



Review Article

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

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ABSTRACT

Hydrogen (H₂) 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 storage of hydrogen gas produced from diverse sources in geological reservoirs, known also as Underground Hydrogen Storage (UHS), is a key prerequisite to decouple production from utilization. UHS targets include depleted natural gas reservoirs, salt caverns and deep aquifers. 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.

Contents

1. Introduction 2
2. Hydrogen as a key resource for energy transition 2

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3. Hydrogen as key energy source in microorganisms	4
4. The limits of life in the subsurface and their relevance for an hydrogen economy	6
5. The microbial diversity of potential UHS sites	12
6. Hydrogenotrophic metabolisms in the subsurface	13
7. Metabolic by-products, microbial inhibition and operational risks	16
8. The microbiology of natural hydrogen seeps	17
9. Conclusion and future outlook	18
Declaration of competing interest	19
Acknowledgements	19
Supplementary data	19
Data availability	19
References	19

1. Introduction

As of writing, carbon dioxide levels in the atmosphere have reached 430.51 ppm (Keeling and Keeling, 2017); <https://keelingcurve.ucsd.edu/>, accessed 15 May 2025) 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, hydroelectric 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 (Jafarizadeh et al., 2024; Sayed et al., 2023). 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 (Takach et al., 2022; Zivar et al., 2021; Miocic et al., 2023).

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 larger hydrogen storage capacities (several orders of magnitude) (Hassanpouryouzband et al., 2021; Heinemann et al., 2021; Zivar et al., 2021; Miocic et al., 2023). 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., 2010; Michael et al., 2009). 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 (Ozarlan, 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 (Bar-On et al., 2018; Kallmeyer et al., 2012; Magnabosco et al., 2018). Research carried out in diverse continental and oceanic tectonic settings have revealed the presence of abundant (between 10^2 and 10^8 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 (Barry et al., 2019; Fullerton et al., 2021; Giovannelli et al., 2021; Hinrichs and Boetius, 2003; Timothy J. Rogers et al., 2022) and are well known to impact fossil fuel reservoirs (Hagar et al., 2022; Mcinerney et al., 2007; 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., 2021; Thaysen et al., 2021, 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., 2019; Zgonnik, 2020; Wong et al., 2026). Geological manifestations of hydrogen have been acknowledged for millennia (Zhao et al., 2023). While hydrogen 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 (Everts et al., 2025). Preliminary investigations indicate that the characterization of potential hydrogen sources, the depths at which hydrogen is likely to be generated, the mechanisms governing its migration, and the factors influencing its 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-rich natural seeps (Etiope, 2023; Frery et al., 2021; Larin et al., 2015; Milkov, 2022; Prinzhofer et al., 2018; Davies et al., 2024; Roche et al., 2026), consensus over analogies and differences between the petroleum system and the natural hydrogen system and the diversity of potential plays in natural hydrogen exploration 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 mainly delve into the diversity of hydrogenotrophic metabolism in the subsurface, discussing their potential role in UHS in diverse reservoirs. We set the scene by introducing the role of H_2 as a key resource for energy transition. We then pass to 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 H_2 oxidation might be an ancestral metabolism that possibly appeared at the origin of life (Martin and Thauer, 2017; Weiss et al., 2016). We then move to presenting 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.

2. 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 (Hale et al., 2022; United Nations, 2016). 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. 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 ($-252.8^\circ C$ 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 need of underground large storage space (Tarkowski and Uliasz-Misiak, 2021; Thiagarajan 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 (Mioic 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”) align 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 together with Natural hydrogen (see below).

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”,

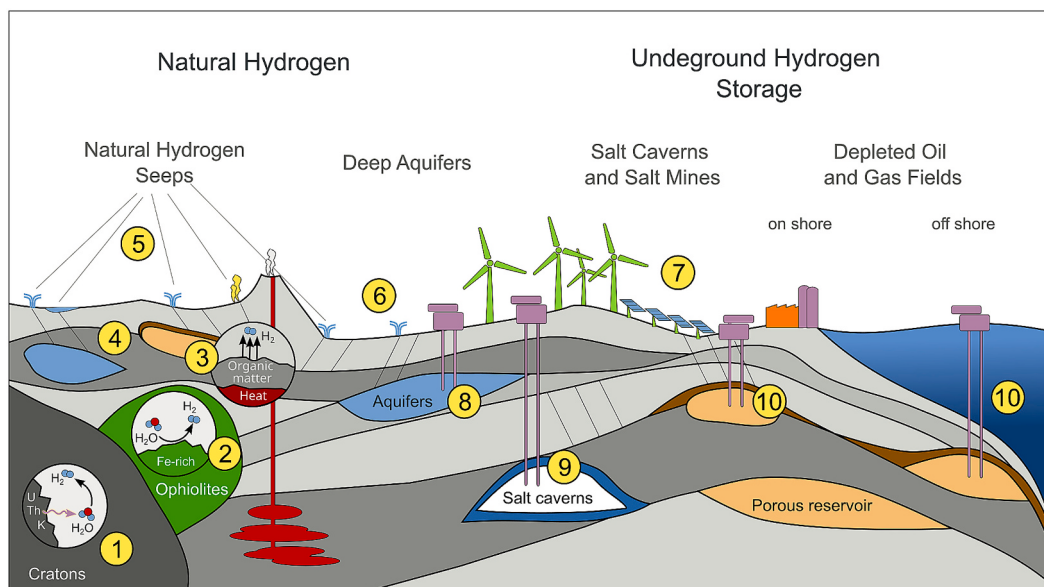


Fig. 1. Summary of the natural processes contributing to the production of hydrogen and target underground storage sites for green hydrogen. Both natural hydrogen exploration and underground hydrogen storage are affected by the ability of subsurface microbes to use hydrogen as an energy source and can 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 as natural analogues for 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 salt mines; and 10. Onshore and offshore depleted oil and gas reservoirs.

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 H_2 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 (Fig. 1) (Wong et al., 2026; Wong et al., 2019). 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 (Epelle et al., 2022; Milkov, 2022; Parnell and Blamey, 2017). While natural H_2 production is currently represented by a single field (Bourabougou, Mali) (Prinzhofer et al., 2018; Maiga et al., 2024), 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; Prinzhofer et al., 2019; Zgonnik et al., 2015; Wong et al., 2026). The natural hydrogen prospecting market is projected to see significant growth with an estimated global market value of 3.41 Billion Euro in 2023 with an anticipated compound annual growth rate (CAGR) of 9.21 % through 2029 (TechSci Research, 2023). Subsurface microbial communities might play a key role also in the natural hydrogen economy as they can alter or consume H_2 in its geological reservoir significantly impacting the H_2 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 and UHS efforts. The knowledge presented in this review is equally relevant to UHS 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 H_2 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., 2021; Miodic et al., 2023; Thaysen et al., 2023; Zivar et al., 2021; Abdellatif et al., 2025). Subsurface geological storage is increasingly considered one of the more suitable spaces for storing H_2 to be used as a carrier of energy (Fig. 1) (Muhammed et al., 2022). UHS 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 UHS facilities is orders of magnitude more economical than building surface-based storage of comparable capacity. Available geological formations for UHS are widespread, making them appealing for decentralized storage, limiting the necessity for long-range transport (Plaet, 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 reservoir, the thickness, porosity and permeability of the reservoir, the geomechanical properties and the sealing characteristics of the insulating caprock (Tarkowski, 2019). The presence of space, either through rock pores or in large cavities, together with an impermeable caprock, is essential for an efficient hydrogen storage and containment in place

(Tarkowski, 2019). The most accredited potential hydrogen storage types are porous rocks reservoirs, that include depleted natural oil and gas 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.

Accumulations of natural gas and petroleum in the subsurface occur in so-called geological traps (Bjørlykke, 2015; de Jager, 2020; Jia, 2012). These consist of a reservoir (a rock that allows the accumulation of hydrocarbons in its pore space, such as sandstones or carbonates), a seal and an underlying aquifer. The rocks that seal the trap (low permeable and non-fractured) keep the hydrocarbons stored in the reservoir and do not allow them to migrate out of the trap. 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 field to be used as an UHS site, where the safety of storage is the key issue (Jackson et al., 2024). One of the concerns posed by the storage of hydrogen into depleted hydrocarbon reservoirs is that large amounts of H_2 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 those in depleted oil and gas deposits. Aquifers are porous and permeable rock formations which have the pore space occupied by brackish or saline waters (Amirthan and Perera, 2023; Jafari Raad et al., 2022). They are common in sedimentary basins all over the world and may represent an alternative for UHS 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 (Jafari Raad et al., 2022; Schultz and Evans, 2020). The reduced geological knowledge in comparison with depleted hydrocarbon fields, means there is greater uncertainty for storage integrity and potential hazards, including possible migration of hydrogen along undetected faults or fractures or poorly defined sealing caprocks, biochemical reactions or reactions of H_2 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 (Caglayan et al., 2020; Li et al., 2024; Ozarslan, 2012). UHS in salt formations 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. These specific properties of salt guarantee long-term stability and the tightness of storage. Salt deposits of suitable thickness, purity and extent, enables the construction of underground storage facilities with multiple caverns hosting significant capacity. Hydrogen has been already successfully stored in several salt caverns in the UK and the US (Caglayan et al., 2020; Evans and Holloway, 2009; Jahanbakhsh et al., 2024). The presence of contaminations of non-salt rocks in the salt beds, as well as of highly soluble potassium-magnesium salts or sulfate evaporite, may provide a migration path for the stored gas or 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), Europe, Asia (mainly China), Oceania and several other areas worldwide. According to more

recent information by CEDIGAZ, the International Association for Natural Gas Information (CEDIGAZ, 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. 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 (<https://www.cedigaz.org/databases/>). Overall, the global landscape of underground gas storage is characterized by ongoing expansion and strategic investments.

3. Hydrogen as key energy source in microorganisms

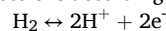
Biology derives energy for its sustenance and growth from thermodynamically favourable chemical redox reactions (Hay Mele et al., 2023). Irrespective of the metabolism employed by the single organisms (not limited to microorganisms, but including animal and plant life), the oxidation of an electron donor 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 (Hay Mele et al., 2023; Jelen et al., 2016), and include a large number of elements in the periodic table. 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 is a key electron donor for microbial redox reactions due to its negative redox potential ($E^{\circ} = -0.42\text{V}$) 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 (SO_4^{2-}) reduction and ferric iron (Fe^{3+}) reduction (Jørgensen et al., 2019). Hydrogen oxidation is also responsible for the production of 20–50 % of the biogenic CH_4 globally (Rosentreter et al., 2021). In several environments, the concentrations of free H_2 are kept at the nanomolar level by the fast consumption of a large number of microbial groups (Adhikari et al., 2016; Chen et al., 2015). 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 H_2 as electron donor for microorganisms, so much so that it 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 H_2 as sole electron donor for their metabolisms (known as obligate hydrogen oxidizers), recent studies have highlighted the utilization of H_2 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 (O_2), which was traditionally considered an inhibitor of their active sites (Horch et al., 2015; Schwartz et al., 2013; Shomura et al., 2011). 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 (Fig. 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., 2024; Greening et al., 2016; 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:



The electrons are transferred one by one to an external electron carrier (such as membrane quinones) via iron-sulfur clusters present within the enzyme (Fig. 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 (Fig. 3 and Table 1). The different groups of hydrogenases are characterized by diverse functions, that include H_2 utilization as electron donor, H_2 sensing and uptake, and H_2 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 (Figs. 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., 2005; Volbeda et al., 1995). [FeFe]-hydrogenase are involved in H_2 production and sensing in Bacteria and Eukaryotes (Fig. 2 and 3) (Bennett et al., 2000; Nicolet et al., 2001; Nicolet et al., 1999; Peters et al., 1998). 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; Birrell et al., 2021). [Fe]-only hydrogenases instead, are present exclusively in methanogens (Archaea) and catalyze H_2 uptake coupled to methenyl-tetrahydromethanopterin (methenyl- H_4MPT^+) reduction (Hartmann et al., 1996; Lyon et al., 2004; Schwörer et al., 1993), a key step in the methane-generation pathway that characterizes these microorganisms. [Fe]-only hydrogenase active site contains a labile light-sensitive

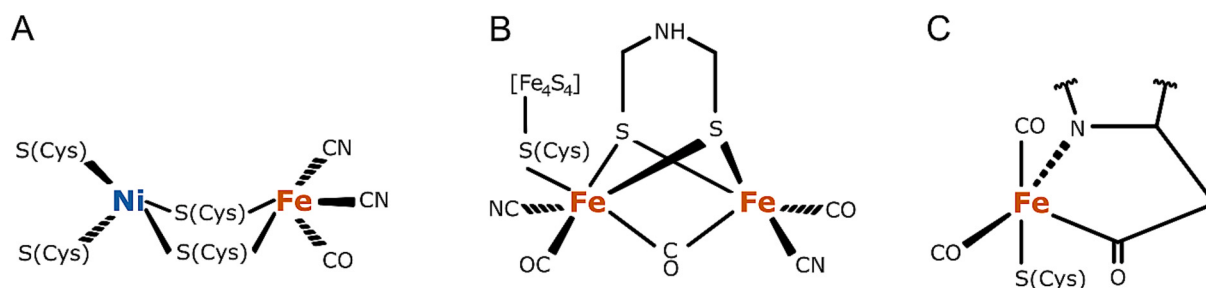


Fig. 2. Cofactor diversity in the 4 main types of hydrogenases: [NiFe] (A), [FeFe] (B) and [Fe] (C) adapted from (Ogo et al., 2020; Peters et al., 2015). While [Fe]-hydrogenases bind a single atom of Fe, [FeFe] and [NiFe] coordinate binuclear centers with the help of different groups: in [FeFe], the 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.

cofactor with a mononuclear low-spin iron that binds two CO ligands, and lacks the iron-sulfur clusters cubic centers (Figs. 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 (Fig. 4 and Table 1). Despite the similarities in structure and functions, phylogenetic analysis suggests that the three hydrogenase types have originated independently (Fig. 4) (Peters et al., 2015; Vignais et al., 2001), 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 (Greening et al., 2016, 2024; Peters et al., 2015), 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 (Greening et al., 2016; Peters et al., 2015; Vignais and Billoud, 2007). 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 O_2 -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; Beimgraben et al., 2014; Constant et al., 2011; 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 (Islam et al., 2020; Ortiz et al., 2021; Podzuweit et al., 1986; Søndergaard et al., 2016), 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

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 Extended Table S1. a. Carbonmonoxide-(dicyano) iron. b. Information not available. c. the di-iron cofactor (dicarbonyl [bis(cyanide-kappaC)]-mu-(iminodimethanethiolato-1kappaS:2kappaS)-mu-(oxomethylidene)di-iron (2+). An extended version of Table 1 is available at 10.5281/zenodo.14606252.

Hydrogenase type	Hydrogenase group	Function	Gene name	Localization	UniProt EC number	Representative organism	Structure accession	Metal cofactors	Reference
NiFe	Group 1a	Respiration	<i>hys</i>	periplasm	1.12.7.2	<i>Desulfomicrobium baculatum</i> (<i>Desulfovibrio baculatus</i>)	4KL8	Ni ²⁺ , FCO ^a , Ca ²⁺ , 4Fe-4S	Volbeda et al., 2013
	Group 1b	Respiration	<i>hyd</i>	cell membrane/periplasm	1.12.2.1	<i>Megalodesulfovibrio gigas</i>	1YQ9	Ni ²⁺ , FCO ^a , Mg ²⁺ , 4Fe-4S, 3Fe-4S	Volbeda et al., 2005
	Group 1c	Respiration/Uptake	<i>hyb</i>	cell membrane	1.12.99.6	<i>Escherichia coli</i>	6EHQ	Ni ²⁺ , FCO ^a , Mg ²⁺ , 4Fe-4S, 3Fe-4S	Pinske et al., 2015
	Group 1d	Respiration	<i>hya</i>	cytoplasmic membrane	1.12.99.6	<i>Escherichia coli</i>	3USE	Ni ²⁺ , FCO ^a , Mg ²⁺ , Li ⁺ , 4Fe-4S, 4Fe-3S, 3Fe-4S	Volbeda et al., 2013
	Group 1e	Respiration/Uptake	<i>hyd</i>	cell membrane	1.12.99.6	<i>Allochrochromatium vinosum</i>	3MYR	Ni ²⁺ , FCO ^a , Mg ²⁺ , 4Fe-4S, 3Fe-4S	Ogata et al., 2010
	Group 1f	Unresolved/Respiration	<i>hya</i>	cell membrane	1.12.99.6	<i>Geobacter sulfurreducens</i>	- ^b	-	Butler et al., 2012
	Group 1g	Unresolved/Respiration	<i>hya</i>	endomembrane system	1.12.99.6	<i>Thermophilum adornatum</i>	AF-A0A3G1A7E9-F1	-	Kochetkova et al., 2020
	Group 1h	Aerobic Respiration	<i>hya</i>	cell membrane (?)	1.12.99.6	<i>Ralstonia eutropha</i>	7ODH	Ni ²⁺ , FCO ^a , Mg ²⁺ , 4Fe-4S, 4Fe-3S	Schäfer et al., 2016
	Group 1i	Respiratory/H ₂ -uptake	-	cell membrane (?)	-	<i>Eggerthella lenta</i>	AF-A0A369MVM6-F1	-	Koppel et al., 2018
	Group 1j	Respiratory/H ₂ -uptake	<i>hyp</i>	cell membrane	-	<i>Archaeoglobus fulgidus</i>	AF-A0A075WGP0-F1	-	Birkeland et al., 2017
	Group 1k	Respiratory/H ₂ -uptake	<i>hyp</i>	cell membrane	-	<i>Methanosarcina acetivorans</i>	AF-A0A832W891-F1	-	Rinke et al., 2021
	Group 1l	Respiration/Energy production and conversion	-	cell membrane	-	<i>Rubrobacter indicocoeani</i>	-	-	Ortiz et al., 2021
	Group 2a	Aerobic/Redox Balance	<i>hyb</i>	endomembrane system	1.12.99.6	<i>Mycolicobacterium smegmatis</i>	8DQV	Ni ²⁺ , FCO ^a , Mg ²⁺ , 4Fe-3S	Grinter et al., 2023
	Group 2b	H ₂ -Sensing	<i>hya</i>	cell membrane	-	<i>Bradyrhizobium diazoefficiens</i>	AF-H7C6M7-F1	-	Kaneko et al., 2002
	Group 2c	Unresolved/Sensing	-	cell membrane	-	<i>Paramagnetospirillum magneticum</i>	AF-Q2W8A7-F1	-	Matsunaga et al., 2005
	Group 2d	Unresolved/Carbon Fixation	<i>hya</i>	cytoplasm	-	<i>Aquifex aeolicus</i>	AF-O66988-F1	-	
	Group 3a	F420 Reduction	<i>frh</i>	-	1.12.98.1	<i>Methanothermobacter marburgensis</i>	4OMF	Ni ²⁺ , FCO ^a , Mg ²⁺ , H ₂ S ₂ Zn, 4Fe-4S	Alex et al. 1990
	Group 3b	H ₂ -production	<i>hyd</i>	cytoplasm	1.12.1.3	<i>Pyrococcus furiosus</i>	AF-E7FI44-F1	-	Pedroni et al. 1995
	Group 3c	Bidirectional	<i>mvh</i>	cytosolic	1.12.99.-	<i>Methanothermobacter marburgensis</i>	AF-P60227-F1	-	Stojanowic et al. 2003
	Group 3d	NAD redox	<i>hyp</i>	cytosolic	-	<i>Methylocella silvestris</i>	AF-B8ERX1-F1	-	Chen et al. 2010
	Group 4a	H ₂ -production	<i>hyp</i>	cell membrane	1.-.-.-	<i>Yersinia enterocolitica</i>	AF-A0A7U7FL32-F1	-	Batzilla et al.
	Group 4b	H ₂ -production	<i>hyc</i>	cell membrane	-	<i>Thermococcus onnurineus</i>	-	-	Strang, 2020
	Group 4c	H ₂ -production	<i>hyd</i>	cell membrane	-	<i>Carboxydotherrmus hydrogenoformans</i>	3B52	Fe ²⁺ , 2Fe-2S, 3Fe-Ni-4S, 4Fe-4S	Wu et al., 2005
	Group 4d	H ₂ -production	<i>hyd</i>	cell membrane	1.12.7.2	<i>Pyrococcus furiosus</i>	6CFW	Ni ²⁺ , FCO ^a , 4Fe-4S,	Yu et al., 2018
	Group 4h1	H ₂ -uptake	<i>eha/ehb</i>	cell membrane	-	<i>Methanococcus aeolicus</i>	AF-A6UTY6-F1	-	Fomenkov et al. 2023

(continued on next page)

Table 1 (continued)

Hydrogenase type	Hydrogenase group	Function	Gene name	Localization	UniProt EC number	Representative organism	Structure accession	Metal cofactors	Reference
FeFe	Group 4e	Bidirectional	<i>hyp</i>	cell membrane	-	<i>Caldicellulosiruptor hydrothermalis</i>	AF_E4QC73-F1	-	Blumer-Schuette et al., 2011
	Group 4f	Respiratory/ H ₂ -evolving	-	cytoplasm	1.6.5.9	<i>Desulfotobacterium dehalogenans</i>	AF-I4AD88-F1	-	-
	Group 4g	Respiratory/ H ₂ -evolving	<i>hyc</i>	cell membrane	-	<i>Anaerolinea thermophila</i>	AF-E8MXZ8-F1	-	Matsuura et al., 2015
	Group 4h2	Respiratory/ H ₂ -evolving	<i>hyc</i>	cell membrane	1.6.99.5	<i>Methanobacterium paludis</i>	AF-F6D2P4-F1-F1	-	Cadillo-Quiroz et al., 2014
	Group 4i	Respiratory/ H ₂ -evolving	<i>hyc</i>	cell membrane	1.6.99.5	<i>Methanococcus aeolicus</i>	AF-A6UTS2-F1-F1	-	Fomenkov et al. 2023
	Group A1	H ₂ -production	<i>hyd</i>	cytoplasm	1.12.7.2	<i>Clostridium pasteurianum</i>	6N59	402°C, 4Fe-4S, 2Fe-2S	Artz et al., 2020
	Group A2	Unresolved/ H ₂ -uptake	<i>hyd</i>	cytoplasm	-	<i>Adlercreutzia equolifaciens</i>	-	-	Horner et al., 2000
	Group A3	Electron Bifurcation	<i>hyd</i>	cytoplasm	1.12.1.4	<i>Thermotoga maritima</i>	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	<i>Rhodospirillum rubrum</i>	AF_Q2RXN0-F1	-	Peters et al., 1998
	Group B	Unresolved/ H ₂ -production	<i>hydA</i>	cytoplasm	1.12.7.2	<i>Lancefieldella parvula</i>	AF_C8W9Q4-F1	-	Copeland et al., 2009
	Group C1	Unresolved/ Sensing	-	cytoplasm	-	<i>Thermoanaerobacterium saccharolyticum</i>	AF_I3VX92-F1	-	Peters et al., 1998
	Group C2	Unresolved/ Sensing	<i>hyd</i>	membrane/ cytoplasm	-	<i>Roseburia hominis</i>	AF-G2SXD3-F1	-	Copeland et al., 2009
Fe	Group C3	Unresolved/ Sensing	<i>hyp</i>	cytoplasm	-	<i>Clostridium sporogenes</i>	AF-A0A7U9GK79-F1	-	Bradbury et al., 2012
	[Fe]	Respiration	<i>hmd</i>	cytoplasm	1.12.98.2	<i>Methanocaldococcus jannaschii</i>	3F47	Fe ²⁺ , Na ⁺	Hiromoto et al., 2009

strategies in energy research. For example, biologically produced H₂ 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 H₂ in trace amounts in the atmosphere might provide a solution to the potential threats generated by the leak of H₂ 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 (Bertagni et al., 2022; Constant et al., 2009; Ocko and Hamburg, 2022; Sand et al., 2023). However data on the actual utilization of H₂ by soil microorganisms, by ocean and subsurface microorganisms as well, is severely limited, and the actual fate of H₂ leaked into the atmosphere as a result of the transition toward an hydrogen economy remains to be investigated.

4. 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 reasons: 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 reasons 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 °C (Takai et al., 2008) in the span of 50 years. Environmental observation always preceded the isolation of new record holder microorganisms, with observation of the presence of bacteria at 89 °C at Yellowstone in 1903 (Setchell, 1903), bacteria living in boiling pools at 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), and

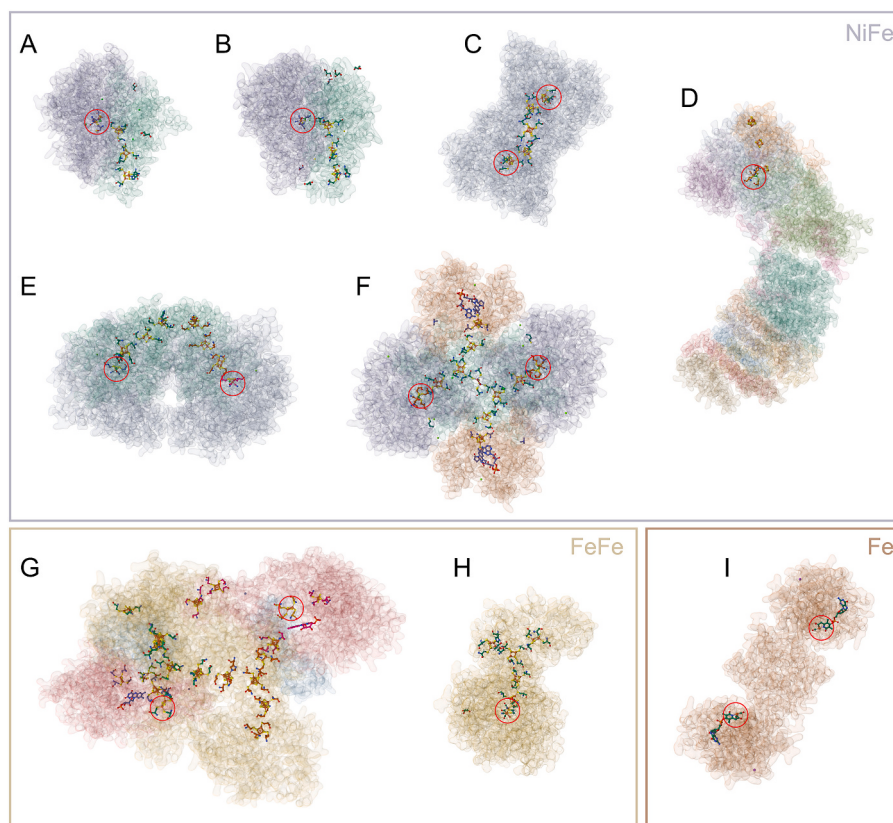


Fig. 3. Structural diversity of selected hydrogenase groups from Table 2. Representative NiFe, FeFe and Fe structures are reported with the iron-sulfur clusters and the coordinating amino acids represented by ball-and-stick; the catalytic site highlighted with a red circle. A. the O₂-tolerant respiratory [NiFeSe]-hydrogenase from *Desulfomicrobium baculatum* (PDB 4KL8); B. the prototypical respiratory O₂-sensitive [NiFe]-hydrogenase from *Megalodesulfovibrio gigas* (PDB 1YQ9); C. the carbon monoxide-respiring [NiFe]-hydrogenase from *Carboxydotherrmus 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).

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) (Cowan, 2004; Daniel and Cowan, 2000; 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 (Belilla et al., 2019; Yakimov et al., 2015). 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., 2021) 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 (Boyd et al., 2024; Zhao et al., 2023). Similarly, microorganisms

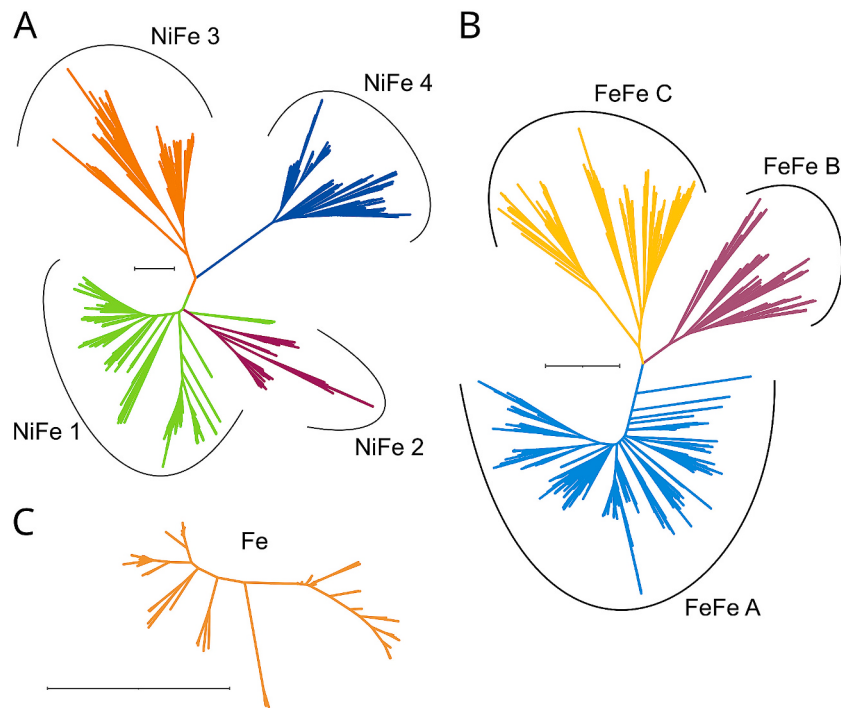


Fig. 4. Phylogenetic trees for NiFe (A), FeFe (B) and Fe (C) hydrogenases major subunits colored according to the major hydrogenases supergroups. The trees are unrooted ML trees obtained from 5,058, 2,242 and 80 sequences for NiFe, FeFe and Fe hydrogenases respectively. Scale bars are equal to a branch length of 1.

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 reservoir	Sand and Sandstone, Limestone, Dolostone	500 – 5,500	4 – 85	24 – 196	4.5 – 9	0 – 34
Subsurface aquifers	Sand and Sandstone, Limestone, Dolostone, other sedimentary rocks	200 – 3,000	3 – 25	10 – 90	5.3 – 9	0.5 – 10
Salt caverns	Halite	350 – 2,000	4 – 32	40 – 100	~6 – 8	saturation

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 UHS (Table 2), culture based and environmental based limits of life far exceed the average reservoir conditions found in the literature (Fig. 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., 2021). 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 (Fig. 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. and depth

Life in the subsurface has been demonstrated to occur in every known lithology, with cell concentrations between 10² and 10⁸ cell g⁻¹ of sediment/rock 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, 2026), 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

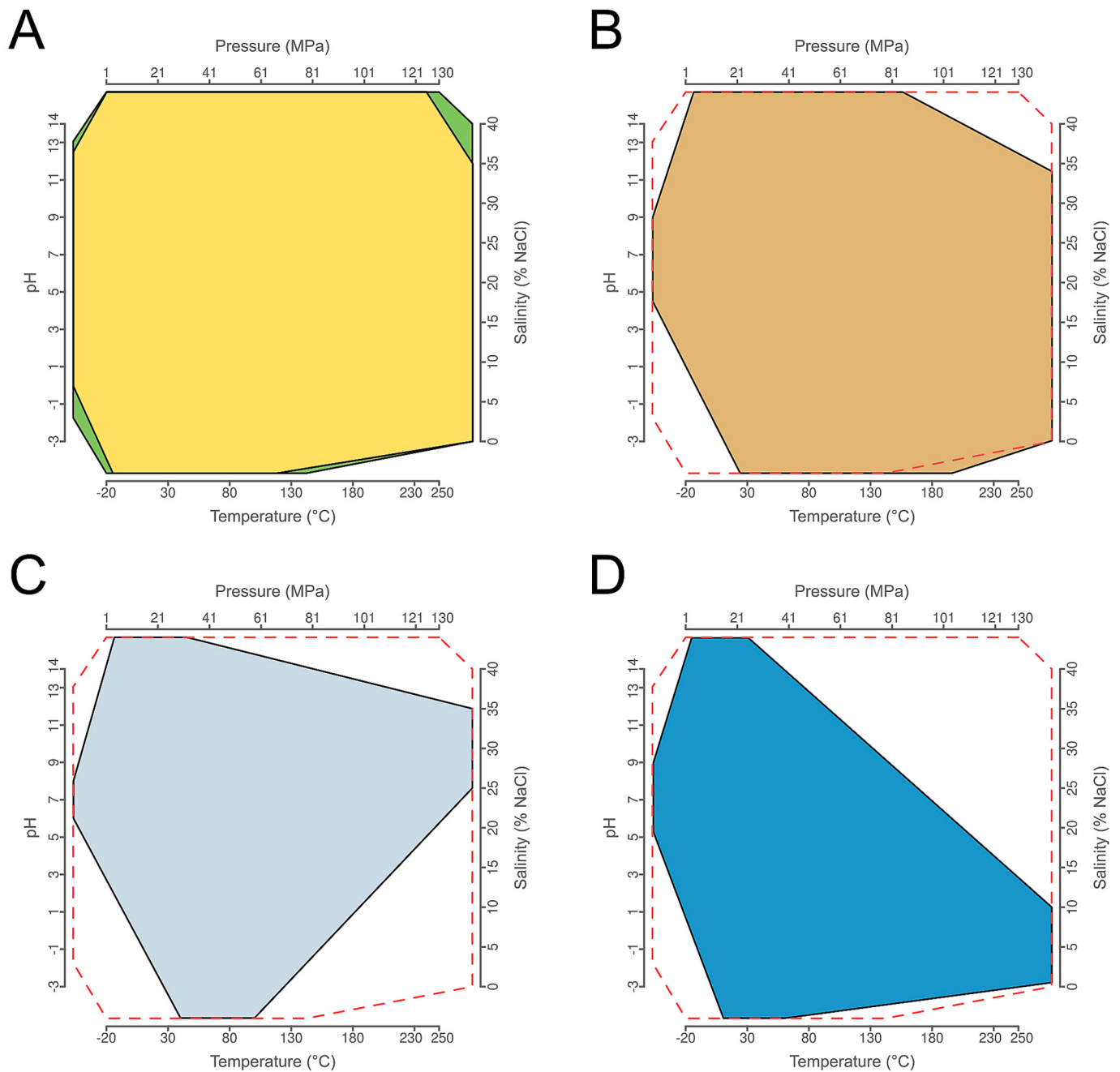


Fig. 5. The temperature, pH, pressure and salinity limits of life compared to the range of conditions in geological hydrogen storage reservoirs. Polygon plots are designed to represent ranges in multidimensional space (e.g. minimum and maximum values) and do not represent the combinatorics of existing conditions. 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.

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).

Storage temperatures for H_2 in depleted oil/gas fields, saline aquifers and caverns range from about 20 to 100 °C, within a depth range of 500

to 2,000 m (Fig. 5) (Hassanpouryouzband et al., 2020; Matos et al., 2019; 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 (Bu et al., 2018; Hagen et al., 2016; Holden and Sistu, 2023; Kurr et al., 1991; Liu and Whitman, 2008; Maus et al., 2016; Takai et al., 2008); (Kurr et al., 1991; Takai et al., 2008). Homoacetogens show optimum growth

temperatures from 20 to 70–72 °C (Basen et al., 2018; Basen and Müller, 2017; Leigh et al., 1981). 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 (Merino et al., 2019; Yakimov et al., 2015). It appears to be the brine composition, rather than the salinity alone, that puts a hard limit on microbial growth (Payler et al., 2019; Singh, 2016). 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 H₂ storage sites, such as depleted oil/gas reservoirs and deep aquifers, ranges from 0 to 3 M NaCl (Buttinelli et al., 2011; Delshad et al., 2023; Shi et al., 2020), at which highly diverse microbial communities can live (Merino et al., 2019; Rothschild and Mancinelli, 2001; Telesh et al., 2013). 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 thrive optimally at low salinities between 0 and 0.4 M NaCl. Nonetheless, some sulfate-reducing mesophiles strains, including thirty-four mesophiles, exhibit the capability to grow at NaCl concentrations up to 1.7 M (Jakobsen et al., 2006; Krekeler et al., 1997; Ollivier et al., 1991). 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 et al., 1996; Zhilina and Zavarzin, 1990). 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 (Bräuer et al., 2011; Ganzert et al., 2014; Stewart et al., 2015; Takai et al., 2002). Sulfate-reducing mesophiles are considered more widely adapted to different pH conditions, ranging from 1 to 10.7 values of pH (Thaysen et al., 2021). 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 H₂ 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 more 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 H₂ storage microbial life has not been established (Dopffel et al., 2021; Merino et al., 2019), 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 (Merino et al., 2019; Rothschild and Mancinelli, 2001). 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 sulfate-reducing *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 (CO₂) can be toxic for some microorganisms, including sulfate reducers 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).

5. The microbial diversity of potential UHS sites

The assessment of the risks associated with H₂ loss requires the knowledge of biotic and abiotic processes occurring in storage sites, in order to estimate H₂ production and consumption rates (S. Gregory et al., 2019). When H₂ 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., 2018) 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, H₂ concentration can control the speed of microbial metabolic reactions occurring: this suggests that the high amount of H₂ injected in a storage site could accelerate the metabolic reactions (Zivar et al., 2021).

Microorganisms in the environment can exist in two primary states: free-living (planktonic) or attached to solid surfaces (sessile). Microorganisms in sessile forms can organize into biofilms, which are aggregates of cells embedded in self-produced extracellular polymeric substances (EPS) (Hall-Stoodley et al., 2004; Toyofuku et al., 2016). Biofilm formation can be triggered by low-nutrient or stressful conditions, as a survival and protection mechanism, or to maintain favourable positioning in environments dominated by strong gradients (Fazeli-Nasab et al., 2022). Within biofilms, microorganisms are highly interconnected, sharing and exchanging soluble molecules and nutrients that contribute to their higher metabolic activity compared to their free-living counterparts (Wu et al., 2019). In porous reservoirs, biofilms can influence rock porosity, permeability, and wettability (Ali et al., 2023). Microbial populations in these environments may exist as biofilms on rock surfaces or as free-floating cells in the fluid phase. This distribution is shaped by nutrient availability, microbial mobility, and competition. The coexistence of the same microbial population both in biofilms and the free-living state is probable due to the dynamics of biofilm formation and disaggregation over time (Yao et al., 2022). In other cases, heterogeneous biofilms enable survival of different microbial groups under adverse conditions (Stewart and Franklin, 2008). Biofilm dynamics, controlled by a variety of physio-chemical and metabolic factors, make reservoirs and more in general subsurface biofilms a central player in UHS and natural hydrogen research. Given that the metabolic and functional traits of the diverse population are distinct between biofilms and the fluid phase at a given time, the predictions that rely only on brine samples from the reservoirs might provide limited informations on the subsurface dynamics (Brablcová et al., 2013). Therefore, biofilm microbial diversity could constitute a black box for UHS, not only for the negative effect on the rock structure of the storage site, but also because potential hydrogenotrophic microorganisms forming it could be more metabolically active than the counterparts in the free state (Dopffel et al., 2021).

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 (Bach et al., 2003; van Beilen et al., 1994). Bacteria related to the genera *Xanthobacter polyaromaticivorans* and *Stenotrophomonas* sp. have been 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 O_2 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 (NO_3^-) reducers under anoxic conditions. The great majority of the oil field microbial community is dominated by strictly anaerobic microorganisms such as *Methanobolus*, *Archaeoglobus*, *Desulfosarcina*, *Deferribacter*, *Oceanospirillum* and *Thiomicrospira* (Greene et al., 1997; Hubert et al., 2003; Li et al., 2017; Voordouw et al., 1996).

Although we know that these microbial groups mainly include fermentative bacteria, sulfate reducing bacteria, iron reducing bacteria, syntrophic bacteria and methanogens (Beeder et al., 1994; Dopffel et al., 2021; Orphan et al., 2001), 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 (Etiope et al., 2011; Truche et al., 2010; Zivar et al., 2021). The possible negative implications are H_2 flow path blockage, pores clogging, corrosion of the infrastructures by sulfide (H_2S), loss of caprock integrity and the subsequent H_2 leakage (Dopffel et al., 2021; Heinemann et al., 2021; Thaysen et al., 2021; Zivar et al., 2021). 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 (Haddad et al., 2022a; Shafahi and Vafai, 2009).

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 suggests 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 (Jehanthon et al., 2005; Magot, 2005) 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 decennial 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, sul, homo-acetogens, and iron reducing bacteria), could be stimulated during UHS operations (Haddad et al., 2022a,

2022b; Heinemann et al., 2021; Thaysen et al., 2021).

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 (Lowenstein, 2012; Park et al., 2009; Vreeland et al., 1998), it is relatively easy to find them during the process of solution mining. The sulfate minerals in evaporite formations 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. 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 reducing bacteria (Foti et al., 2007), can be enriched from such environments (Blum et al., 2012; Sorokin, 2011; Sorokin et al., 2017). Coherently with these studies, a 250 million-year-old halotolerant bacterium has been isolated

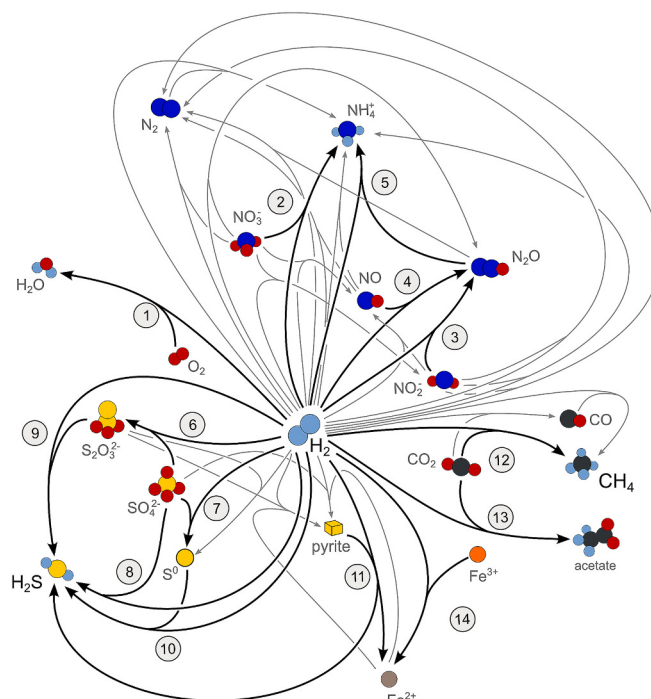


Fig. 6. Hydrogenotrophic metabolism of interest in underground hydrogen storage sites and in natural hydrogen reservoirs. Lines for exemplificative reactions of relevance for each principal electron acceptors are in bold and numbered according to Table 3. The other reactions can be found in the supplementary 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.

Table 3

Hydrogenotrophic microbial metabolisms of relevance in diverse subsurface hydrogen reservoirs. Reactions are numbered according to Fig. 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 Extended Table S3. The threat to UHS operations and the relevance to different reservoir settings has been assessed after an extensive review of the literature (see supplementary extended Table S3). 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 S3 is available at 10.5281/zenodo.14606252.

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	$H_2(aq) + \frac{1}{2}NO_3^- + \frac{1}{2}H^+ \leftrightarrow \frac{1}{2}NH_4^+ + \frac{1}{2}H_2O$	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	$H_2(aq) + \frac{1}{2}SO_4^{2-} + \frac{1}{2}H^+ \leftrightarrow \frac{1}{2}S_2O_3^{2-} + \frac{1}{2}H_2O$	8	-74.04	Sulfate content of the hosting rocks, contaminations from evaporite deposits, entrapment of seawater (especially offshore), microbial and abiotic oxidation of reduced sulfur species	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 well known in oil and gas reservoirs	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	$H_2(aq) + \frac{1}{3}SO_4^{2-} + \frac{2}{3}H^+ \leftrightarrow \frac{1}{3}Sulfur(cr) + \frac{4}{3}H_2O$	6	-92.66	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
8	$H_2(aq) + \frac{1}{2}SO_4^{2-} + \frac{1}{2}H^+ \leftrightarrow \frac{1}{2}H_2S(aq) + H_2O$	8	-77.27	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
9	$H_2(aq) + \frac{1}{2}S_2O_3^{2-} + \frac{1}{2}H^+ \leftrightarrow \frac{1}{2}H_2S(aq) + \frac{1}{2}H_2O$	8	-80.50					
10	$H_2(aq) + sulfur \leftrightarrow H_2S(aq)$	2	-31.10					

(continued on next page)

Table 3 (continued)

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
11	$\text{H}_2(\text{aq}) + \text{pyrite} + 2\text{H}^+ \leftrightarrow \text{Fe}^{+2} + 2\text{H}_2\text{S}(\text{aq})$	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	$\text{H}_2(\text{aq}) + \frac{1}{4}\text{CO}_2(\text{aq}) \leftrightarrow \frac{1}{4}\text{CH}_4(\text{aq}) + \frac{1}{2}\text{H}_2\text{O}$	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 ₂ S production, H ₂ SO 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	$\text{H}_2(\text{aq}) + \text{CO}_2 \leftrightarrow \frac{1}{4}\text{CH}_3\text{COO}^- + \frac{1}{2}\text{H}_2\text{O} + \frac{1}{4}\text{H}^+$	8	160.71	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 significant amount as impurity in the evaporite	Medium to high. Iron oxides are abundant in many sediments and aquifers
14	$\text{H}_2 + 3\text{Fe}_2\text{O}_3 \leftrightarrow 2\text{Fe}_3\text{O}_4 + \text{H}_2\text{O}$	6	-153.96					

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.

6. Hydrogenotrophic metabolisms in the subsurface

Microorganisms are known to couple the oxidation of H₂ to the reduction of a large number of oxidized elements and molecules (Fig. 6, Table 3 and Supplementary Extended Table S3). The diversity of possible electron acceptors that can be coupled to H₂ oxidation is astonishing, and goes well beyond the classically investigated examples in UHS environments such as CO₂, SO₄²⁻ and Fe³⁺ (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 Extended 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 (TeO₄²⁻), selenate (SeO₄²⁻), arsenate (AsO₄³⁻), chromate (CrO₄²⁻), uranate (UO₄²⁻) and dozens of minerals (Heim, 2011). These electron acceptors are deemed to be of secondary relevance to UHS and natural H₂ 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 (Sn²⁻), tetrathionate (S₄O₆²⁻), sulfite (SO₃²⁻) and dimethylsulfide (C₂H₆S) and many others that are not explicitly included in this review.

The oxidation of H₂ using a variety of electron acceptors yields different energy depending on the redox couple and the environmental conditions (Fig. 6, Table 3 and Supplementary Extended 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 Extended Table S3), it appears evident that sulfate reduction, iron reduction and hydrogenotrophic methanogenesis play a potential key role in the consumption of stored H_2 .

Sulfate reducing bacteria and Archaea are capable of reducing a large number of compounds in addition to sulfate, sharing substrates with elemental sulfur (S^0) and thiosulfate ($S_2O_3^{2-}$) 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, 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., 2021). 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 (Jelen et al., 2018; Rickard and Luther, 2007).

Given the high abundance of iron in the crust, Fe^{3+} reduction is among the most important metabolism for UHS sites. Ferric 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 (Chen et al., 2008; Colleran et al., 1995; Lackner et al., 2020; Paulo et al., 2015; Stefanie et al., 1994). 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 CO_2 to CH_4 through H_2 oxidation, and are key player in several subsurface ecosystems (Beaver and Neufeld, 2024; Chapelle et al., 2002; S. Gregory et al., 2019; Milkov, 2022) and in UHS reservoirs (Dopffel et al., 2021; Pannekens et al., 2019). They are known to consume large amounts of H_2 in diverse conditions, and global estimates suggest that between 10 and 50 % of the biogenic CH_4 is derived from H_2 oxidation (Wong et al., 2026). By contrast, the role of homoacetogens, bacteria capable of coupling the oxidation of H_2 to CO_2 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, oil and gas reservoirs, and deep aquifers remains largely unexplored (Zgonnik, 2020; Wong et al., 2026). In oil and gas depleted reservoirs, the most likely electron acceptors are SO_4^{2-} , $S_2O_3^{2-}$, pyrite, CO_2 , Fe^{3+} and iron-oxide minerals (Table 3 and Supplementary Extended 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 minerals in the reservoir, either as depositional phases or as cements. 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). CO_2 , 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 (Baines and Worden, 2004; Cardoso and Andres, 2014; Head et al., 2003; Mao et al., 2024; Muhammed et al., 2023).

In deep aquifers, the most likely electron acceptors are O_2 , pyrite, CO_2 , Fe^{3+} and iron-oxide minerals (Table 3 and Supplementary Extended Table S3). Oxygen traces added during the process of desulfurization are often co-injected in the gas network and can be stored in

UGS, causing unclear consequences (Haddad et al., 2022a, 2022b). 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 ($\sim 25^\circ C$) (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 $S_2O_3^{2-}$, SO_4^{2-} and CO_2 (Table 3 and Supplementary Extended 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 intercalations of sulfate-minerals (e.g. gypsum) in the evaporite sequence or as minor phase in the salt 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 O_2 , NO_3^- 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 O_2 production in the subsurface independent from oxygenic photosynthesis have been described (Ruff et al., 2023), as well as the presence of abiotic production of O_2 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 of sulfur-oxidizing bacteria known as *Thiobacilli*, widely present in potential UHS sites like petroleum reservoirs (Al-Khazaali and Ataei, 2023; Tian et al., 2017), plays a significant role in the production of sulfuric acid through the oxidation of various sulfur compounds, including H_2S , S^0 , SO_3^{2-} , and $S_2O_3^{2-}$ (Ehrlich, 1981). The resulting acidity is recognized as a key factor in the solubilization of carbonates in soils (Dommergues and Mangelot, 1970; Duchaufour, 2012), in the weathering of limestone (Kaufmann, 1960; Winkler, 1978) 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 H_2 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 H_2 (Ebrahimiyejta, 2017), is considered the major abiotic mineral dissolution process during H_2 storage.

7. Metabolic by-products, microbial inhibition and operational risks

All the hydrogenotrophic metabolisms discussed above generate a number of chemical by-products, some of which are of particular relevance for UHS operations: i) CH_4 ; ii) H_2S ; iii) organic acids. Microbial production of CH_4 could be either considered a desirable process 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, H_2S , a toxic gas produced by SSRM, is highly toxic and corrodes steel infrastructures in a process called Microbial Induced Corrosion (MIC) causing economic damages (Dopffel et al., 2021; Hemme and van Berk, 2017). Ferrous-ferric oxide (also called “magnetite”) produced in the iron-reduction carried out by iron reducing bacteria, 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 Garrels, 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., 2021). The major organic acid produced in fuel-oil/aqueous systems is acetic acid (CH_3COOH) (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 H_2S concentrations of 3.3 mM, respectively, stop the growth (Choi and Rim, 1991; Ntagia et al., 2020). In systems characterized by circum-neutral 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 microorganisms (Albina et al., 2019; Reis et al., 1992).

Hydrogen might also directly inhibit its own utilization by microorganisms 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 (Liu et al., 2023; Osman et al., 2022). A range of studies investigated the metabolism of methanogens at excess H_2 concentrations and ambient pressure, with contrasting results. 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. (2019) and 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 (Bassani et al., 2023; Ben Hania et al., 2013) and in natural gas field (Dohrmann and Krüger, 2023). Several studies demonstrate that Thermotogales tolerance to H_2 differs greatly between species depending on H_2 partial pressure and that hydrogen excess can inhibit the growth of most of them, with few exceptions such as *Mesotoga spp.* (Dohrmann and Krüger, 2023; Huber and Stetter, 2001). 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; Soares et al., 2023; Xie et al., 2021), therefore the response to variable *in situ* H_2 concentrations remains unknown.

While inventories of microbial metabolisms occurring in subsurface environments and relevant to UHS have been attempted before (Boyd et al., 2024; Dopffel et al., 2024; Dopffel et al., 2021; Thaysen et al., 2021; 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 metabolisms 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 (Fig. 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 CH_4 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 organic carbon transported in the subsurface or deposited in the sedimentary sequence—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 (Al-Yaseri et al., 2023; Aslannezhad et al., 2023; Kieft, 2021; 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 (Freedman et al., 2017; Grabowski et al., 2005). 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 CH_4 -producing Archaea are found in depleted oil reservoirs during oil biodegradation processes, where SO_4^{2-} 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 H_2 (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 H_2 -rich and UHS potential sites (Amils et al., 2023; Brazelton et al., 2012; Chapelle et al., 2002; 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.

8. 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 narrowed by logistic and operational factors; and iii) direct access to subsurface communities is often costly 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 H_2 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⁺, Th⁴⁺, and U-bearing minerals decay leading to H_2O radiolysis, and H_2O reaction with surface radicals during the mechanical breaking of the rock Si-O bond (Klein et al., 2020; Langhi and Strand, 2023). Biological processes might also contribute to H_2 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 (Suzuki et al., 2024; Tissot and Welte, 1984), 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 hydrocarbons and H_2 (Hanson and Hanson, 2024; Tissot and Welte, 1984).

Hydrogen produced in the subsurface migrates through the crust from its source and might accumulate in geologic traps (Maiga et al., 2024; O'Sullivan et al., 2025; Sekar et al., 2023). 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 (Leila et al., 2021; McMahon et al., 2023; Québécois et al., 2023; Wang et al., 2023; Zgonnik, 2020; Wong et al., 2026). 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, 2026). 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 H_2 in a wide range of physico-chemical, geological and biological conditions, allowing to obtain fundamental data to interpret and model

UHS operations and inform natural H_2 prospecting.

Evidence is growing that each H_2 -generating geological process supports microbial communities, even if determining to what extent is not trivial (Gregory et al., 2019). 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 (Blair et al., 2007; Lin et al., 2005; Sauvage et al., 2021). One plausible explanation is that the redox disequilibria generated via radiolysis supports chemolithoautotrophic microbial communities (Dzaugis et al., 2016; Türke et al., 2015). 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 known to rely on H_2 as electron donor (Adam and Perner, 2018; Holden and Sistu, 2023; Petersen et al., 2011; Reveillaud et al., 2016). 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 H_2 is not only present, but represents a key electron donor (Colman et al., 2016; Rogers et al., 2022; Upin, 2020).

Given the diversity of microbial niches present on Earth and how these map to UHS sites and subsurface environments (Fig. 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 H_2 production, migration and accumulation in a variety of ways, and while the amount of information available on this regard is currently very limited, our knowledge of their interaction with geologic H_2 will play a key role in the economy of natural hydrogen.

9. Conclusion and future outlook

The role of hydrogenotrophic microorganisms and their associated hydrogenase enzymes in UHS 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, H_2 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 H_2 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 H_2 oxidation and the complex trophic interactions arising in subsurface ecosystems. 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, we suggest 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 H_2 consumption or utilize microbial processes to enhance storage stability.

Considering the current knowledge on subsurface biosphere and limits of life as discussed in this review, it is practically certain life is present in all sites suitable (technically and economically) for UHS and natural H_2 accumulation. Thus the interactions between microorganisms and H_2 are not only theoretically possible in the subsurface, but practically certain, and this is something we will have to address as we transition to a hydrogen based economy. Unveiling the role that microorganisms play in H_2 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.

Declaration of competing interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.earscirev.2026.105620>.

Data availability

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/>). Sequences and extended versions of Tables 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.14606252.

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