegradation processes, where sulfate reduction and methanogenesis dominate (Jiménez et al., 2016).

The complexity of the microbial trophic networks is often underestimated in UHS studies, leading to simplified microbial models that might negatively impact our ability to predict microbial effects on UHS operations. System level approaches to microbial trophic networks are necessary to elucidate the effects of repeated hydrogen (and cushion gas) injections in diverse UHS reservoirs. Currently, a limited number of studies have used shotgun metagenomic to investigate the functional diversity of microorganisms in both subsurface hydrogen-rich and UHS potential sites (Chapelle et al., 2002; Brazelton et al., 2012; Amils et al., 2023; Ruff et al., 2023). Functional approaches to the investigation of UHS communities are paramount to dissect the activity of hydrogenotrophic microorganisms, and need to be coupled with chemical network modelling to provide predictive tools for UHS operations.

The microbiology of natural hydrogen seeps

The state space of the different conditions controlling microbial diversity and its interaction with hydrogen as electron donor is enormous. Relying on studies investigating microbial diversity in UHS reservoir might severely limit our understanding of the role of microbiology in UHS for a number of reasons: i) the number of operating UHS sites is extremely limited; ii) the accessibility to UHS potential sites to collect high quality microbial diversity samples is narrow by logistic and operational factors; and iii) direct access to subsurface communities is often constantly and logistically challenging. This limits

our ability to thoroughly investigate the effects of diverse environmental factors in controlling the diversity and distribution of hydrogenotrophic metabolism in the subsurface. Additionally, understanding the factors controlling microbial utilization of hydrogen in the subsurface has profound implications not only for UHS operations but also for natural hydrogen prospecting.

Hydrogen is naturally abundant in the subsurface and it is generated through a diverse suite of biotic and abiotic processes. The main geological processes leading to H_2 formation include H_2O reduction during Fe oxidation in minerals (*i.e.* serpentinization of ultramafic rocks), H_2S oxidation coupled with low-pressure magma degassing, K^-, Th^-, and U-bearing minerals decay leading to H_2O radiolysis, and H_2O reaction with surface radicals during the mechanical break of the rock Si-O bond (Klein et al., 2020; Langhi and Strand, 2023). Biological processes might also contribute to hydrogen formation in the subsurface together with thermogenesis from organic carbon, hydrocarbons and coal (Langhi and Strand, 2023). On Earth's surface, one of the main H_2-releasing microbial processes is photosynthesis, as H_2 evolves from NADH (Nandi and Sengupta, 1998). Furthermore, microbial metabolism plays a crucial role in the transition of organic matter (and thus H_2) from the biosphere to the geosphere taking place in the soils, sediments and upper crust. Aerobic respiration, anaerobic respiration and fermentation allow the degradation of biotic macromolecules (Tissot and Welte, 1984; Suzuki et al., 2024), and excess electrons are disposed through the catalytic activity of the H_2-evolving hydrogenases, resulting in H_2 production (Nandi and Sengupta, 1998). The remaining residues are polycondensed into refractory organic matter and kerogens. Further burial results in a progressive increase in the heat and pressure to which organic matter is subjected, eventually leading to the formation of oil, gas, hydrocarbons (mostly methane) and H_2 (Tissot and Welte, 1984; Hanson and Hanson, 2024).

Hydrogen produced in the subsurface migrates through the crust from its source and might accumulate in geologic traps (Sekar et al., 2023; Maiga et al., 2024; O'Sullivan et al., 2025). During its generation, migration and accumulation, hydrogen will be used by the *in situ* subsurface microbial communities through the same metabolic pathways described in the preceding sections. Recent estimates of potential H_2 microbial consumption rates and its loss from natural or stimulated geologic reservoirs suggest that up to >90 % of geologic H_2 produced could be lost due to microbial oxidation (Boyd et al., 2024) and hydrogenase gene abundance is almost an order of magnitude higher in subsurface environments, compared to surface environments (Löffler et al., 1999). Hydrogen leaking from the subsurface to the surface has been detected in a number of natural deeply-sourced springs globally (Zgonnik, 2020a; Leila et al., 2021; McMahon et al., 2023; Quéméneur et al., 2023; Wang et al., 2023). The same plumbing and migration system allowing subsurface hydrogen to reach the surface can transport to the surface microbial communities present at depth (Giovannelli and Lloyd, 2025). These communities can be sampled at the

surface, using naturally advecting springs as a window into the subsurface (Giovannelli et al., 2022). Given the diversity of conditions present in natural deeply-sourced springs, these can be used as model to investigate the subsurface microbial diversity and its interaction with hydrogen in a wide range of physico-chemical, geological and biological conditions, allowing to obtained fundamental data to interpret and model UHS operations and inform natural hydrogen prospecting.

While determining to what extent each hydrogen-generating geological process supports microbial communities is not trivial, evidence is growing (Gregory et al., 2019a). For instance, in radiolytic environments, measured H_2 concentrations are significantly lower than expected, and this discrepancy suggests that radiolytic H_2 production almost equals its consumption (Lin et al., 2005; Blair et al., 2007; Sauvage et al., 2021). One plausible explanation is that the redox disequilibria generated via radiolysis supports chemolithoautotrophic microbial communities (Türke et al., 2015; Dzaugis et al., 2016). Likewise, estimates of earthquake-derived H_2 suggest this energy source is sufficiently high to support subsurface microbial ecosystems, while the deuterium-depleted H_2 observed in natural faults systems hints to the presence of lithoautotrophic H_2-metabolizing organisms (Hirose et al., 2011; Suzuki et al., 2015). To the best of our knowledge, no evidence is available about microbial diversity fuelled by thermogenic H_2. In contrast, the microbial community associated with hydrothermal vent fields has been widely described, and it is to rely on hydrogen as electron donor (Petersen et al., 2011; Reveillaud et al., 2016; Adam and Perner, 2018; Holden and Sistu, 2023). Likewise, metagenomic-based investigation of the microbial functional diversity in a marine serpentinite-hosted hydrothermal chimney (at the Lost City hydrothermal field) and two continental serpentinite-hosted alkaline seeps (at the Tablelands Ophiolite, Newfoundland) revealed novel [NiFe]-hydrogenase sequences. In both marine and terrestrial sites, phylogenetic analyses suggested aerobic, potentially autotrophic Betaproteobacteria of the Burkholderiales order as the most likely H_2-oxidizers. Besides, microbial H_2 synthesis catalyzed by [FeFe]-hydrogenases in anaerobic Gram-positive bacteria of the Clostridiales order was indicated by metagenomics evidence (Brazelton et al., 2012). Several microbial metabolism have been identified in serpentinization areas. Among these, H_2 oxidation has been mainly attributed to the *Serpentinimonas* and *Hydrogenophaga* genera, hydrogenotrophic methanogenesis to the archaeal *Methanobacteriales* and *Methanosarcinales* orders, and sulfate reduction to the *Desulfovibrionales* and *Dethiobacter* orders (Popall et al., 2023). The above mentioned findings point to the widespread availability of differently-originated natural H_2 and to the opportunity for this energetic currency to be exploited by chemolithoautotrophic microorganisms as an electron donor in diverse natural ecosystems. On land, metagenomic investigations of geologically diverse deeply-sourced springs suggest that hydrogen is not only present, but represents a key electron donor (Colman et al., 2016; Upin, 2020; Rogers et al., 2022a).

Given the diversity of microbial niches present on Earth and how these map to UHS sites and subsurface environments (Figure 5), a careful reconstruction of the metabolic networks present in diverse naturally rich, deeply-sourced springs might provide a powerful tool to explore the state space of microbial hydrogenotrophic diversity. Subsurface microorganisms might impact natural hydrogen production, migration and accumulation in a variety of ways, and while the amount of information available on this regard is severely limited, our knowledge of their interaction with geologic hydrogen will play a key role in the economy of natural hydrogen.

Conclusion and future outlook

The role of hydrogenotrophic microorganisms and their associated hydrogenase enzymes in underground hydrogen storage and in natural environments presents both challenges and opportunities for the future of energy storage. As the global community seeks to reduce reliance on fossil fuels and transition towards sustainable energy solutions, hydrogen stands out as a promising alternative, offering high energy density and the potential for carbon-free energy. However, the success of these innovative technologies also depends on a comprehensive understanding of the microbial processes that can affect hydrogen storage. While the awareness toward the potential role played by subsurface microorganisms in UHS operations is growing, available studies and models severely underestimate the complexity of subsurface microbial communities. Additionally, subsurface microbiology is surprisingly absent in publications dealing with natural hydrogen prospecting. This review highlights the diversity of hydrogenotrophic metabolisms relevant to UHS operations and subsurface ecosystems, discussing the diversity of the key enzymes responsible for hydrogen oxidation and the complex trophic interactions arising in subsurface ecosystems. It discusses the importance of investigating the limits of life with respect to different geological and geochemical settings, which can strongly affect and diversify the microbial ability to survive and/or grow both in natural and engineered environments. Advances in molecular biology, metagenomics, and bioinformatics provide tools to identify and characterize the microbial populations present in potential UHS sites and subsurface environments, helping in assessing the risks these communities pose to the whole UHS ecosystem. Finally, it suggests the use of hydrogen-rich natural deeply-sourced springs as a model for extrapolating the hydrogenotrophic microbial diversity and its controlling factors to operational settings. By mapping the diversity and distribution of hydrogenotrophic metabolisms and hydrogenase genes in association with environmental conditions, it may be possible to design strategies that either inhibit microbial hydrogen consumption or utilize microbial processes to enhance storage stability. Unveiling the role that microorganisms play in hydrogen cycling has broader implications beyond UHS, contributing to our understanding of subsurface microbial ecology, biogeochemical cycling, as well as the evolutionary history of life and limits of life on Earth.

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Contributions

MC and DG conceived the paper. MC, GCG, FM, DC, and DG made the figures and tables. All authors contributed to the writing of the draft and the final editing.

Ethics declarations

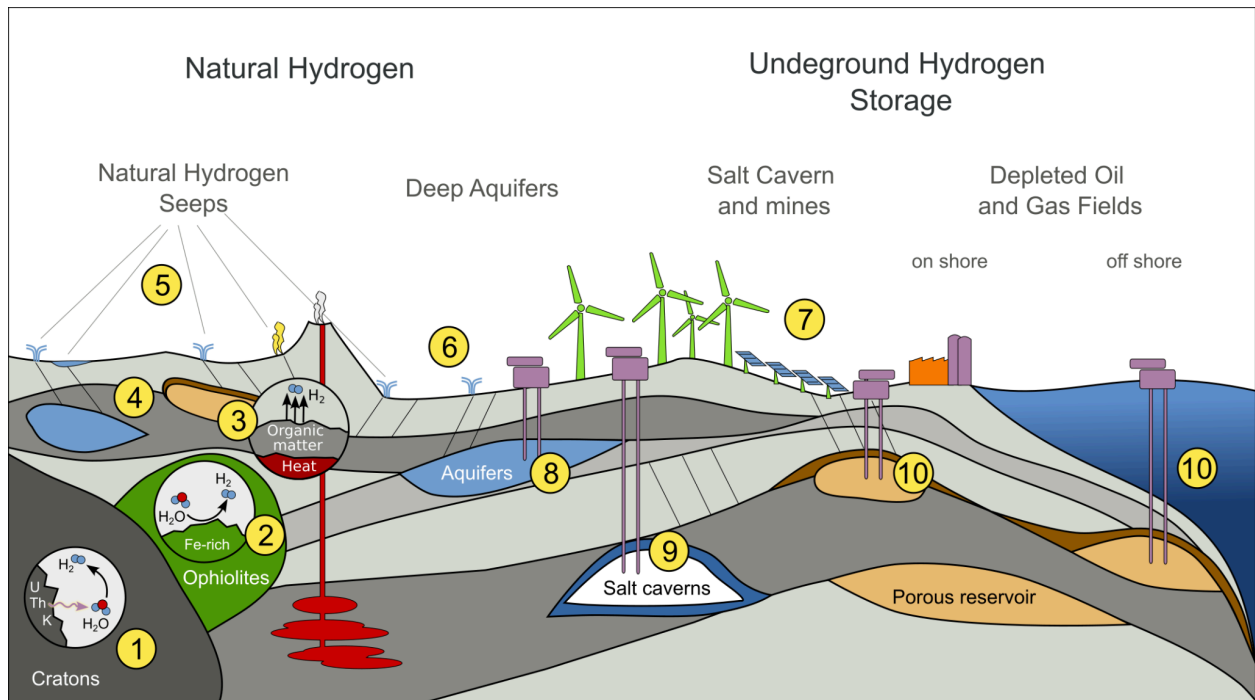The other authors declare no competing interests.

Figures

Figure 1. Summary of the natural processes contributing to natural hydrogen in the environments and target storage sites for green hydrogen. The two hydrogen colors are affected by the ability of subsurface microbes to use hydrogen as an energy source and can be approached together from a microbiological standpoint. 1. radiolytic H_2 production in stable cratons; 2. serpentinization reaction in Fe-Rich mantle rocks; 3. Thermogenic and biogenic production of hydrogen in organic rich basins; 4. Natural hydrogen produced through a variety of processes can accumulate in deep aquifers and porous reservoirs similar to those targeted for UHS; 5. Natural hydrogen deeply-sourced seeps can be used to research and explore the interaction of subsurface microbes with hydrogen as well as enable natural hydrogen prospecting; 6. Deeply-sourced seeps can also be used to monitor potential leakages from UHS sites; 7. Renewable energy production facilities are used to electrolyze water and produce hydrogen during peak production times. Produced hydrogen can then be stored in: 8. Deep aquifers; 9. Salt caverns and mines; and 10. onshore and offshore depleted oil and gas reservoirs.
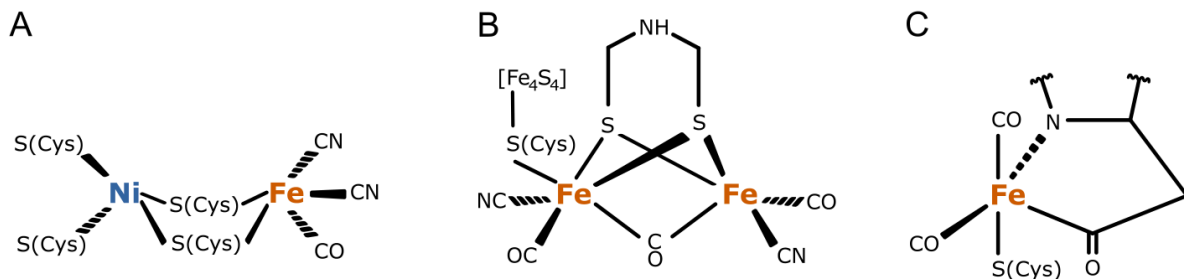
Figure 2. Cofactor diversity in the 4 main types of hydrogenases: [NiFe] (A), [FeFe] (B) and [Fe] (C). While [Fe]-hydrogenases bind a single atom of Fe, [FeFe] and [NiFe] coordinate binuclear centers with the help of different groups: in [FeFe], the dithiolate ligand as well as several carbon monoxide (CO) and cyanide (CN) groups stabilize the binuclear center; in [NiFe], the same CO and CN groups stabilize the Fe atom, while the Ni atom is exclusively coordinated by the thiol groups of cysteines (selenocysteine in the case of NiFeSe). [Fe]-hydrogenases also lack CN groups and differ mechanistically to the rest, as explained in the text.
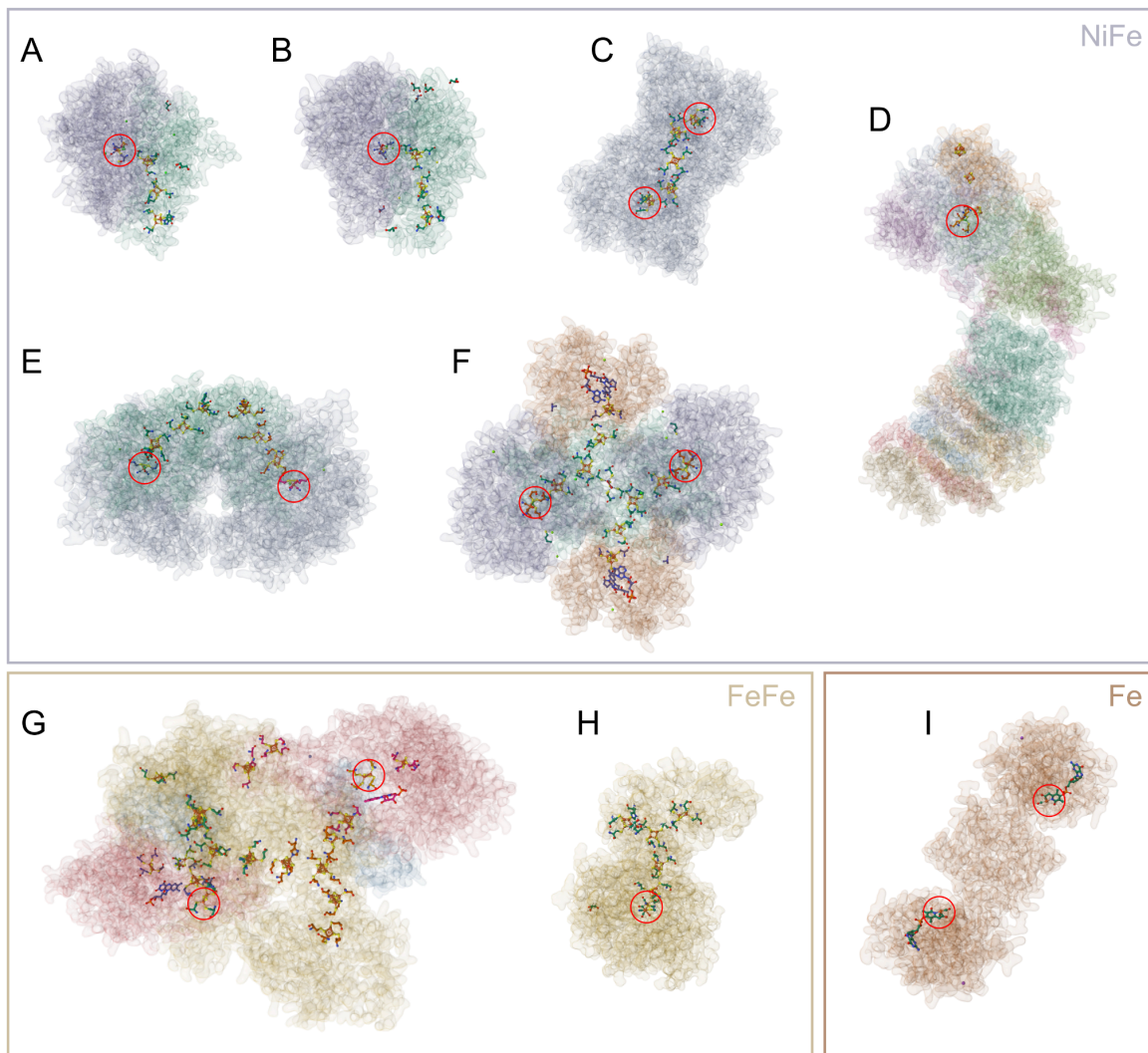
Figure 3. Structural diversity of selected hydrogenase groups from Table 2. Representative NiFe, FeFe and Fe structures are reported with the iron-sulfur clusters and the coordinating amino acids represented by ball-and-stick; the catalytic site highlighted with a red circle. A. the O_2-tolerant respiratory [NiFeSe]-hydrogenase from *Desulfomicrobium baculatum* (PDB 4KL8); B. the prototypical respiratory O_2-sensitive [NiFe]-hydrogenase from *Megalodesulfovibrio gigas* (PDB 1YQ9); C. the carbon monoxide-respiring [NiFe]-hydrogenase from *Carboxydothermus hydrogenoformans* (PDB 3B52); D. the Mrp-linked [NiFe]-hydrogenase from *Pyrococcus furiosus* (PDB 6CFW); E. the Hyb-type [NiFe]-hydrogenase from *Escherichia coli* (PDB 6EHQ); F. the F420-coupled [NiFe]-hydrogenase from *Methanothermobacter marburgensis* (PDB 4OMF); G. the bifurcating [FeFe]-hydrogenase from *Thermotoga maritima* (PDB 7P8N; H. the prototypical [FeFe]-hydrogenase from *Clostridium pasteurianum* (PDB 6N59); I. the methenyl-H4MPT dehydrogenase [Fe]-hydrogenase from *Methanocaldococcus jannaschii* (PDB 3F47).
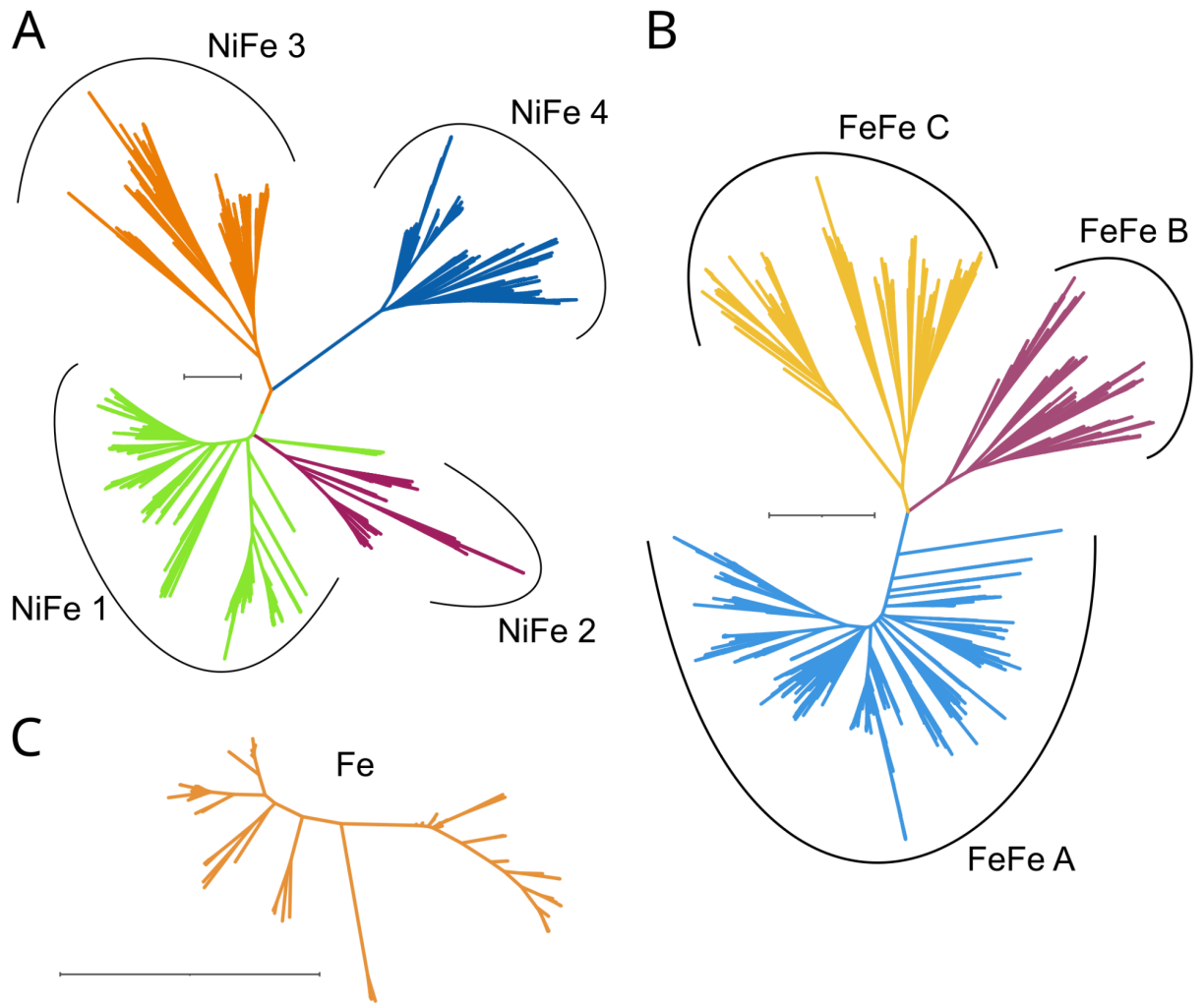
Figure 4. Phylogenetic trees for NiFe (A), FeFe (B) and Fe (C) hydrogenases major subunits colored according to the major hydrogenases supergroups. The trees are unrooted ML trees obtained from 5,058, 2,242 and 80 sequences for NiFe, FeFe and Fe hydrogenases respectively. Scale bars are equal to a branch length of 1.
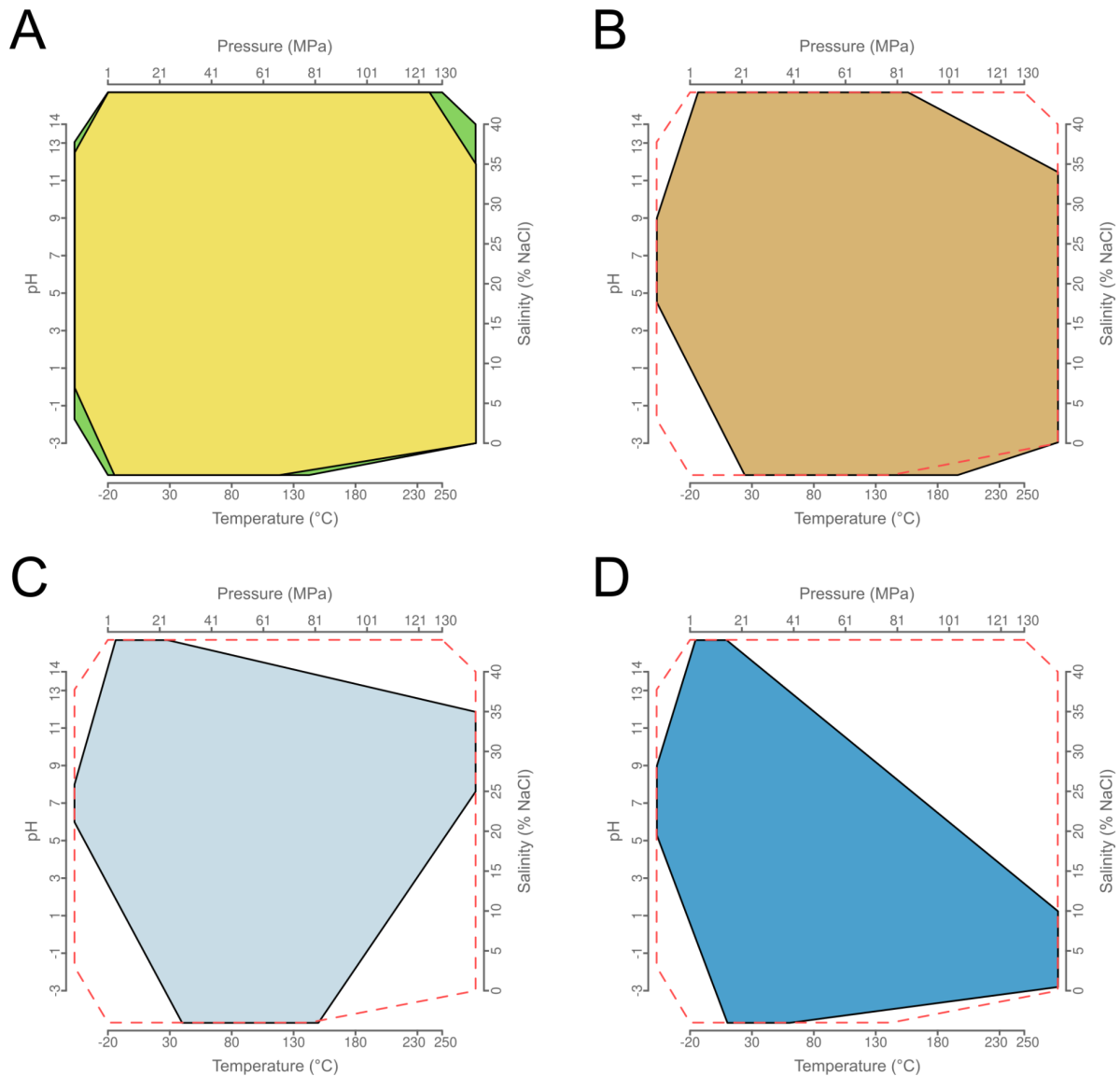
Figure 5. The temperature, pH, pressure and salinity limits of life compared to the range of conditions in geological hydrogen storage reservoirs. Polygon plots are designed to represent ranges in multidimensional space (e.g. minimum and maximum values) and do not represent the combinatorics of existing conditions (see Giovannelli et al, 2024 for explanations). A. Limits of life as defined by laboratory cultures (in yellow) and environmental surveys (in green). The actual limits of life have been a moving target, and have been changing regularly over the years as new knowledge accumulates. B. Range of conditions present in depleted porous reservoirs. C. Range of conditions present in salt cavern reservoirs. D. Range of conditions present in deep aquifer reservoirs. The boundary of the limits of life obtained from environmental surveys are reported in the reservoir's plot as a dashed red line for comparison.
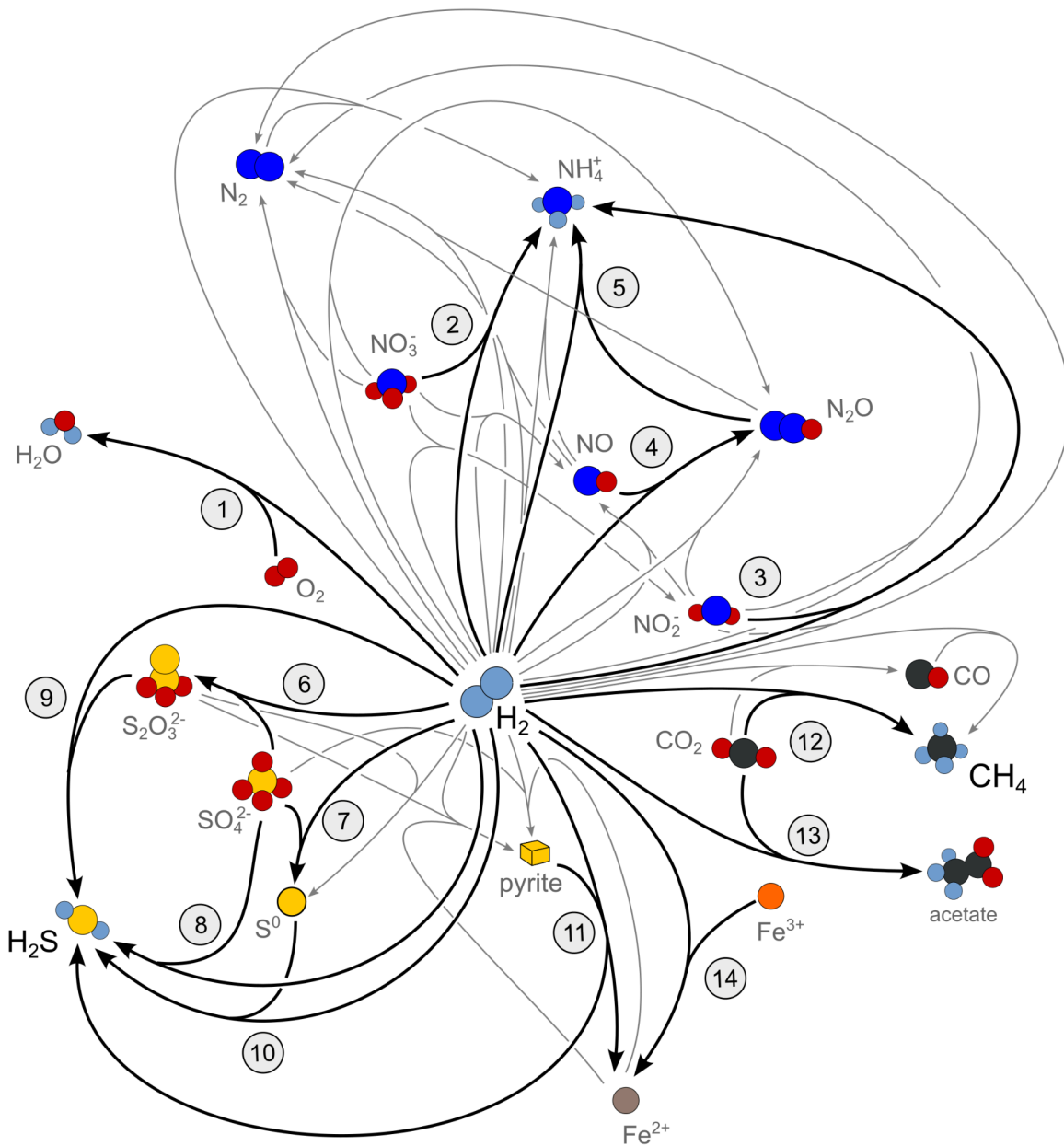
Figura 6. Hydrogenotrophic metabolism of interest in underground hydrogen storage sites and in natural hydrogen reservoirs. Lines for exemplificative reactions of relevance for each principal electron acceptors are in bold and numbered according to Table 3. The other reactions can be found in the supplementary extended version of Table 3 (available at https://doi.org/10.5281/zenodo.14606251), and are marked with light gray lines. Reactions using minerals as electron acceptors are omitted, with the exception of pyrite. Key compounds of interest (H_2, H_2S and CH_4) are highlighted.

Tables

Table 1. Major groups and classes of hydrogenases and their biological function, representative organisms and protein structures. A complete table containing additional information is provided in the supplementary online material. a. Carbonmonoxide-(dicyano) iron. b. Information not available. c. the di-iron cofactor (dicarbonyl[bis(cyanide-kappaC)]-mu-(iminodimethanethiolatato-1kappaS:2kappaS)-mu-(oxomethylidene)di-iron (2+). An extended version of Table 1 is available at 10.5281/zenodo.14606251.

Hydrogenase type	Hydrogenase group	Function	Gene name	Localization	UniProt EC number	Representative organism	Structure accession	Metal cofactors	Reference
NiFe	Group 1a	Respiration	*hys*	periplasm	1.12.7.2	*Desulfomicrobium baculatum (Desulfovibrio baculatus)*	4KL8	Ni^{2+}, FCO^a, Ca^{2+}, 4Fe-4S	Volbeda et al. 2013
	Group 1b	Respiration	*hyd*	cell membrane/ periplasm	1.12.2.1	*Megalodesulfovibrio gigas*	1YQ9	Ni^{2+}, FCO^a, Mg^{2+}, 4Fe-4S, 3Fe-4S	Volbeda et al. 2005
	Group 1c	Respiration/ Uptake	*hyb*	cell membrane	1.12.99.6	*Escherichia coli*	6EHQ	Ni^{2+}, FCO^a, Mg^{2+}, 4Fe-4S, 3Fe-4S	Pinske et al. 2015
	Group 1d	Respiration	*hya*	cytoplasmic membrane	1.12.99.6	*Escherichia coli*	3USE	Ni^{3+}, FCO^a, Mg^{2+}, Li^+, 4Fe-4S, 4Fe-3S, 3Fe-4S	Volbeda et al. 2013
	Group 1e	Respiration/ Uptake	*hyd*	cell membrane	1.12.99.6	*Allochromatium vinosum*	3MYR	Ni^{2+}, FCO^a, Mg^{2+}, 4Fe-4S, 3Fe-4S	Ogata et al. 2010
	Group 1f	Unresolved/ Respiration	*hya*	cell membrane	1.12.99.6	*Geobacter sulfurreducens*	_[b]	-	Butler et al. 2012
	Group 1g	Unresolved/ Respiration	*hya*	endomembrane system	1.12.99.6	*Thermofilum adornatum*	AF-A0A3G1A7E9-F1	-	Kochetkova et al. 2020
	Group 1h	Aerobic Respiration	*hya*	cell membrane (?)	1.12.99.6	*Ralstonia eutropha*	7ODH	Ni^{2+}, FCO^a, Mg^{2+}, 4Fe-4S, 4Fe-3S	Schäfer et al. 2016
	Group 1i	Respiratory/ H2-uptake	-	cell membrane (?)	-	*Eggerthella lenta*	AF-A0A369MVM6-F1	-	Nitzan et al. 2018
	Group 1j	Respiratory/ H2-uptake	*hyp*	cell membrane	-	*Archaeoglobus fulgidus*	AF-A0A075WGP0-F1	-	Birkeland et al. 2017
	Group 1k	Respiratory/ H2-uptake	*hyp*	cell membrane	-	*Methanosarcina acetivorans*	AF-A0A832W891-F1	-	Rinke et al. 2021
	Group 1l	Respiration/ Energy production and conversion		cell membrane	-	*Rubrobacter indicoceani*	-	-	Ortiz et al. 2021
	Group 2a	Respiration Aerobic/ Redox Balance	*hyb*	endomembrane system	1.12.99.6	*Mycolicibacterium smegmatis*	8DQV	Ni^{2+}, FCO^a, Mg^{2+}, 4Fe-3S	Grinter et al. 2023
	Group 2b	H_2-Sensing	*hya*	cell membrane	-	*Bradyrhizobium diazoefficiens*	AF-H7C6M7-F1	-	Kaneko et al., 2002
	Group 2c	Unresolved/ Sensing	-	cell membrane	-	*Paramagnetospirillum magneticum*	AF-Q2W8A7-F1	-	Matsunaga et al. 2005

	Group	Function	gene	localization	EC	Organism	ID	Cofactors	Reference
	Group 2d	Unresolved/Carbon Fixation	*hya*	cytoplasm	-	*Aquifex aeolicus*	AF-O66988-F1	-	
	Group 3a	F420 Reduction	*frh*	-	1.12.98.1	*Methanothermobacter marburgensis*	4OMF	Ni^{2+}, FCO^a, Mg^{2+}, H2S2Zn, 4Fe-4S	Alex et al 1990
	Group 3b	H$_2$-production	*hyd*	cytoplasm	1.12.1.3	*Pyrococcus furiosus*	AF-E7FI44-F1	-	Pedroni et al. 1995
	Group 3c	Bidirectional	*mvh*	cytosolic	1.12.99.-	*Methanothermobacter marburgensis*	AF-P60227-F1	-	Stojanowic et al. 2003
	Group 3d	NAD redox	*hyp*	cytosolic	-	*Methylocella silvestris*	AF-B8ERX1-F1	-	Chen et al. 2010
	Group 4a	H$_2$-production	*hyp*	cell membrane	1.-.-.-	*Yersinia enterocolitica*	AF-A0A7U7FL32-F1	-	Batzilla et al.
	Group 4b	H$_2$-production	*hyc*	cell membrane	-	*Thermococcus onnurineus*	-	-	Strang, 2020
	Group 4c	H$_2$-production	*hyd*	cell membrane	-	*Carboxydothermus hydrogenoformans*	3B52	Fe^{2+}, 2Fe-2S, 3Fe-Ni-4S, 4Fe-4S	Wu et al, 2005
	Group 4d	H$_2$-production	*hyd*	cell membrane	1.12.7.2	*Pyrococcus furiosus*	6CFW	Ni^{2+}, FCO^a, 4Fe-4S,	Yu et al., 2018
	Group 4h1	H$_2$-uptake	*eha/ehb*	cell membrane	-	*Methanococcus aeolicus*	AF-A6UTY6-F1	-	Fomenkov et al. 2023
	Group 4e	Bidirectional	*hyp*	cell membrane	-	*Caldicellulosiruptor hydrothermalis*	AF_E4QC73-F1	-	Blumer-Schuette et al., 2011
	Group 4f	Respiratory/H$_2$-evolving	-	cytoplasm	1.6.5.9	*Desulfitobacterium dehalogenans*	AF-I4AD88-F1	-	-
	Group 4g	Respiratory/H$_2$-evolving	*hyc*	cell membrane	-	*Anaerolinea thermophila*	AF-E8MXZ8-F1	-	Matsuura et al., 2015
	Group 4h2	Respiratory/H$_2$-evolving	*hyc*	cell membrane	1.6.99.5	*Methanobacterium paludis*	AF-F6D2P4-F1-F1	-	Cadillo-Quiroz et al., 2014
	Group 4i	Respiratory/H$_2$-evolving	*hyc*	cell membrane	1.6.99.5	*Methanococcus aeolicus*	AF-A6UTS2-F1-F1	-	Fomenkov et al. 2023
FeFe	Group A1	H$_2$-production	*hyd*	cytoplasm	1.12.7.2	*Clostridium pasteurianum*	6N59	402^c, 4Fe-4S, 2Fe-2S	Artz et al., 2020
	Group A2	Unresolved/H$_2$-uptake	*hyd*	cytoplasm	-	*Adlercreutzia equolifaciens*	-	-	Horner et al., 2000
	Group A3	Electron Bifurcation	*hyd*	cytoplasm	1.12.1.4	*Thermotoga maritima*	7P8N	402^c, 4Fe-4S, 2Fe-2S, Zn^{2+}	Furlan et al. 2022
	Group A4	H$_2$-production/Electron Bifurcation	-	cytoplasm	1.12.7.2	*Rhodospirillum rubrum*	AF_Q2RXN0-F1	-	Peters et al., 1998
	Group B	Unresolved/H$_2$-production	*hydA*	cytoplasm	1.12.7.2	*Lancefieldella parvula*	AF_C8W9Q4-F1	-	Copeland et al., 2009
	Group C1	Unresolved/Sensing	-	cytoplasm	-	*Thermoanaerobacterium saccharolyticum*	AF_I3VX92-F1	-	Peters et al., 1998
	Group C2	Unresolved/Sensing	*hyd*	membrane/cytoplasm	-	*Roseburia hominis*	AF-G2SXD3-F1	-	Travis et al., 2015
	Group C3	Unresolved/Sensing	*hyp*	cytoplasm	-	*Clostridium sporogenes*	AF-A0A7U9GK79-F1	-	Bradbury et al., 2012
Fe	[Fe]	Respiration	*hmd*	cytoplasm	1.12.98.2	*Methanocaldococcus jannaschii*	3F47	Fe^{2+}, Na$^+$	Hiromoto et al, 2009

Table 2. Classification of the different types of reservoir and their principal physico-chemical characteristics influencing microbial metabolism.

Type of reservoir	Dominant host rocks	Depth range (m)	Pressure range (MPa)	Temperature range (°C)	pH range	Salinity range (% NaCl)
Depleted oil and gas porous reservoir	Sandstone, Mudstone, Shale, Limestone, Dolostone	500 – 5,500	4 – 110	24 – 196	4.5 – 9	0 – 34
Subsurface aquifers	Silicic rocks, carbonates, sedimentary rocks	200 – 3,000	3 – 30	10 – 100	5.3 – 9	0.5 – 10
Salt caverns	Halite	350 – 2,000	4 – 24	40 – 90	~7 – 9	saturation

Table 3. Hydrogenotrophic microbial metabolisms of relevance in diverse subsurface hydrogen reservoirs. Reactions are numbered according to Figure 5. ΔG° are calculated at 60 °C and 30 MPa. A complete table with the reactions involving CHNOS and common Fe mineral is provided in the Supplementary online material. The threat to UHS operations and the relevance to different reservoir settings has been assessed after an extensive review of the literature (see supplementary online material). a - Gibbs free energies for N_2O and NO have not been calculated since these compounds are not present in the thermodynamic database of the Water-Organic-Rock-Microbe (WORM) Portal. An extended version of Table 3 is available at 10.5281/zenodo.14606251.

n.	Hydrogenotrophic reactions	e⁻ transferred	ΔG° (kJ/mol)	Origin of the electron acceptor	Threat to UHS operations	Likelihood in porous reservoirs	Likelihood in salt caverns	Likelihood in deep aquifers
1	$H_2(gas) + \frac{1}{2}O_2(gas) \leftrightarrow H_2O(aq)$	4	-227.71	Possible contamination during gas injection or due to underground aquifers mixing	H_2 consumption, biofilm formation, clogging and steel corrosion	**Low**. Oxygen presence is potentially connected with infiltrations from surface sources and aquifers	**Low**. Oxygen presence is potentially connected with infiltrations from surface sources and aquifers	**Medium**. Oxygen presence could be due to entrainment of fresh surface waters as result of the geological setting of the reservoir
2	$H_2(aq) + \frac{1}{4}NO_3^- + \frac{1}{2}H^+ \leftrightarrow \frac{1}{4}NH_4^+ + \frac{3}{4}H_2O$	8	-187.59	Nitrate content in injected fluids, contaminations from surface reservoir/surface water from agricultural origins, organic matter decay, underground aquifers mixing	H_2 consumption, generation of highly reactive nitrogen species intermediate, biofilm formation, pore clogging, changes in pH, can lead to MICP when a organic carbon source is degraded	**Low**. Nitrate presence could be linked to organic matter degradation and underground aquifers infiltration	**Low**. The nitrate content could be only associated to percolation of meteoric water or presence of aquifers into the cave	**Low to medium**. Nitrate could be due to entrainment of fresh surface waters, agricultural contaminations and organic matter decay as result of the geological setting of the reservoir
3	$H_2(aq) + \frac{1}{3}NO_2^- + \frac{2}{3}H^+ \leftrightarrow \frac{1}{3}NH_4^+ + \frac{2}{3}H_2O$	6	-191.45	Nitrite is generally a byproduct of microbial denitrification	Same as above	**Low**. depends on presence of nitrate and microbial activity	**Low**. depends on presence of nitrate and microbial activity	**Low**. depends on presence of nitrate and microbial activity
4	$H_2(aq) + 2NO(aq) \leftrightarrow N_2O(aq) + H_2O$	2	-ᵃ	Nitric oxide is generally a byproduct of microbial denitrification	H_2 consumption, generation of highly reactive nitrogen species intermediate, biofilm formation, pore clogging, changes in pH if reduced to ammonia	**Low**. depends on presence of nitrate and microbial activity	**Low**. depends on presence of nitrate and microbial activity	**Low**. depends on presence of nitrate and microbial activity
5	$4H_2(aq) + N_2O(aq) + 2H^+ \leftrightarrow 2NH_4^+ + H_2O$	8	-	Nitrous oxide is generally a byproduct of microbial denitrification	H_2 consumption, generation of highly reactive nitrogen species intermediate, biofilm formation, pore clogging, changes in pH if reduced to ammonia	**Low**. depends on presence of nitrate and microbial activity	**Low**. depends on presence of nitrate and microbial activity	**Low**. depends on presence of nitrate and microbial activity
6	$H_2(aq) + \frac{1}{2}SO_4^{-2} + \frac{1}{2}H^+ \leftrightarrow \frac{1}{4}S_2O_3^{-2} + \frac{5}{4}H_2O$	8	-74.04	Sulfate content of the hosting rocks, contaminations from evaporite deposits, entrainment of seawater (especially offshore),	H_2 consumption, H_2S formation, pyrite precipitation, pH reduction, iron corrosion, mineral precipitation	**Medium to high**. Sulfate-reducing microorganisms are widespread and active in subsurface ecosystems and are	**Medium**. Sulfate is possibly present as part of the evaporite and sulfate reducing bacteria have been reported	**Low to medium**. Sulfur might be present in deep aquifers depending on the origin and mixing of the aquifer
7	$H_2(aq) + \frac{1}{3}SO_4^{-2} + \frac{2}{3}H^+ \leftrightarrow \frac{1}{3}sulfur(cr) + \frac{1}{3}H_2O$	6	-92.66					
8	$H_2(aq) + \frac{1}{4}SO_4^{-2} + \frac{1}{2}H^+ \leftrightarrow \frac{1}{4}H_2S(aq) + H_2O$	8	-77.27					

				microbial and abiotic oxidation of reduced sulfur species		well known in oil and gas reservoirs		
9	$H_2(aq) + ¼S_2O_3^{-2} + ½H^+ ↔ ½H_2S(aq) + ¾H_2O$	8	-80.50	Thiosulfate might be present as intermediate of reduced sulfur species microbial and abiotic oxidation and it will depend on availability of reduced sulfur and its possible oxidation reactions	H_2 consumption, H_2S formation, pyrite precipitation, pH reduction, iron corrosion, mineral precipitation	**Medium to high**. Likely if a sulfur cycle is established either starting from sulfate reduction or from the oxidation of reduced sulfur species	**Medium to high**. Likely if a sulfur cycle is established either starting from sulfate reduction or from the oxidation of reduced sulfur species	**Low to medium**. Likely if a sulfur cycle is established either starting from sulfate reduction or from the oxidation of reduced sulfur species
10	$H_2(aq) + sulfur ↔ H_2S(aq)$	2	-31.10	Elemental sulfur might be present due to the presence of hydrothermal and volcanic deposits or as the byproduct of sulfur microbial metabolism either in the oxidative or reductive direction	H_2 consumption, H_2S formation, pyrite precipitation, pH reduction, iron corrosion, mineral precipitation	**Low to medium**. Likely if a sulfur cycle is established either starting from sulfate reduction or from the oxidation of reduced sulfur species	**Low to medium**. Likely if a sulfur cycle is established either starting from sulfate reduction or from the oxidation of reduced sulfur species	**Low**. Likely if a sulfur cycle is established either starting from sulfate reduction or from the oxidation of reduced sulfur species
11	$H_2(aq) + pyrite + 2H^+ ↔ Fe^{+2} + 2H_2S(aq)$	2	-2.88	Pyrite might be present as sedimentary or hydrothermal pyrite or might be microbially or abiotically precipitated	H_2 consumption, mineral dissolution causing mechanical weakening of the reservoir rocks, biofilm formation	**Medium**. Could be present in the reservoir rock or result from microbial metabolism. Pyrite dissolution has been identified as the major abiotic reaction that can happen in a reservoir during hydrogen storage	**Low**. Could be present as impurity in the evaporite	**Medium**. Some H_2-induced pyrite reduction and re-precipitation of pyrrhotite may be significant in some sites at low temperatures (about 25 °C), e.g : town gas storage in an aquifer at Beynes in France
12	$H_2(aq) + ¼CO_2(aq) ↔ ¼CH_4(aq) + ½H_2O$	8	-47.56	Carbon dioxide can be present as cushion gas or due to the dissolution of carbonates at lower pH, might be induced microbially through acidification of the environments (both through H_2S production, H_2SO_4 and organic acids from fermentation), or as a result of the microbial degradation of organic matter	H_2 consumption, biofilm formation, pore clogging, methanation	**Medium to high**. CO_2 is often injected as cushion gas before the H_2 storage. CO_2 can also be produced by subsurface microbial activity and carbonate dissolution	**Medium to high**. CO_2 is often injected as cushion gas before the H_2 storage. CO_2 can also be produced by subsurface microbial activity which, in these environments, seems to be not so high	**Medium to high**. CO_2 is often injected as cushion gas before the H_2 storage. CO_2 can also be produced by subsurface microbial activity, due to carbonate dissolution and or due to mixing with meteoric waters
13	$H_2(aq) + CO_2 ↔ ¼CH_3COO^- + ½H_2O + ¼H^+$	8	160.71					
14	$H_2 + 3Fe_2O_3 ↔ 2Fe_3O_4 + H_2O$	6	-153.96	Iron oxide is provided by the host rock and dependent on the host rock composition. It can additionally be provided by mixing with iron rich aquifers and subsequent microbial iron oxidation. Iron from the infrastructure might locally support this reaction	H_2 consumption, mineral dissolution and reprecipitation, metal corrosion, carbon steel corrosion, pores clogging	**Medium to high**. Might be an important reaction if iron oxide is available in the reservoir rock	**Low to medium**. Iron is generally limited, but there are cases where it might be present in significative amount as impurity in the evaporite	**Medium to high**. Iron oxides are abundant in many sediments and aquifers

Supplementary online material

Methods

Sequences of proteins corresponding to [NiFe]/[FeFe]/[Fe-only]-hydrogenases were retrieved from the HydDB Aarhus University hydrogenase gene database (http://services.birc.au.dk/hyddb/) (Søndergaard et al., 2016). Representatives of each group (reported in the extended online version of Table 1) were blasted against the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/) using the Basic Local Alignment Search Tool (Altschul et al., 1990). Amino acid sequences belonging to the different enzymatic subgroups were assembled into a manually curated comprehensive dataset, including specific related information such as the accession numbers, PFAM ID, operone structure and others and provided in Table 2 and Table S2 for the extended version. A number of hydrogenase subgroups were manually added to the database following an extensive literature search. All data in the table was manually curated.

Phylogenetic trees of amino acid sequences translated from the gene specifying the large subunit of [NiFe]-hydrogenases as well as the catalytic subunits of [FeFe] and [Fe-only] hydrogenases were constructed as follows: sequences for each overarching group (i.e. [NiFe]/[FeFe]/[Fe-only]-hydrogenases) were aligned using MAFFT (v7.526) with the flags "--auto", "--anysymbol" (to account for selenocysteine) and with "--thread" set to 80 (Katoh and Standley, 2013); the resulting alignment was further trimmed using ClipKIT (v2.3.0) with default parameters (Steenwyk et al., 2020); finally, IQ-TREE (v2.3.4) was used for reconstructing the phylogenies, with "-nt" set to 80 and "-mem" to 200G (Minh et al., 2020). Phylogenetic tree display and annotation was performed through iTOL v7 (Letunic and Bork, 2021). The whole pipeline was run on the HPC IBiSco (Infrastructure for BIg data and Scientific COmputing; project PON R&I 2014-2020 dell' Avviso 424/2018 - Azione II. 1.) at the University of Naples Federico II, using SLURM (v20.11.5) (Jette and Wickberg, 2023; Callaghan et al., 2024).

Hydrogenase structures were investigated using the Protein Data Bank structure visualization tool, and the structure of the catalytic site for each hydrogenase type visualized using Molsketch v0.8.1 (Pirhadi et al., 2016). All figures were edited using the open source vectorial software Inkscape (v1.4, https://inkscape.org/). Sequences and extended versions of Table 1 and 3 are available through the GitHub repository https://github.com/giovannellilab/hydrogenotrophy_UHS_review and released through ZENODO with permanent DOI: 10.5281/zenodo.14606251.

Supplementary Tables

Supplementary tables are available at 10.5281/zenodo.14606251.