



Molecular Hydrogen, a Neglected Key Driver of Soil Biogeochemical Processes

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ABSTRACT The atmosphere of the early Earth is hypothesized to have been rich in reducing gases such as hydrogen (H₂). H₂ has been proposed as the first electron donor leading to ATP synthesis due to its ubiquity throughout the biosphere as well as its ability to easily diffuse through microbial cells and its low activation energy requirement. Even today, hydrogenase enzymes enabling the production and oxidation of H₂ are found in thousands of genomes spanning the three domains of life across aquatic, terrestrial, and even host-associated ecosystems. Even though H₂ has already been proposed as a universal growth and maintenance energy source, its potential contribution as a driver of biogeochemical cycles has received little attention. Here, we bridge this knowledge gap by providing an overview of the classification, distribution, and physiological role of hydrogenases. Distribution of these enzymes in various microbial functional groups and recent experimental evidence are finally integrated to support the hypothesis that H₂-oxidizing microbes are keystone species driving C cycling along O₂ concentration gradients found in H₂-rich soil ecosystems. In conclusion, we suggest focusing on the metabolic flexibility of H₂-oxidizing microbes by combining community-level and individual-level approaches aiming to decipher the impact of H₂ on C cycling and the C-cycling potential of H₂-oxidizing microbes, via both culture-dependent and culture-independent methods, to give us more insight into the role of H₂ as a driver of biogeochemical processes.

KEYWORDS H₂ oxidation, anaerobic processes, biogeochemical processes, carbon cycle, environmental microbiology, hydrogen, soil

Several prebiotic chemistry models have hypothesized that the primary atmosphere of the early Earth was rich in reducing gases, such as molecular hydrogen (H₂), carbon monoxide (CO), and methane (CH₄) (1–4). The ubiquitous availability of H₂, its low activation energy requirements, and its ability to diffuse through microbial cells led to the hypothesis that H₂ was the first electron donor as a mean to generate an ion gradient and, consequently, ATP synthesis (5, 6). In this regard, the iron-sulfur world hypothesis stipulates that NiFe/FeS active centers similar to the active site of hydrogenase enzymes, catalyzing the interconversion of H₂ into protons and electrons, could have emerged a billion years ago and have led to the first biotic sources and sinks of H₂ (7). The availability of elevated mixing ratios of H₂ in many ecological niches might represent a type of selection pressure maintaining this ancient metabolism within today's microbes, including environmental isolates (e.g., newly cultivated *Verrucomicrobia*) (8) and human pathogens (e.g., *Helicobacter pylori*-associated gastric carcinogenesis) (9). Indeed, hydrogenases are well represented within the tree of life, as they are found in thousands of genomes from over 30 phyla (10, 11) (Fig. 1–3). They support autotrophic and mixotrophic lifestyles in various ecosystems encompassing soil, aquatic, and animal-associated niches (6).

In soils, microbial growth is often limited by the scarcity of carbon sources and nutrients (6), which translates to bursts of growth between intervals of starvation and

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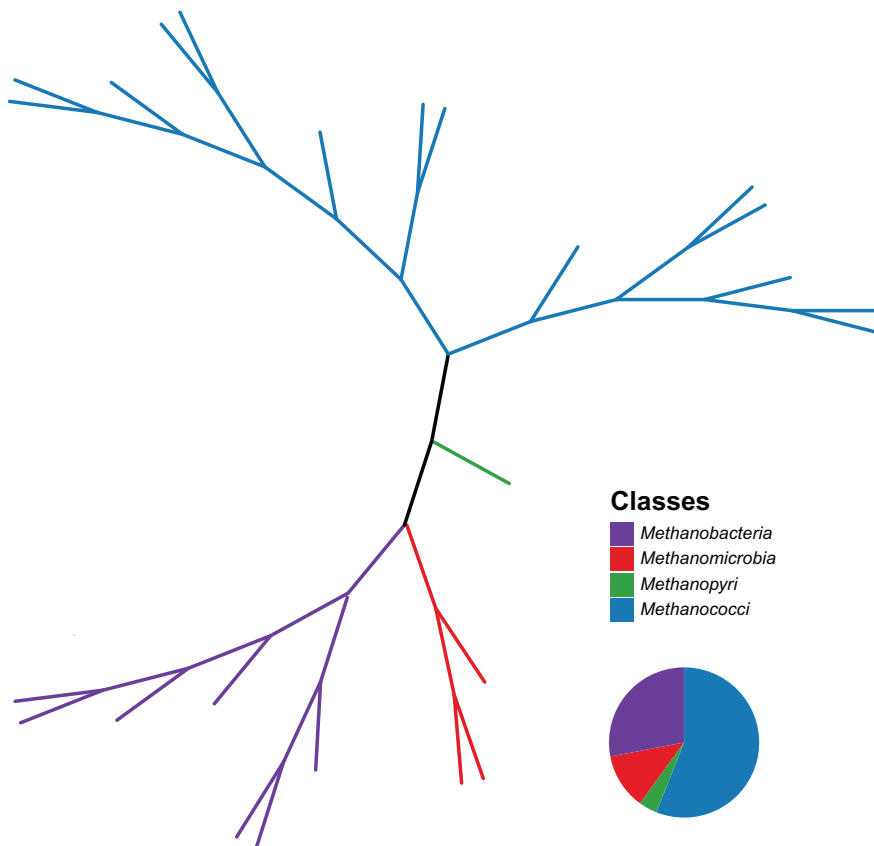
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[Fe]-Hydrogenases

FIG 1 Consensus tree of 25 [Fe]-hydrogenase sequences, with an alignment length of 389 amino acids. Protein sequences were imported from a study by Greening et al. (10), which included nonredundant putative hydrogenase catalytic subunits from cultured and environmental metagenomes sourced from NCBI RefSeq and Joint Genome Institute, Microbial Dark Matter (JGI, MDM) databases. More details are found in the article by Greening et al. (10). See Fig. S1 for complete taxa names and a more detailed phylogenetic tree. Hydrogenase amino acid sequences were aligned with MUSCLE (198) and clustered into phylogenetic trees using the following algorithms: maximum likelihood (Jones-Taylor-Thornton substitution model) using RAxML version 8.2.10 (199), maximum parsimony using PAUP 4.0 (200), and neighbor-joining (Jones-Taylor-Thornton substitution model) using BIONJ (201). CIPRES Science Gateway version 3.3 (202) servers were used for phylogenetic trees construction. Tree branches supported by over 50% of the 1,000 bootstrap replications were represented in the final consensus tree. The consensus tree was built using the “ape” package (203) in R (204). Branch colors represent taxonomic classification at the class level, since all 25 sequences are part of the *Euryarchaeota* phylum, while pie charts show the relative abundance of each class within *Euryarchaeota*.

survival. On the other hand, H_2 is ubiquitous, requires a low activation energy, and can easily permeate microbial cells (6). The ubiquity of H_2 is notably due to its high penetrating power through materials and its continuous abiogenic production in Earth's upper mantle and biogenic production by fermentation, N_2 fixation, and photoproduction (6). H_2 oxidation can be combined with the reduction of a wide variety of oxidants, and therefore, it can be performed in most ecosystems. This combination provides a quick energy fix, via H_2 , to overcome those short-to-long intervals of starvation-survival. Several studies have calculated this minimal energy requirement from thermodynamic and bioenergetic perspectives (12–14).

When environmental conditions are not suitable for growth, H_2 oxidation could provide energy to undergo anabiosis, i.e., long-term-stationary phase or even dormancy (6, 15). Such a lifestyle might combine starvation-survival with another survival mechanism, such as anhydrobiosis (i.e., desiccation) or cryobiosis (i.e., freezing) (16), not unlike microbes trapped in amber or frozen in permafrost. Metabolically inactive

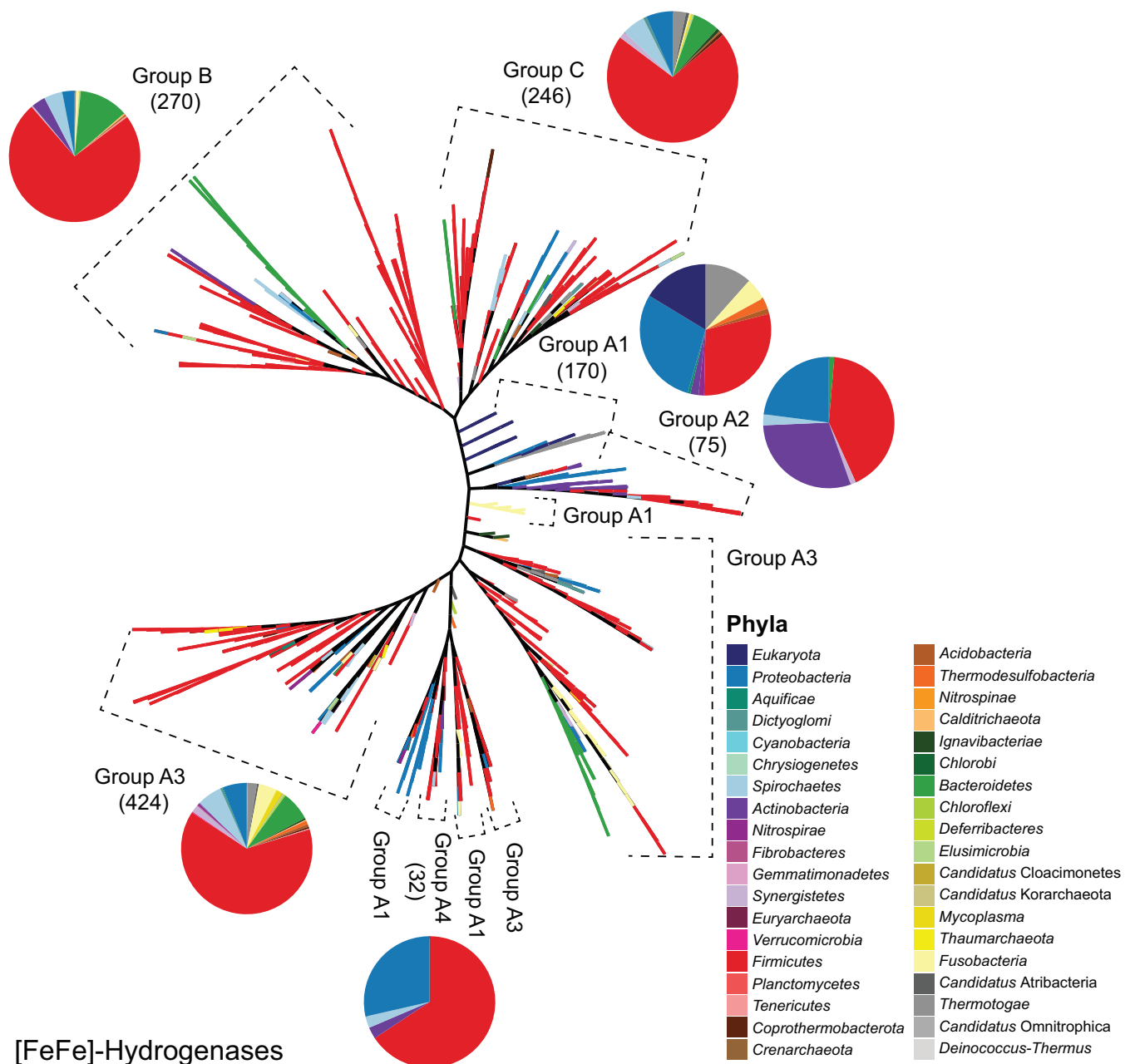
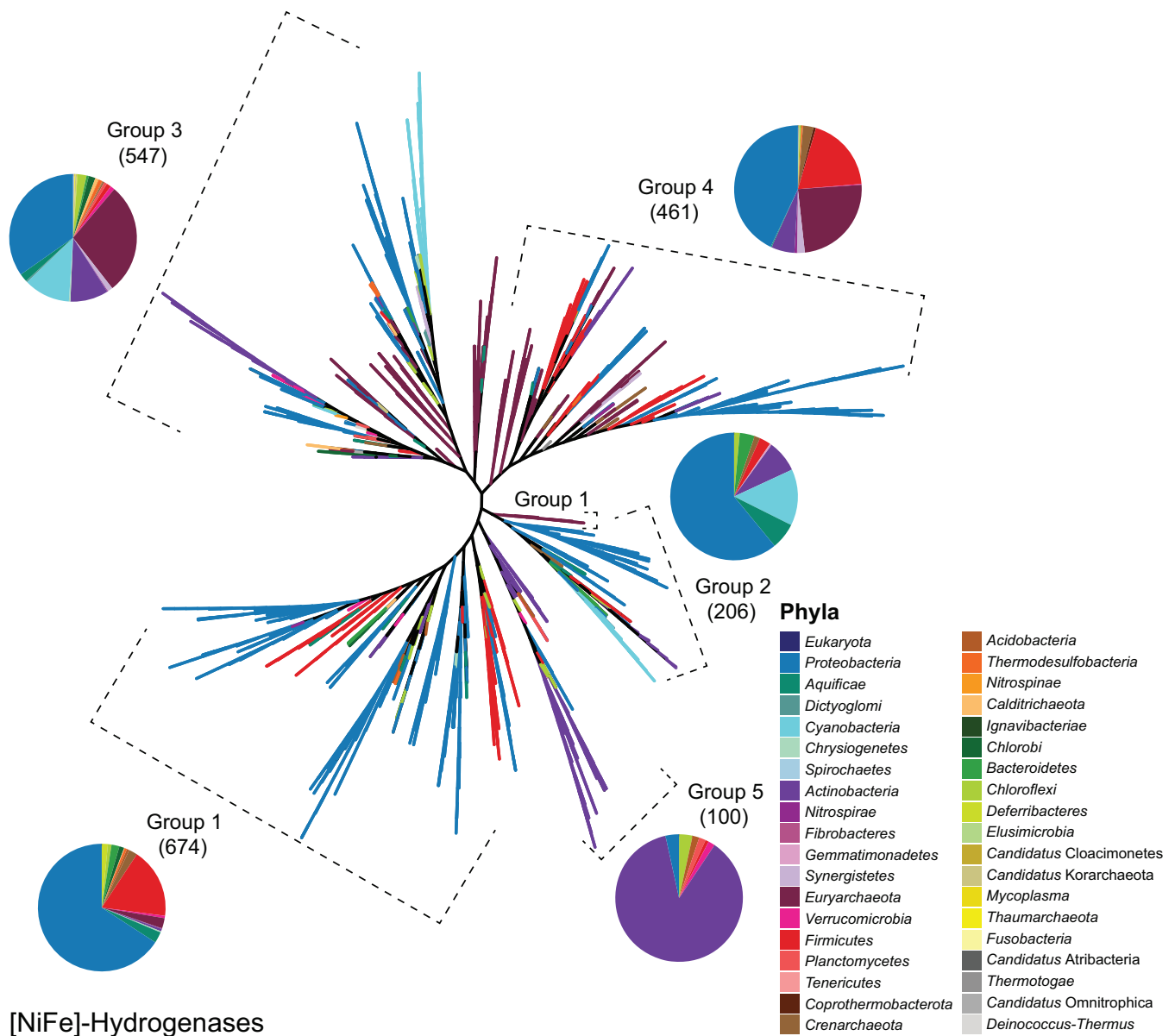


FIG 2 Consensus tree of 1,217 [FeFe]-hydrogenase sequences, with an alignment length of 3,525 amino acids, computed as described in Fig. 1. See Fig. S2 for complete names of taxa and a more detailed phylogenetic tree. Branch colors represent taxonomic classification at the phylum level, while pie charts show the relative abundance of each phylum within hydrogenase subgroups (except for Eukaryota being a superkingdom). Dashed brackets show which tree branches belong to a specific hydrogenases group. Numbers between parentheses depict the amount of sequences within that group.

lifestyles drastically reduce energy demands, yet microbial cells still need to prevent amino acid racemization (i.e., spontaneous change of isomeric form) and DNA depurination over extended periods of time by constantly repairing cellular damage or resynthesizing DNA (13, 14, 17, 18), requiring a continuous input of energy. When organic matter and H₂ abound, H₂-oxidizing microbes (HOM) can switch from short- or long-term dormancy to a more metabolically active lifestyle (6).

While recent studies have shown the ubiquity and importance of H₂ in natural and engineered ecosystems, this field of study has been fairly overlooked since few research groups have focused on biotic H₂ dynamics at the ecosystem level (19). The aim of this review is to examine and discuss the potential role of H₂ as an energy source



[NiFe]-Hydrogenases

FIG 3 Consensus tree of 1,988 [NiFe]-hydrogenases sequences, with an alignment length of 1,850 amino acids, computed as described in Fig. 1. See Fig. S3 for complete names of taxa and a more detailed phylogenetic tree. Branch colors represent taxonomic classification at the phylum level, while pie charts show the relative abundance of each phylum within hydrogenase subgroups (except for Eukaryota being a superkingdom). Dashed brackets show which tree branches belong to a specific hydrogenases group. Numbers between parentheses depict the amount of sequences within that group.

maintaining microbial viability and activity and, in turn, sustaining soil biogeochemical processes. First, a brief overview of hydrogenase enzymes is provided, including their classification and physiological roles, with emphasis on their kinetic properties and O₂ tolerance. The following section describes functional groups producing and oxidizing H₂ across oxic and anoxic soil habitats. The last section integrates the biochemical properties of hydrogenases to propose succession patterns of HOM along theoretical gradients of O₂ and H₂ prevailing in soils, as well as their potential implications in the carbon cycle. This review concludes with research perspectives aiming to evaluate the contribution of HOM in soil biogeochemical processes.

HYDROGENASE CLASSIFICATION: AN OVERVIEW

Hydrogenases are metalloenzymes catalyzing the interconversion of H₂ to protons and electrons according to the following reaction: H₂ ⇌ 2H⁺ + 2e⁻ (20). These

enzymes are used either to provide energy, to reduce cofactors, or to disperse reducing equivalents generated through anaerobic processes (11). The conformation of electron bridges between structural subunits and the localization of the protein, membrane-bound or soluble, determine their physiological role (20). Their classification into [Fe]-hydrogenases, [FeFe]-hydrogenases, and [NiFe]-hydrogenases is based on differences between their active sites and amino acid sequences (21). These classes probably originated from converging rather than sequential evolution, since their tertiary structure, catalytic activity, and taxonomic distribution are clearly distinct (22, 23). Horizontal gene transfer is also common in hydrogenases due to their modular structure (24–27). Consequently, several microbes, such as *Cupriavidus necator* (phylum *Proteobacteria*) and *Mycobacterium smegmatis* (phylum *Actinobacteria*), have the genomic capability to express multiple hydrogenases with distinct physiological roles, resulting in an increased metabolic flexibility (28, 29).

(i) [Fe]-hydrogenases. [Fe]-hydrogenases are homodimers consisting of two 38-kDa subunits with a single Fe atom in their catalytic center (20). In contrast with [FeFe]- and [NiFe]-hydrogenases, [Fe]-hydrogenases do not contain Fe-S clusters, are restricted to hydrogenotrophic methanogenic archaea within the *Euryarchaeota* phylum (30) (Fig. 1; see also Fig. S1 in the supplemental material), and are not inactivated by O₂ (31, 32). Under nickel-limiting conditions, [Fe]-hydrogenases work along with F₄₂₀-dependent methylene tetrahydromethanopterin (methylene-H₄MPT) dehydrogenases to redirect electrons originating from H₂ oxidation toward H₂⁻ and CO₂-dependent methanogenesis (33).

(ii) [FeFe]-hydrogenases. [FeFe]-hydrogenases are mono-, di-, tri-, or tetrameric enzymes containing an active center with two Fe atoms, called H-cluster, as well as Fe-S clusters (34). They are strictly anaerobic since their active site is denatured by O₂ exposure (20). [FeFe]-hydrogenases almost exclusively produce H₂ (28, 35), with a few exceptions, such as in *Desulfovibrio vulgaris* (phylum *Proteobacteria*), whose [FeFe]-hydrogenase can oxidize H₂ under nickel-limited conditions to circumvent [NiFe]-hydrogenase synthesis (36). [FeFe]-hydrogenases are mostly found in anaerobic *Firmicutes* and sulfate reducers (Fig. 2 and S2) due to their O₂ sensitivity (23, 37, 38). They consist of the only class of hydrogenases found in eukaryotes, namely, in the unicellular green alga *Chlamydomonas reinhardtii* (phylum *Chlorophyta*) (39), where they are located within membrane-limited organelles like chloroplasts and hydrogenosomes (40).

(iii) [NiFe]-hydrogenases. [NiFe]-hydrogenases are the most widespread hydrogenase class (11). Several structural and accessory genes are necessary for their maturation (41). While [NiFe]-hydrogenases can be multimeric, they contain at the very least a core heterodimeric component comprising a large α -subunit (ca. 60 kDa) and a small β -subunit (ca. 30 kDa) (20). [NiFe]-hydrogenases represent a heterogeneous group of hydrogenases divided into 5 subgroups according to their phylogeny (Fig. 3 and S3) and physiology (20). Among these physiological traits, O₂ tolerance and low catalytic affinity are the most common (20). Indeed, only a few subgroups are sensitive to O₂, and a single subgroup has a high-affinity toward H₂, i.e., it can oxidize atmospheric H₂ (0.53 ppmv [42]). This classification scheme supported by concurrence between gene sequences and physiological roles of hydrogenases has been revisited recently using structural gene sequences similarity networks, suggesting a higher number of putative subclasses within each group (10, 43). Such reclassifications will not be covered here owing to the lack of experimental observations supporting their coherence with genetic, biochemical, and physiological features of hydrogenases.

(a) Group 1 [NiFe]-hydrogenases. Group 1 [NiFe]-hydrogenases (membrane-bound hydrogenases [MBH]) are H₂ uptake hydrogenases attached to the cytoplasmic membrane (periplasmic side) (20). They are intimately linked to the electron transport chain by supplying the quinone pool with electrons via cytochrome *b*, leading to chemiosmosis and the subsequent generation of ATP (44). MBH are used for energy generation by coupling the oxidation of H₂ with the reduction of several electron acceptors, such

as CO_2 , SO_4^{2-} , NO_3^- , Fe(III) oxides, or O_2 (44). They are mostly found in *Proteobacteria* and *Firmicutes* (62.5% and 17.3%, respectively) (Fig. 3 and S3). Ancestral MBH found in methanogenic archaea and sulfate-reducing bacteria are O_2 sensitive (44). In contrast, those associated with the Isp complex, in obligate and facultative autotrophs, and with HybA-MBH, in *Gammaproteobacteria*, have a low tolerance toward O_2 (44). O_2 -tolerant MBH are used to perform the Knallgas reaction, i.e., oxidation of H_2 using O_2 as terminal electron acceptor, or other reactions involving high-potential electron acceptors such as nitrogen oxides or bacteriochlorophylls (44).

(b) *Group 2 [NiFe]-hydrogenases*. Group 2 [NiFe]-hydrogenases consist of cytoplasmic enzymes divided into 2 distinct functional groups, 2a and 2b (20). [NiFe]-hydrogenases encompassing group 2a are soluble uptake hydrogenases oxidizing H_2 endogenously produced by N_2 fixation (45) or NADH/NADPH oxidation (29) and are mainly found in *Cyanobacteria* and other N_2 -fixing bacteria (45, 46) (Fig. 3 and S3). Their role is to channel electrons to the electron transport chain and supply energy to the cell (34). They are often located inside cyanobacterial heterocysts devoid of oxygenic photosynthesis and are upregulated under anoxic conditions due to their O_2 sensitivity (47). In contrast, group 2b [NiFe]-hydrogenases are H_2 -sensing regulatory hydrogenases (RH) acting as two-component signal transduction systems (20). The specific shape and size of the hydrophobic gas channel leading exogenously produced H_2 to the active site confer O_2 tolerance to the enzyme (48). When H_2 is present, RH induce the transcription of group 1 and group 3 uptake hydrogenase operons in Knallgas bacteria (49, 50).

(c) *Group 3 [NiFe]-hydrogenases*. Group 3 [NiFe]-hydrogenases are cytoplasmic enzymes called bidirectional heteromultimeric [NiFe]-hydrogenases or soluble hydrogenases (SH) (20). Their role is to provide redox balance within the cell in both bacteria and archaea (11). The structural subunits of those enzymes are in close association with protein modules binding soluble cofactors (20). Group 3 [NiFe]-hydrogenases are O_2 tolerant due to two extra cyanide ligands bound to the Ni and Fe atoms of their active site (51). These hydrogenases, mainly found in *Proteobacteria* (37.4%) and *Euryarchaeota* (27.4%) (Fig. 3 and S3), are further divided into 4 subgroups (20). Group 3a [NiFe]-hydrogenases are heterotrimeric enzymes initially found in methanogenic archaea (52), but more recently in some bacterial phyla (53), and are used to reduce the F_{420} cofactor. Group 3b [NiFe]-hydrogenases are tetrameric enzymes acting like bifunctional sulfhydrogenases (20). They were initially found in hyperthermophilic archaea yet have also been detected in bacterial phyla more recently (10). They reduce S^0 to H_2S or oxidize NAD(P)H and produce H_2 to disperse reducing equivalents generated through fermentation, or, alternatively, to produce NADPH by using H_2 (54). Group 3c [NiFe]-hydrogenases are heterodisulfide reductase-associated hydrogenases that catalyze the reduction of compounds such as methyl viologen and coenzyme M-disulfide bond-coenzyme B (CoM-S-S-CoB) (55) and are mostly found in methanogens and sulfate reducers. Group 3d [NiFe]-hydrogenases consist of a heteromeric hydrogenase and a NADH-dehydrogenase module that binds to NAD(P)H (20). The amount of hydrogenase subunits varies greatly, consisting of, for example, a heterodimeric enzyme in *C. necator* (56) or a heteropentameric enzyme in *Cyanobacteria* (57). These hydrogenases are used to balance the NAD^+/NADH pool by reducing NAD^+ using H_2 (20).

(d) *Group 4 [NiFe]-hydrogenases*. Group 4 [NiFe]-hydrogenases or membrane-associated energy-converting hydrogenase are oxygen-sensitive (58) multimeric enzymes comprising two proteins embedded within the cytoplasmic membrane as well as hydrophilic subunits (20). Group 4 [NiFe]-hydrogenases are mostly found in methanogenic archaea, chemolithoautotrophic bacteria, and hyperthermophilic archaea (Fig. 3 and S3). In facultative chemolithoautotrophs, group 4 hydrogenases disperse reducing equivalents generated through fermentation by coupling the reduction of protons from water with the anaerobic oxidation of C_1 compounds like formate or carbon monoxide (59). In acetoclastic methanogens, energy-converting hydrogenases (Ech) perform the H_2 -dependent reduction of ferredoxin, leading to the generation of a proton gradient through the cytoplasmic membrane and the generation of ATP (60). In

methanogens without a cytochrome, group 4 [NiFe]-hydrogenases (Eha and Ehb) reduce ferredoxin and catalyze H₂ oxidation, which is then coupled with the generation of a sodium ion motive force (20). Group 4 [NiFe]-hydrogenases also include energy-converting Mbh-type hyperthermophilic enzymes producing H₂ in order to reestablish oxidized ferredoxin levels (61).

(e) *Group 5 [NiFe]-hydrogenases.* Group 5 [NiFe]-hydrogenases are O₂-tolerant (62) uptake hydrogenases harboring a high affinity toward H₂ enabling them to oxidize atmospheric H₂ (63). Their activity was first discovered in spores of *Streptomyces* spp. (phylum *Actinobacteria*) (64). These enzymes are likely associated with the cytosolic side of the membrane through an electron acceptor, but conflicting results have been reported (62, 65). Genomic database mining demonstrated that group 5 [NiFe]-hydrogenases are mostly found in *Actinobacteria* (84.0%, Fig. 3 and S3) (10, 66). The proposed role of these hydrogenases is to provide a growth advantage (67) or a supplementary energy source within a survival-mixotrophic lifestyle (68) to sustain microbial seedbanks in oligotrophic environments (66). However, *C. necator* has a weakly expressed group 5 [NiFe]-hydrogenase that cannot oxidize atmospheric H₂ (62). Indeed, biochemical properties conferring high affinity for H₂ are unknown. While there is a high sequence similarity between the active site of group 5 [NiFe]-hydrogenases, such hydrogenases display a wide spectrum of affinities, e.g., *K_m* over 1,000 ppmv in *C. necator* in contrast to 11 ppmv in *Streptomyces* sp. strain PCB7 (64). This suggests that either a lateral gene transfer in *C. necator* (69) or the evolution of unknown biochemical attributes in hydrogenases were found in high-affinity HOM. Alternatively, other factors, such as the currently unknown physiological electron acceptor of group 5 [NiFe]-hydrogenases (65, 70), could be responsible for their affinity toward H₂. Their affinity has not been tested extensively *in vitro*; thus, we cannot assume that *C. necator* is an exception to the rule. Also, diverging phylogenetic analyses spawned confusion in hydrogenase terminology, yet group 5 (62, 66, 71) and group 1h (10) refer to the same enzymes.

OVERVIEW OF THE H₂ BIOGEOCHEMICAL CYCLE

Biological sources and sinks of H₂ are common in oxic and anoxic ecosystems. In anoxic ecosystems, such as wetlands, freshwaters under the chemocline, marine sediments, and animal gastrointestinal tracts, H₂ is mainly produced as a reaction intermediate of organic matter degradation by organisms from the three domains of life. The main biological sinks of H₂ in anoxic ecosystems are acetogens, methanogens, sulfate-reducing microbes, iron oxide-reducing microbes, and nitrate-reducing microbes (35). In oxic environments, such as upland soils, freshwaters above the chemocline, and open oceans, H₂ is mainly generated by N₂ fixation and is consumed by Knallgas bacteria and high-affinity H₂-oxidizing bacteria (35). Due to its energetic potential, H₂ is quickly consumed by microbes within the same microenvironment, indicating that H₂ production is likely the limiting step of the H₂ biogeochemical cycle (33). Microbial functional groups responsible for H₂ production and utilization in anoxic and oxic ecosystems will be presented in the following subsections to showcase their vast diversity and their involvement in the C biogeochemical cycle.

(i) H₂ production in anoxic ecosystems. In anoxic ecosystems, photoproduction (or biophotolysis), dark fermentation, and photofermentation are responsible for most of the H₂ production (35).

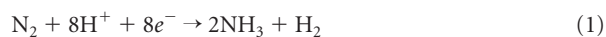
(a) *Photoproduction.* Photoproduction of H₂ in aquatic sediments is performed by unicellular algae such as *Chlorella fusca* (phylum *Chlorophyta*), *Tetradismus obliquus* (formerly *Scenedesmus obliquus*; phylum *Chlorophyta*) (72), and *Chlamydomonas reinhardtii* (73), as well as *Cyanobacteria*. In soils, several purple nonsulfur bacteria perform photoproduction, including *Rhodobacter capsulatus* (phylum *Proteobacteria*) (74) and *Rhodospseudomonas rutila* (phylum *Proteobacteria*) (75). This process occurs under oxygen and CO₂ limitation (76, 77). Light-excited electrons are transferred across photosystems to reduce ferredoxin, and then ferredoxin is reoxidized in order to produce H₂ via an [FeFe]-hydrogenase (78). This process maintains the electron flow

within the electron transport chain to resume oxygenic photosynthesis as soon as better conditions arise (77), such as changing tides or the next photoperiod.

(b) *Photofermentation and dark fermentation.* Unicellular algae such as *Fragilariopsis* spp. (phylum *Bacillariophyta*) and *Pyramimonas* spp. (phylum *Chlorophyta*) (78), strict anaerobes like *Clostridium acetobutylicum* (phylum *Firmicutes*) (79), and even facultative anaerobes like *Escherichia coli* (phylum *Proteobacteria*) (80) from both aquatic and terrestrial environments can produce H₂ through dark fermentation. Glycolysis, followed by the decarboxylation of pyruvate into acetyl-coenzyme A (acetyl-CoA), reduces ferredoxin/NAD⁺ into oxidized ferredoxin/NADH, supplying electrons to a hydrogenase and generating H₂ by their reoxidation (78, 81, 82). Alternatively, the conversion of CoA and pyruvate into formate and acetyl-CoA followed by the cleavage of formate also leads to the generation of H₂ and CO₂ (83). Oxidized ferredoxin and NADH are regenerated by discarding reducing equivalents, and glycolysis can generate ATP anew. Photofermentation is performed by purple nonsulfur bacteria (e.g., *Rhodospirillum rubrum* [phylum *Proteobacteria*] or *Rhodobacter sphaeroides*) that use light energy to convert organic substrates, like lactic acid, into H₂ and CO₂ (83). These processes can be performed sequentially, given that there are dark and light cycles. According to genomic surveys and culture-dependent measurements, in aquatic environments, H₂-evolving fermenters include unicellular algae, *Firmicutes*, *Bacteroidetes*, *Spirochaetes*, and *Proteobacteria* (78, 84–86). In anoxic soils, such as paddy fields, molecular surveys targeting [FeFe]-hydrogenases have shown that *Firmicutes* (mostly *Clostridia*), *Proteobacteria* (mostly *Deltaproteobacteria*), and *Chloroflexi* are the most abundant H₂ producers (87, 88). Similarly, in a moderately acidic fen slurry enrichment, fermenters were identified as *Firmicutes* (*Clostridia* and *Negativicutes*) and *Deltaproteobacteria* (89).

(c) *Nitrogen fixation.* Various microbes, such as *Clostridium* spp. and *Bacillus* spp. (phylum *Firmicutes*) as well as methanogenic archaea and *Cyanobacteria* can fix N₂ and consequently produce H₂ in anoxic or microoxic environments (90, 91). Since current estimates suggest that most of the biological nitrogen fixation is performed in oxic ecosystems (92), especially in surface waters (93, 94) and in the rhizosphere of leguminous and actinorhizal plants (95), it will be discussed in the next section.

(ii) **H₂ production in oxic ecosystems.** *Nitrogen fixation.* The main biological process responsible for H₂ production in oxic ecosystems is biological N₂ fixation (BNF). BNF is performed by the nitrogenase complex according to the following reaction (96–98):



BNF requires a tremendous amount of energy, i.e., a minimum of 16 ATP per fixed N₂ molecule (2 ATP per electron transferred [98]); thus, it is strictly regulated and used only in the absence of bioavailable N (99). Since nitrogenases are irreversibly inactivated by O₂ (98, 100), microbes can avoid O₂ via temporal or spatial separation strategies, as follows: separation of N₂ fixation and oxygenic photosynthesis (100), avoidance of O₂, high respiration rates, and the use of O₂ transporters to buffer local O₂ concentrations (98, 101–103). H₂ is an obligate by-product of the BNF reaction, requiring approximately 35% of the electron flux used toward BNF (104) and leading to local accumulations in environments such as the legume rhizosphere (105, 106). BNF and hydrogen oxidation are often linked due to their complementary outcomes, since H₂ oxidation coupled to O₂ reduction recovers part of the ATP used toward N₂ fixation and leads to a reduction in O₂ pressure and H₂ buildups, both of which would prevent nitrogen fixation (46, 107). Furthermore, three BNF strategies are used by microbes, including symbiotic N₂ fixation (99, 108), plant-associated N₂ fixation (109), and free-living N₂ fixation (97, 110). Unlike other nitrogen fixers, most leguminous plants symbionts do not possess the genetic capability to recycle H₂ produced through nitrogen fixation (i.e., absence of Hup activity) (111). Studies have hypothesized that the additional energy input provided by H₂ exerts a fertilization effect promoting growth of the host plant (112, 113).

(iii) H₂ uptake in anoxic ecosystems. In anoxic ecosystems, numerous microbial functional groups use H₂ as an electron donor to reduce terminal electron acceptors according to their availability and underlying thermodynamic constraints (33). This section discusses some of them in increasing redox potential order, from acetogenesis to denitrification, although other electron acceptors can be coupled to H₂ oxidation, such as MnO₂ and CrO₄. Many of those microbes are facultative anaerobes, yet these processes are only performed in anoxic ecosystems since the reduction of O₂ would be favored thermodynamically over alternative electron acceptors. These functional groups are not mutually exclusive, i.e., numerous bacteria and archaea are able to perform H₂ oxidation using more than one electron acceptor.

(a) *Acetogens.* Acetogenesis is performed by several strict anaerobes from the bacterial and archaeal domains of life (114). Acetogens are found in a wide variety of ecosystems: wetlands (115), the gastrointestinal tract of animals (116, 117), oceans, subsurface sediments (118), hypersaline waters (119), and oxic soils (120). While the latter sounds counterintuitive, several acetogens and methanogens can survive in the presence of O₂ due to different mechanisms (121, 122). Most acetogens, namely, *Clostridium* spp., are comprised within the *Firmicutes* (123), yet some are also found among other phyla, including the archaeal phylum *Bathyarchaeota* (114). The acetogenesis reaction (equation 2) follows the Wood-Ljungdahl pathway to convert C₁ inorganic compounds (CO₂) into C₂ organic compounds (acetate) (124):



Acetogens scavenge H₂ generated through the degradation of organic matter. Acetogens face fierce competition from methanogens, methanogens having a lower threshold for H₂ utilization and a higher energy yield (125, 126). Acetogens can overcome methanogens in some ecosystems, such as in the termite gut (127), since the production of acetate consists of an ecological advantage in its animal host. Acetogens are inherently involved in H₂ and C-cycling processes by coupling the utilization of CO₂ and H₂. In turn, this leads to the generation of acetate, a bioavailable C source for the surrounding microbial community. As strict or facultative anaerobes, acetogens can also use dissolved organic matter (DOM, also known as labile) through fermentation and produce their own H₂ and CO₂. Additionally, some of them, e.g., *Clostridium carboxidivorans* and *Clostridium drakei* (128, 129), are also involved in CO oxidation to CO₂, crystalline cellulose and hemicellulose degradation, polymeric organic matter (POM, also known as recalcitrant) turnover (130), and humic acid decomposition (131).

(b) *Methanogens.* Methanogenesis is only performed by methanogenic archaea, which are common in wetlands, in the intestinal tract of animals, in extreme environments, such as hot springs and hydrothermal vents (132), and even in oxic soils (120). Methanogens consist of the only functional group harboring [Fe]-hydrogenases, which enables them to use H₂ as a reducing agent for the ferredoxin-dependent reduction of CO₂ to CH₄, according to the following simplified reaction (133, 134):



Methanogens thrive in ecosystems or layers within those ecosystems where electron acceptors with higher redox potential, such as sulfate and nitrate, are depleted due to the low redox potential of CO₂ (33). They scavenge CO₂ and H₂ derived from the degradation of organic matter, which they can ferment on their own. Hydrogenotrophic methanogens using electron bifurcation are considered obligate autotrophs (135). Methylotrophic methanogens using oxidative phosphorylation consume a wider variety of substrates (136). Recent studies, however, have shown that hydrogenotrophic methanogens such as the marine methanogen *Methanococcus maripaludis* (phylum *Euryarchaeota*) (137) might in fact have a greater flexibility than initially thought by using a combination of CO and formate rather than only CO₂ and H₂. The assumption that methanogens are strict anaerobes has also been challenged with the discovery of "*Candidatus* Methanotherix paradoxum" (phylum *Euryarchaeota*) (nonhydrogenotrophic) (138). The involvement of

methanogens in the C cycle also includes their ability to reduce humic acids, e.g., in *Methanosarcina barkeri* (phylum *Euryarchaeota*) (139), which temporarily decreases their methanogenesis potential. Studies even suggest that anaerobic methanotrophic archaea (ANME) could be methanogens as well (140, 141).

(c) *Sulfate-reducing bacteria and archaea.* Sulfate-reducing bacteria (SRB), such as *Desulfovibrio* spp. and *Desulfotomaculum* spp. (phylum *Firmicutes*), as well as sulfate-reducing archaea (SRA), such as *Archaeoglobus* spp. (phylum *Euryarchaeota*), are anaerobic microbes coupling the oxidation of H₂ (or organic compounds) to the reduction of sulfate (SO₄²⁻) into sulfide compounds (142, 143).

While they are found in oxic environments, such as coastal sediments (144), sulfate-reducing microbes are more common in marine and freshwater sediments, deep subsurface environments, and the gastrointestinal tract of animals (143). They are also found in acid mine drainages and alkaline environments, such as soda lakes (143). H₂-utilizing sulfate reducers can outcompete acetogens and methanogens within the same ecosystem due to their higher growth kinetics (143). Species of *Desulfitobacterium* (phylum *Firmicutes*) are also able to perform the reduction of humic acids and polyaromatic compounds (145, 146). Studies have also shown their potential ability to fix CO₂ (147, 148) and oxidize CO (149). Sulfate-reducing microbes are thought to be the main drivers of carbon cycling in marine sediments (143). H₂-oxidizing SRB also act as syntrophs in consortia performing the anaerobic oxidation of CH₄ (150).

(d) *Iron oxide reducers.* Iron oxide-reducing bacteria and archaea [Fe(III) reducers] are found in soils, acid mine effluents (151), aquatic sediments (152), hot springs (153), and subsurface ecosystems. Since Fe(III) is insoluble in water, microbes need direct contact with Fe(III) to reduce it (154). Microbes such as *Shewanella* spp. (phylum *Proteobacteria*), *Geobacter* spp. (phylum *Proteobacteria*), and *Geothrix* spp. (phylum *Acidobacteria*) can produce chelators to solubilize Fe(III) in water or even produce electrically conductive bacterial nanowires to reach it (154). They use Fe(III) as an electron acceptor for the oxidation of organic matter or H₂ (155–157). Many Fe(III) reducers use other electron acceptors, such as O₂, SO₄²⁻, NO₃⁻, or even CO and CO₂ (139, 155). Those Fe(III) reducers include bacteria, such as *Geobacter* spp. and *Shewanella* spp. (152, 155), but also methanogens (139) and other archaea, like *Pyrobaculum islandicum* (phylum *Crenarchaeota*) (158). Fe(III)-reducing processes are more efficient at scavenging H₂ than are sulfate-reducing processes (159). Several Fe(III) reducers can use humic substances as electron acceptors to anaerobically oxidize organic compounds (e.g., acetate produced by acetogens) as well as H₂ to support their growth (160). Such electron transfers also chelate Fe(III) oxides and make them bioavailable to Fe(III) reducers such as *Geobacter metallireducens*, which also contribute to pollutant degradation (e.g., benzene) (160).

(e) *Denitrifying bacteria and archaea.* Denitrifying bacteria and archaea couple the reduction of nitrate (NO₃⁻) and subsequently reduced nitrogen compounds to the oxidation of H₂ (or organic compounds) (161). Denitrification is performed in aquatic and terrestrial ecosystems mostly by heterotrophic microbes, such as *Paracoccus denitrificans* (phylum *Proteobacteria*), but is also quite common in autotrophic microbes, such as *Thiobacillus denitrificans* (phylum *Proteobacteria*), or within the *Hydrogenophiales* order (phylum *Proteobacteria*) (162–164). It is the most energetically favored anaerobic respiration process and is used by a wide variety of microbes (10, 33). Since it is performed in numerous sequential reduction reactions, typically NO₃⁻ → NO₂⁻ → NO → N₂O → N₂, many denitrifying microbes do not possess the whole metabolic machinery to perform complete denitrification. Within a single ecosystem, different microbial strains often complement their denitrifying capabilities for more efficient energy retrieval and avoidance of toxic N intermediates (165). *Pseudomonas* spp. (phylum *Proteobacteria*) can perform denitrification as well as POM degradation, including degradation of lignin (166) and aromatic compounds (167). Like SRB, some denitrifying bacteria are also syntrophs within AOM consortia (168).

(iv) H₂ uptake in oxic ecosystems. In aerated soils, the oxidation of elevated H₂ mixing ratios is performed by Knallgas bacteria, whereas high-affinity H₂-oxidizing bacteria (HA-HOB) are able to oxidize much lower H₂ mixing ratios, such as atmospheric H₂ (ca. 0.53 ppmv [42]).

(a) *Knallgas bacteria.* Knallgas bacteria are aquatic and terrestrial obligate or facultative chemolithoautotrophs that use O₂ as a final electron acceptor to oxidize H₂ according to the Knallgas reaction:



Model Knallgas microbes include *C. necator* (formerly *Ralstonia eutropha*), which possesses 4 [NiFe]-hydrogenases from groups 1, 2, 3, and 5 (28, 62, 169), yet not all Knallgas bacteria have several hydrogenases. While *C. necator* possesses a group 5 [NiFe]-hydrogenase, it cannot oxidize atmospheric H₂ mixing ratios (62) and is thus not a HA-HOB, as opposed to *Mycobacterium smegmatis* (65). When H₂ concentrations are sufficiently high, *C. necator* fixes CO₂ and oxidizes H₂ as its sole energy source using its group 1 [NiFe]-hydrogenase (51). The group 3 [NiFe]-hydrogenase is rather used to maintain redox balance within the cell by coupling H₂ oxidation to NAD⁺ reduction (28). To avoid wasting energy by using group 1 and 3 hydrogenases constitutively when H₂ mixing ratios are low, *C. necator* also produces a group 2 hydrogenase acting as a sensor regulating the transcription of structural and auxiliary genes of the two other hydrogenases (28). Finally, the group 5 hydrogenase generates ATP at a lower rate than the MBH (group 1) but is entirely insensitive to O₂ (62). Other Knallgas bacteria have similar H₂ uptake mechanisms and encompass various phylogenetic backgrounds (e.g., *Proteobacteria*, *Firmicutes*, *Cyanobacteria*, *Chloroflexi*, and *Acidobacteria*). Furthermore, many N₂-fixing microbes, namely, the actinorhizal plant symbionts *Frankia* spp. (phylum *Actinobacteria*) and *Cyanobacteria*, can oxidize H₂ produced through N₂ fixation (170). Various Knallgas bacteria are also known plant growth-promoting rhizobacteria (PGPR) (112, 171), while many more perform crucial C-cycling steps. Several Knallgas bacteria can break down polymeric compounds. For instance, *Bacillus pseudofirmus* and *Bacillus thermotolerans* can reduce humic acids when oxidizing Fe(III) oxides (172, 173), while *Burkholderia* spp. (phylum *Proteobacteria*) can break down lignin (166). Furthermore, many methanotrophs, such as *Methylosinus trichosporium* (phylum *Proteobacteria*) and *Methylacidiphilum fumariolicum* (phylum *Verrucomicrobia*), can oxidize H₂ and CH₄ under oxic conditions (174, 175) and are thus also Knallgas bacteria. Various Knallgas bacteria, such as *Hydrogenomonas facilis* (phylum *Proteobacteria*) (176) and *C. necator* (177), can also fix CO₂ or oxidize CO (178).

(b) *Atmospheric H₂ oxidation.* The oxidation of atmospheric H₂ has long been considered to originate from abiotic enzymes due to the failure to isolate microbes responsible for this process at low H₂ partial pressure (179, 180). Bacteria responsible for the oxidation of H₂ at low mixing ratios have only been recently isolated (64), hence their small representation in the scientific literature. Recent genomic database surveys (10) have shown that most microbes possessing group 5 [NiFe]-hydrogenases are *Actinomycetes*, such as *Streptomyces* spp. and *Mycobacterium* spp. Only a few high-affinity H₂-oxidizing microbes have been isolated and characterized so far, including *Rhodococcus equi* (phylum *Actinobacteria*) (181), various *Streptomyces* spp. (64, 68, 181), and *Mycobacterium smegmatis* (65). In this regard, the role of group 5 [NiFe]-hydrogenases within the cell is not understood. HA-HOB are mostly found in soils, and they represent a major (70%) sink of atmospheric H₂ (182). While atmospheric H₂ provides substantial amounts of energy, it is insufficient for bacterial growth (63). So far, HA-HOB have been shown to use H₂ as an additional energy source for enhanced growth and survival (67, 181) or within a mixotrophic lifestyle called survival mixotrophy (68). HA-HOB are also involved in C cycling. For instance, *Rhodococcus jostii* and *Rhodococcus equi* can degrade lignin and several other POM compounds (166, 183). *Streptomyces* species are also known to perform the breakdown of humic acids and even coal, thus contributing to POM cycling (184–186). Other *Actinobacteria*, such as *Mycobacterium smegmatis*, can also oxidize atmospheric CO and H₂ (187). Candidate

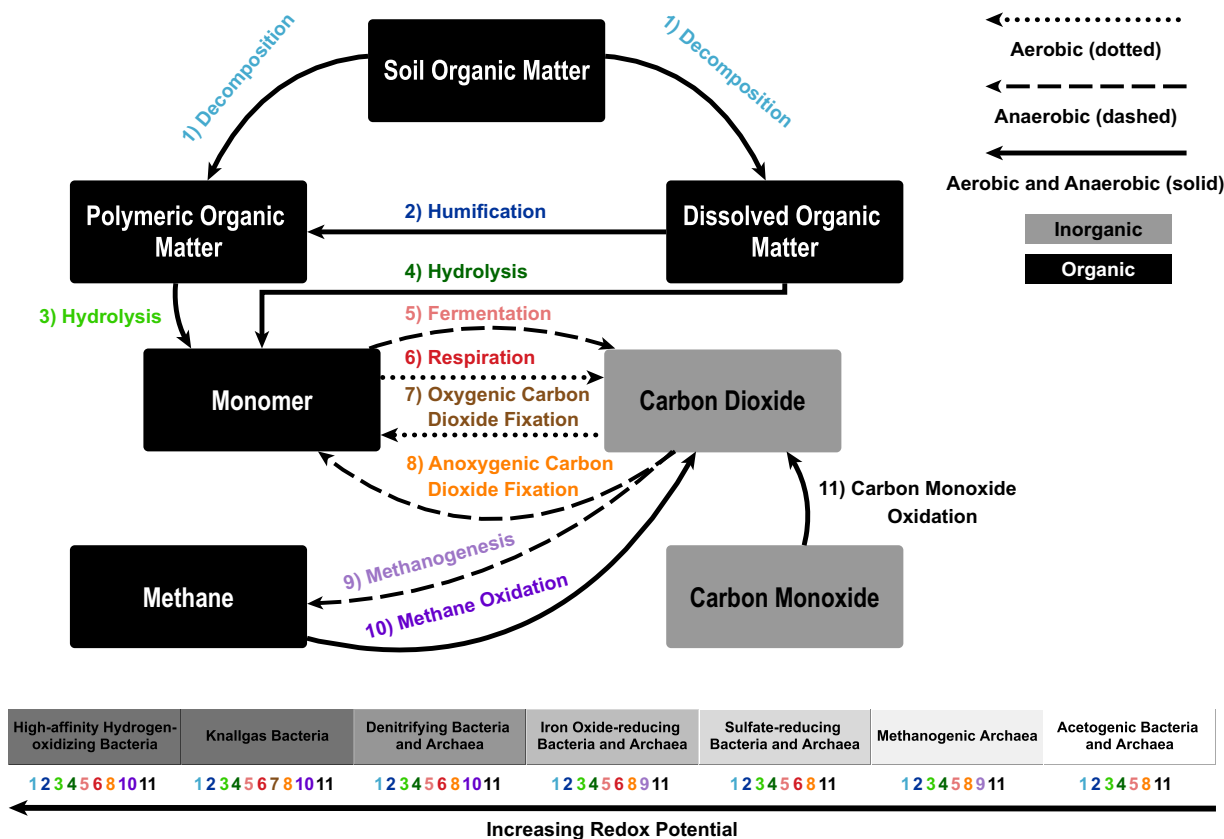


FIG 4 Juxtaposition of the carbon cycle and main H₂-oxidizing functional groups. HOM contribute to all key steps of the C cycle. All numbers below microbial functional groups consist of reactions performed by these groups. While the oxidation of CH₄ can be performed in anoxic ecosystems, the anaerobic oxidation of CH₄ (AOM) is not performed by HOM, only by their syntrophic bacterial partners (i.e., sulfate- or nitrate-reducing bacteria). H₂-utilizing processes occur in a thermodynamically favored fashion according to available substrates, where O₂ is used first, followed by nitrate, iron oxides, sulfate, and carbon dioxide, as shown in the gradient at the bottom of the figure. The only key C-cycling process missing in oxic ecosystems is methanogenesis, yet residual CH₄ diffuses from anoxic to oxic layers in various ecosystems. An exception to this is that nonhydrogenotrophic methanogens are also active in oxic layers (138), thus providing CH₄ directly to oxic ecosystems.

taxa might also have the genetic potential to oxidize atmospheric H₂ and perform CO₂ fixation (188).

JUXTAPOSITION OF THE H₂ AND C BIOGEOCHEMICAL CYCLES IN SOILS ALONG THEORETICAL O₂ GRADIENTS

A wide variety of HOM can hydrolyze DOM but also take part in the breakdown of POM, including cellulose, hemicellulose, lignin, humic substances, and polycyclic aromatic compounds (189), and also catalyze the oxidoreduction of CO, CO₂, and CH₄ (Fig. 4). HOM are able to switch between different modes of nutrition, both in terms of carbon and energy sources (6), which confers them great metabolic versatility when subject to various C inputs. Many of these C-cycling processes also lead to the generation of C sources usable by the broader microbial community, such as acetate. Moreover, in microenvironments rich in H₂, e.g., the legume rhizosphere, termite gut, and serpentinizing systems, metabolites released by HOM might also prime the decomposition and turnover of organic matter, namely, in soils. Recent studies have shown that the exposure of soil to elevated H₂ concentrations representative of those found in nature (i.e., 10,000 ppmv) increased the amount of C sources used, as well as the intensity of their consumption within a defined time period (190, 191). However, it is not known if HOM involved in H₂ oxidation are responsible for this priming-effect-like response due to their mixotrophic growth strategy or if positive interactions between HOM and heterotrophic non-HOM led to it. With this in mind, it could be expected that

other C-cycling processes performed by HOM, such as decomposition, humification, and hydrolysis, could also increase in H₂-rich ecosystems (Fig. 4).

As for trace gases, a study has shown that atmospheric CO and H₂ deposition velocities are positively correlated in both arable and forest soils (192, 193). While the initial hypothesis was that diffusion was ultimately responsible for this response, a more recent study has shown that H₂, CO, and CH₄ oxidation potential by microbes covaries as well. Indeed, along an increasing gradient of H₂ exposure from 0.5 to 10,000 ppmv H₂, low-affinity H₂ oxidation increased, net CO₂ production decreased, and high-affinity H₂, CO, and CH₄ oxidation decreased (194). CO₂ production results were not published but were measured at the same time and with the same instrument as the other trace gases (194). Taken together, those studies imply that ecosystems rich in H₂ could be hubs of DOM and POM cycling; HOM oxidize H₂ preferentially if available at elevated mixing ratios instead of using other trace gases.

While potential activity does not guarantee that *in situ* measurements will show the same behavior, examples of H₂-driven C cycling exist. Two studies have shown that CO₂ dynamics in soil systems continuously fed with air or air supplemented with H₂ change from net CO₂ production to net CO₂ fixation during H₂ exposure (195, 196). A recent metagenomic survey has hypothesized similar outcomes in Antarctic desert soils (188). Fixed CO₂ would increase the input of organic carbon in the system, which can then be distributed to the rest of the microbial community after cell death or production of secondary metabolites. Responsible strains in these studies have not been identified, yet several Knallgas bacteria are known to oxidize H₂ and fix CO₂. While these hypotheses are applied to H₂-oxidizing CO₂-fixing microbes, it is expected that H₂-oxidizing methanotrophs could also favor certain metabolic pathways in the presence of H₂ by decreasing CH₄ oxidation and thus increasing net C input by methanogens (Fig. 4) (194).

While HOM are involved in various C cycling processes, their relative contribution to each of them is unknown. Two different types of approaches could be used to increase our understanding of H₂-C dynamics, community-level and individual-level approaches. As for community-level approaches, a good starting point would be to expose soil (i.e., microcosms, mesocosms, or even fields) to a baseline mixing ratio of H₂ (e.g., 0.53 as control [42]), as well as to lower and higher mixing ratios representative of natural conditions. Soil H₂ exposure could be provided by synthetic gas mixtures or through the manipulation of N₂-fixing H₂-producing rhizobia (with or without Hup activity as isogenic control and treatment strains, respectively) recruited by legume plants. This would enable a direct comparison between H₂ availability and C turnover. As for individual-level approaches, three experiments could be performed, as follows: culturing novel HOM, single-cell sequencing of novel uncultured HOM, or analyzing metagenome-assembled genomes (MAGs) of uncultured HOM. Traditional cultivation would allow for a more direct testing of HOM C-cycling capabilities, while culture-independent methods would give us more insight into their overall versatility. Furthermore, several H₂ and C-cycling processes seem to covary in soil, such as atmospheric CH₄ and elevated H₂ oxidation, yet it is not known if these processes are performed by the same microbes or simply within the same ecological niches. The three proposed individual-level methods could help solve this conundrum, all the while reducing current biases in genomic databases. For example, some taxa have more sequenced representatives and are consequently more represented in databases regardless of their involvement in the cycle (e.g., *Actinomycetales* and high-affinity H₂ oxidation). Further work in these directions would shed light on the importance of the overlap between the C and H₂ cycle at the species and ecosystem level as well as provide further insights into the overall biogeochemical potential of HOM. In turn, this knowledge could be used to develop techniques that take advantage of H₂ hot spots in soils, e.g., for potential increases in C turnover or for agricultural benefits, namely, in legume rhizosphere rich in H₂ (197). Last, this review exemplifies the idea that HOM, as a functional group, is a reductionist concept. That is, HOM should not be reduced to their H₂-oxidizing function; they are highly diverse, and many of them perform crucial ecosys-

tem functions that could be more important at the ecosystem level than their individual energy-retrieving potential.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.02418-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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