Anaerobic dihydrogen consumption of nutrient-limited aquifer sediment microbial communities examined by stable isotope analysis

Michaela Löffler[a], Laura Schwab[a], Frank Dethlefsen[b], Louisa Lagmöller[b], Carsten Vogt[a] and Hans-Hermann Richnow[a,c]

[a]*Department Isotope Biogeochemistry, Helmholtz Centre for Environmental Research – UFZ, Leipzig, Germany;* [b]*Aquatic Geochemistry & Hydrogeology, Applied Geosciences, University of Kiel, Kiel, Germany;* [c]*Isodetect GmbH, Leipzig, Germany*

Contact Carsten Vogt Email carsten.vogt@ufz.de
Helmholtz Centre for Environmental Research - UFZ
Department of Isotope Biogeochemistry
Permoserstrasse 15
04318 Leipzig, Germany

Supplementary material for this article can be accessed at DOI…

Abstract

The biogeochemical consequences of H_2 underground storage in porous aquifers are poorly understood. Here, the effects of nutrient limitations on anaerobic H_2 oxidation of an aquifer microbial community in sediment microcosms were determined in order to evaluate possible responses to high H_2 partial pressures. Dihydrogen isotope analyses of H_2 yielded isotope depletion in all biotic setups indicating microbial H_2 consumption. Carbon isotope analyses of carbon dioxide (CO_2) showed isotope enrichment in all H_2-supplemented biotic setups indicating H_2-dependent consumption of CO_2 by methanogens or homoacetogens. Homoacetogenesis was indicated by detection of acetate and formate. Consumption of CO_2 and H_2 varied along the differently nutrient-amended setups, as did the onset of methane production. Plotting carbon against dihydrogen isotope signatures of CH_4 indicated that CH_4 was produced hydrogenotrophically and fermentatively. The putative hydrogenotrophic *Methanobacterium* sp. was the dominant methanogen. Most abundant phylotypes belonged to typical ferric iron reducers, indicating that besides CO_2, Fe(III) was an important electron acceptor. In summary, our study provides evidence for the

adaptability of subsurface microbial communities under different nutrient-deficient conditions to elevated H_2 partial pressures.

Keywords: Anaerobic H_2 consumption; aquifer microbial communities; carbon-13; carbon dioxide; hydrogen-2; isotope ecology; methane; methanogenesis; microcosms

1. Introduction

Green dihydrogen (H_2) is one of the key components of a green, sustainable energy system in Europe in the future [1,2]. However, the production level of renewable energy is dependent on the weather and varies with the seasons. Therefore, the availability of green H_2, generated with renewable energy, can fluctuate and infrastructure for transport and storage is needed. Similar to natural gas, H_2 might be stored underground in porous rock or salt caverns [3] to balance these fluctuations. In contrast to methane (CH_4) which is rather persistent when being stored at anoxic conditions in the subsurface [4,5], H_2 is an excellent electron donor for many aerobic and anaerobic microbes and is expected to be oxidized at various environmental conditions using a variety of different terminal electron acceptors such as Fe(III), sulphate, nitrate or carbonate [6,7]. An overview over common microbial H_2-oxidising processes is shown in Table 1. This widespread use of H_2 can be explained by its general availability in the subsurface due to its ubiquitous formation in the fermentation of organic compounds, e.g. ethanol or lactate [8,9], and through abiotic processes in the subsurface[10,11]. It is also visible in the genetic diversity of H_2-dependent enzymes, hydrogenases [12]. Thus, it is no surprise that H_2 was shown to be utilized by indigenous microbial communities during underground gas storage in porous rock reservoirs [13,14].

[Table 1 here with Citations [7,10,15–17].]

In natural sediments and aquifers the partial pressure of H_2 is very low due to competition for H_2 as an energy rich electron donor [7]. In contrast, underground storage is characterized by high H_2 concentrations, which are barely occurring at natural conditions [10,11]. This has consequences for the microbial metabolism and existing H_2-based syntrophic interactions; the latter do not operate above a certain, comparatively low H_2 threshold value [18]. For this reason, the sediment used in this study was used in previous experiments with higher partial pressures of H_2 to obtain a H_2-oxidizing community adapted to higher H_2 concentrations [19,20].

In this study, we tried to elucidate microbial processes coupled to the oxidation of H_2 at elevated H_2 pressures by an enriched microbial community from typical aquifer sediments under nutrient-deficient conditions. We assume H_2-oxidisers of natural subsurface microbial communities would consume H_2 if limitations of nutrients and trace elements would not hinder their metabolisms and growth. Considering the excess of H_2 within underground storages, such limitations and subsequent inhibition of microbiological consumption of H_2 in such systems may be crucial for medium- to long-term storage in porous sediments and wells as to predict the fate of H_2 in aquifers potentially used as groundwater resources. The sediment from a shallow North German aquifer was used in flow-through column experiments previously and the native microbial community was supplied with H_2 and carbon dioxide (CO_2) [20,21].

Limitations often lead to decreased growth rates [22–24], even though microorganisms can adapt in various ways: increasing scavenging and uptake efficiency, recycling of nutrients, switching substrates, or changes in protein expression with nutrient demand [23–25]. In our study, we tested groundwater cations (Na, K, Mg,

Ca), trace elements (Fe, Zn, Mn, B, Co, Cu, Ni, Mo) and macro nutrients (SO_4, NH_4, PO_4) to elucidate potential effects of nutrient deficiencies.

To monitor processes, we used compound-specific isotope analyses (CSIA) of hydrogen and carbon of H_2, CO_2 and CH_4. Isotopes are measured as ratio of the heavy to light element. There are two main types of isotope effects: kinetic and equilibrium isotope effects [26–28]. Equilibrium isotope effects aim to establish an isotope equilibrium between two substances, e.g. molecular H_2 and water [29–31]. Kinetic isotope fractionation is usually coupled to irreversible bond change reactions, for example consumption of substrate or formation of products. Since microorganisms need to invest less activation energy for breaking or formation of bonds of lighter isotopes, these are preferentially used. This leads to an isotopically heavier, also called enriched, remaining fraction and an isotopically lighter, or depleted, product.

Kinetic isotope effects can be used to distinguish between microbial metabolic pathways, as different processes are associated with specific isotope enrichment. With dihydrogen and carbon isotope analyses of methane for example, one can differentiate acetoclastic from hydrogenotrophic methanogenesis [32–36].

When all other electron acceptors are depleted, CO_2 can be considered as the relevant terminal electron acceptor. Therefore, we used H_2 and carbon isotope analyses to track the utilization of H_2 and CO_2 and the H_2-driven formation of CH_4 in nutrient-limited aquifer microbial communities, which was previously adapted to H_2 as electron donor and nutrient-deficient conditions. With this set of experiments, we aimed to see the influence of different nutrient limitations on the microbiome and its metabolism.

2. Materials and methods

All chemicals until otherwise stated have been bought from Merck, Germany, through

Th. Geyer GmbH, Germany. The sandy sediment we used stemmed from a pristine aquifer and has previously been used in flow-through column experiments [20,21], which solely relied on H_2 and CO_2 added to groundwater diluted with *Aqua dest*.. Before use in the column, the medium-grained sandy sediment entailed 161.6 mmol/kg Na, 226.2 mmol/kg K, 578.6 mmol/kg Ca, 1.8 mmol/kg Mn_{tot}, 69.8 mmol/kg Fe_{tot}, <0.4 mmol/kg Fe(II), 7.2 mmol/kg Fe(III), 15.6 mmol/kg S_{red}, 25.0 mmol/kg C_{org}, 408.3 mmol/kg C_{inorg} [21]. The sediment stemmed from a pristine, shallow aquifer in Northern Germany with a typical groundwater composition for 'neutral' shallow aquifers in the area [21,37] used in the microcosm experiment presented in this study was taken from the last quarter (25–40 cm) of the flow-through column [21] and was not homogenized before start of the experiment. It was stored with a water phase above the sediment in the dark at 8 °C until the start of the experiment. A day prior to the setup of the microcosms, the sediment was removed from the fridge, filled into a 250 ml autoclaved Schott bottle with 100 ml filter-sterilized tap water (Merck Millipore, Germany) that had been sparged with H_2/CO_2 (25 %/75 % (v/v)), and shaken overnight at 30 °C and 120 rpm.

2.1. Experimental setups – microcosms

Abiotic controls contained about 6 g of wet sediment, 5 ml of the sediment's water phase and 10 ml filter-sterilized tap water (Merck Millopore, Germany), sparged with 25 %/75 % (v/v) H_2/CO_2, and subsequently autoclaved before the start of the experiment. Then, part of the headspace was exchanged with 12 ml CO_2 and amended with 3 ml H_2, leading to about 6 mmol/l H_2 and 35 mmol/l CO_2 (at slight overpressure) in total. The pH was between 5.5 and 6.0.

Afterwards, four experimental setups with three replicates were prepared in a consecutive way: 1) biological control (SC), 2) amended with groundwater ions (GW), 3) amended with trace elements (TE) and 4) amended with macro nutrients (MN). First, roughly 6 g of wet sediment were added into 50 ml autoclaved serum bottles previously flushed with helium, as well as 5 ml of the water phase above the sediment and 10 ml filter-sterilized tap water (Merck Millipore, Germany), which had been sparged with 25 %/75 % (v/v) H_2/CO_2. To minimize interaction with air further, the headspace was sparged with a short burst of helium before the vials were stoppered. Subsequently, bottles were crimped close by gas-tight butyl rubber stopper and aluminium rings. Then 12 ml headspace were exchanged with CO_2 (20 mmol/l) via syringe and an empty hollow needle. Afterwards, 3 ml (5 mmol/l) H_2 were added, leading to slight overpressure. From time-to-time H_2 (2, 14, 29, 64, 71, 80, 92, 102, 105 d) and CO_2 (105 d) was re-supplied after sampling (see SI).

2.1.1. Biological control (SC)

For the sediment control experiment (SC), the microcosms were amended with H_2 but no additional nutrients were added to this setup.

2.1.2 Experiments amended with typical groundwater ions (GW)

1 ml each of a solution to approximate the ion content of typical groundwater in Northern Germany [37] made from filter-sterilised tap water (Merck Millipore, Germany) sparged with N_2 to remove O_2 was added to each microcosm. Each microcosm therefore contained additionally 0.556 mmol/l $NaHCO_3$, 0.4 mmol/l $KHCO_3$, 0.295 mmol/l $MgCl_2 * 6\ H_2O$ and 0.517 mmol/l $CaCl_2 * 6\ H_2O$.

2.1.3. Experiments amended with trace elements solutions (TE)

A batch experiment of GW setups (see above) was additionally supplemented with trace element solutions. Therefore 15 μl of trace element solution SL-10 (according to DSMZ 320 medium), which contained 10 ml 25% HCl, 7.545 mmol/l $FeCl_2 * 4\ H_2O$, 0.514 mmol/l $ZnCl_2$, 0.108 mmol/l $MnCl_2 * 4\ H_2O$, 0.097 mmol/l H_3BO_3, 0.799 mmol/l $CoCl_2 * 6\ H_2O$, 0.012 mmol/l $CuCl_2 * 2\ H_2O$, 0.1 mmol/l $NiCl_2 * 6\ H_2O$ and 0.138 mmol/l $Na_2MoO_4 * 2\ H_2O$ per litre. The solution was sparged with N_2 and added to each of the three biological replicates. Thus, each microcosm additionally contained 0.0071 mmol/l ferrous iron.

2.1.4. Experiments amended with macro nutrients (MN)

The last batch experiment amended with the trace element solution (see above) was additionally supplemented with nitrogen, sulphur and phosphorus. Therefore, a solution with filter-sterilised tap water (Merck Millipore, Germany) containing sulphate, phosphate, and ammonium to the TE setups. Each microcosm contained additionally: 0.16 mmol/l Na_2SO_4, 0.997 mmol/l NH_4Cl and 0.199 mmol/l K_2HPO_4, which were and aerobicized by sparging with N_2, respectively.

All microcosms were kept at room temperature (25 °C) and shaken at 120 rpm.

2.2. Analyses of stable isotopes and concentrations

In order to compare measurements worldwide, ratios of samples are standardized to a ratio of international standards [26,28,38]. The isotopic composition is expressed in the delta notation relative to the international standards. Differences between delta values, for example between time points, are expressed with a capital delta, shown here

exemplary for carbon:

$$\Delta^{13}C = \delta^{13}C_t - \delta^{13}C_{t=0}\ [‰].$$

2.2.1. Sampling

A H_2- and a CO_2-standard, respectively, (1 ml in 10 ml He each) were run first on all measurement days as external standard to check for stability of the instrument. In order to measure isotope ratios at distinct time points, 1 ml headspace each was transferred with a gastight syringe into a 10 ml vial that was purged with helium beforehand (see Figure S2). 0.25 ml sample volume was injected manually three times to ensure precision with standard deviations below 5 ‰ and 1 ‰ for H_2 and CO_2, respectively.

Samples for carbon isotope analyses were taken every 2 to 3 days and stored until measurement. Changes in hydrogen isotopes of H_2 and CH_4 were observed over the course of four to five days, as the instrument setup is less robust and the samples experience loss of H_2 over longer periods of storage. The first period of sampling started on t = 92 d, where 3 ml H_2 were re-supplied to the headspace. Then, samples were taken daily as described above. A second sampling period started at t = 105 d. Here, each sediment microcosm was re-supplied with 10 ml H_2 and 1 ml CO_2 and then sampled daily. A last sample was collected at the end of the experimental period, at day 121.

2.2.2. Carbon isotope measurements

Carbon isotope signatures of CO_2 and CH_4 were measured with a gas chromatograph (GC) isotope ratio mass spectrometer (IRMS) setup (see Figure S2). Each vial was measured in triplicate and 0.25 to 0.5 ml each were injected manually into the GC (split ratio 1:3; 6890A, Agilent Technologies, Germany) equipped with a PoraBond Q

(Agilent Technologies, Germany) coupled via Conflow II (Thermo Fisher, Germany) to an IRMS (MAT 253, Thermo Fisher, Germany). The combustion reactor in the Isolink system was kept at 1000 °C and the GC at 40 °C during the measurement.

2.2.3. Dihydrogen isotope measurements

Gas samples for H_2 and CH_4 were measured on a GC (split ratio 1:5; 7890A, Agilent Technologies, Germany) with a Carboxen-1010 PLOT Capillary GC Column (30 m, 320 μm, ~ 15 μm; Merck Supelco, Germany) coupled via Conflo IV (Thermo Fisher Scientific, Germany) to a MAT 253 IRMS (Thermo Fisher, Germany) or a MAT 253 Plus IRMS (Thermo Fisher, Germany), as available. The GC oven temperature program for one run was as follows: Hold 40 °C for 3 min, ramp with 20 °C/min, hold at 90 °C for 7 min, and then ramp with 20 °C/min to 220 °C to rid the column of residual CO_2. This allowed the measurement of δ^2H of both H_2 and CH_4 of the same sample within circa 20 min. An empty ceramic tube was placed into the pyrolysis unit and held at 1420 °C. The carrier gas was He with a flow of 1.2 ml/min.

2.2.4. Concentration analyses

During each isotope measurement run, a standard (1 ml H_2 in 10 ml He; 1 ml CO_2 in 10 ml He; 250 μl) was measured additionally in triplicate. This allowed the comparison of intensities at masses 2 and 45 with a known volume of H_2/CO_2 and thus, the calculation of H_2 and CO_2 concentrations in the headspace. Shown estimates of CH_4-concentration were based on the CO_2-standard.

2.3. Chemical analyses

Water phases of single microcosms were analysed for cations and organic acids at the

end of incubation. Cations and organic acids were analysed by ion chromatography (IC). Acetate, formate, chloride, nitrite, nitrate, phosphate and sulphate were determined on a Metrohm-IC 881 (Deutsche Metrohm GmbH & Co. KG, Germany), equipped with a Metrosep A Supp 5 - 150/4.0 column (Deutsche Metrohm GmbH & Co. KG, Germany). Some samples were unfortunately oxidised, and some were lost before analysis due to technical problems. The pH was measured with pH indicator strips, specific for range of 5 to 7 (chemsolute, Th. Geyer, Germany).

2.4. Analysis of microbial communities by 16S rRNA and mcrA gene amplicon sequencing

Sediment with water was collected from all sediment microcosms after the experimentation period and stored at –20 °C. DNA from all samples, as well as the initial sediment (stored frozen at –20 °C) was extracted with QIAGEN DNAeasy PowerSoil Kit (Qiagen, Germany) according to manufacturer's instructions and quantified using a Qubit Fluorometer 3.0 (Thermo Fisher, USA). The DNA-content was usually above 3 ng/μl for all replicates except for replicate 1 of the *SC* setup, which contained little DNA (see SI). A polymerase chain reaction (PCR) was conducted with primer pairs for bacterial 16S rRNA genes and methyl coenzyme M reductase (mcrA) genes of methanogenic archaea (Eurofins, Germany) with the PCR programs (shown in the supplement) on a S1000 thermocycler (Bio-Rad Laboratories GmbH, Germany). Bacterial community DNA was amplified using primers 341f (5' -CTACGGGNGGCWGCAG- 3') and 785r (5' -ACTACHVGGGTATCTAAKCC- 3') for the V3–V4 region of the 16S rRNA gene [39].

Additionally, the microbial methanogenic community was analysed using a primer set designed for mcrA genes [40], i.e. mcrA-fwd (5' -GGT GGT GTM GGD

TTC ACM CAR TA- 3') and mcrA-rev (5' -CGT TCA TBG CGT AGT TVG GRT AGT- 3'). Both amplification reactions consisted of the following reaction mix per sample: 12.5 μl Mytaq^{TM} HS Mix 2x (Bioline, UK), 1 μl of the respective primer extended with the Illumina sequencing adapter to a final concentration of 1 pM, 1 μl BSA (1:20), 15 ng DNA extract and PCR-grade water to a final volume of 25 μl.

Subsequent to amplification, sequencing libraries were prepared according to manufacturer's instructions (Illumina, 2013) using multiplexing barcodes Nextera XT (Illumina XT Kit; Illumina, USA) and AMPure XP beads (Beckman Coulter, USA) for DNA purification. Finally, amplicons (4 nM) were pooled, denatured and sequencing was performed on Illumina's MiSeq platform in paired-end mode (2 x 250 reads). Generated raw, de-multiplexed sequence data were processed with Qiime2 version 2019.10 [41]. Sequence primers were clipped using Cutadapt [42], the sequences were filtered using DADA2 [43] and, finally, taxonomic assignment was based on the SILVA 123 database [44,45].

3. Results

3.1. Concentrations and isotope signals of H_2 and CO_2 in the abiotic controls

The carbon isotope value of CO_2 (see Figure S-3) in both replicates of the abiotic control were –27.5 ± 1.2 ‰ and –28.1 ± 1.3 ‰, respectively. Less CO_2 was measured in the headspace compared to the injected value, probably due to equilibration of gaseous CO_2 with water. Concentrations of CO_2 decreased from 13.2 ± 0.6 % (5.8 mmol/l) in the headspace of the serum bottle to 6.0 ± 0.2 % (2.7 mmol/l) and from 14.5 ± 0.6 % (6.2 mmol/l) to 6.5 ± 0.5 % (2.9 mmol/l) with continuous sampling and with just one re-supplementation of 1 ml pure CO_2 (Figure S-3). The hydrogen isotope signature as

shown in Figure S-4B remained stable over 121 days with δ^2H = –148.4 ± 11.4 ‰ and δ^2H = –155.4 ± 5.0 ‰ for both replicates.

Continuous supplementation of H_2 during the experimental period resulted in high concentrations of H_2 in the headspace of abiotic controls (Figure S-3). Slight overpressure at t = 92 d yielded 80.3 ± 4.3 % (35.6 mmol/l) and 86.8 ± 2.7 % (38.3 mmol/l), then the concentrations declined sharply to 54.7 ± 2.7 % (24.3 mmol/l) and 50.3 ± 0.9 % (22.4 mmol/l). Measured concentrations were slightly higher than the added H_2 at this point (28.5 mmol/l), probably due to the overpressure. Afterwards, concentrations reduced with each sampling, leading to 25.5 ± 2.7 % (11.3 mmol/l) and 35.0 ± 2.8 % (15.6 mmol/l) at t = 121 d.

3.2. Concentrations and isotope signals of H_2, CO_2 and CH_4 in biotic microcosms

Concentrations of CO_2 in all four biological setups, as well as the abiotic control decreased over time due to continuous sampling (see Figure S-4). The remaining amounts of CO_2 at time of the last sacrifice sampling were distinctly different between setups indicating different biogeochemical processes and equilibria. Around 4 % CO_2 remained in the headspaces of TE (4.3 ± 0.8 %; 1.9 mmol/l) and MN (4.0 ± 0.6 %; 1.8 mmol/l), whereas the *SC* and GW differed strongly with concentrations of 2.2 ± 0.3 % CO_2 (0.9 mmol/l) and 2.1 ± 1.4 % (0.9 mmol/l), respectively. In all biological microcosms, carbon isotope signatures of CO_2 showed a general trend towards isotope enrichment over time (Figure 1), in contrast to the abiotic controls (Figure S-3). The strongest enrichment was observed in the SC setup and a lower enrichement in the TE setup. Onset of isotope enrichment was earlier in the SC and MN setups than in the other two. The difference between replicates was strongest in the GW

setup (Figure 1). This indicates that microbial processes consuming CO_2 are responsible for isotope fractionation. This may be either the assimilation of CO_2 for synthesis of biomass or the use of CO_2 as terminal electron acceptor.

[Figure 1 here]

Concentrations of H_2 in the two observation periods decreased considerably in the MN setup and slightly in all others (see Table S-5). Only the TE setup (all replicates) contained residual H_2 (day 92) before the re-spike on day 93. Highest H_2 concentrations were found in the GW setup and the least variance between biological replicates was observed in the MN setup. Loss of H_2, assumed to be due to sampling, could be seen in abiotic controls over time (Figure S-3D).

Isotope analyses of H_2 showed a tendency towards isotope depletion in the biotic microcosms (Figure 2), in contrast to the H_2 signatures of the abiotic controls which remained stable (δ^2H = –152 ‰ and δ^2H = –155 ‰ (Figure S-3, Figure 2). This indicates that autoclaving destroyed the catalytic properties of the microbial community completely.

[Figure 2 here]

In both observation periods, δ^2H-values became isotopically lighter and thus, more negative. Only the TE setup contained residual H_2 at day 92, which was already isotopically depleted. Therefore, re-spiking with isotopically heavier H_2 resulted in a starting value that lied in-between. Least variance between replicates was found in the MN and highest in the GW setup. This indicates that biological activity and its biocatalysts, such as hydrogenases, can alter the isotopic composition of H_2.

No methane could be detected in the abiotic controls, whereas methane was found repeatedly in all replicates of the MN and SC setup. The highest average (day 121: 3.3 ± 0.5 %; 1.5 mmol/l) and individual (replicate 3, day 121: 3.7 ± 0.1 %; 1.6 mmol/l) concentrations were found in the SC setups. On the last day of the incubation period, methane could be detected in all replicates of the TE setup, too (average 1.2 ± 0.8 %; 0.5 mmol/l). Only one replicate of the GW setup produced methane, yielding about 3.6 % CH_4 (1.6 mmol/l) in the headspace at day 121. Onset of methane production varied between setups with MN being the first to yield CH_4 in all three replicates at days 30 (replicate 1) and 45 (replicate 2) and 56 (replicate 3) (see Figure 1). Similar timeframes were observed for the SC setup with methane detected at days 45 (replicates 2 and 3) and 95 (replicate 1). This means that both the presence and absence of macro nutrients, trace elements and groundwater ions yielded an early onset of methanogenesis.

We observed two distinct isotopic signatures for carbon in CH_4 (Figure 1). In both the SC and the TE setup, $\delta^{13}C$-values were lower than those of CH_4 produced in the MN setup. There might be a correlation of the onset of methane production with isotopically heavier carbon isotope composition, but the evidence is not conclusive (cf. Table-2).

[Table 2 here]

This difference in carbon isotopes was not mirrored in $\delta^{2}H$-values of CH_4 (Figure 2). There is a distinction in time, as the CH_4 in the first observation period was heavier ($\delta^{2}H$ = –189 ‰ ± 74 ‰) than the CH_4 that was produced later ($\delta^{2}H$ = -404 ‰± 26 ‰; $\Delta^{2}H$ = 215 ‰).

3.3. Detection of metabolites and analysis of inorganic compounds

The pH of biological samples were around 6.5, abiotic controls showed a lower pH (5.5 to 6.0).

The results of the analyses of the water phases are shown in Table-3. Samples were taken after the end of the experiment.

[Table 3 here]

Acetate was detected in all biotic samples (ranging from 0.041 to 0.215 mmol/l), whereas formate was only found in replicate 2 of the GW setup (0.506 mmol/l) and within the abiotic control (0.036 mmol/l). Sulphate was present before the start of the experiment (see values of the abiotic control, Table S4) but could not be detected at the end of incubation. Nitrite or nitrate were below the detection limit. The phosphate content ranged from 0.024 to 0.131 mmol/l in biotic setups, which therefore was no limiting factor. As the abiotic control was autoclaved, it may have affected stability of some components.

3.4. Microbial community structure

All samples except GW1 showed a similar community structure at the end of the incubation, which was however different from the structure of the original community at the beginning of incubation, indicating a succession of the microbial community during incubation (Figure 3). The most abundantly found bacterial phylotypes in the inoculum and all setups and replicates were affiliated to the genus *Ferribacterium* (see Figure 3). Other abundant phylotypes belonged to the genera *Geothrix* and *Desulfovibrio* and to the family *Burkholderiaceae*. The results of the mcrA analyses showed that *Methanobacterium* sp. was the dominant methanogen in all samples (see

SI, Figure S-1), similar to the results obtained by 16S rRNA gene analysis (Figure 3).

[Figure 3 here]

These GW samples, which did not produce CH_4, differed considerably from the microbial community compositions of all other setups. Phlyotypes belonging to the *Obscuribacterales* and *Nocardioides* were present in higher abundance only in samples GW1, GW2 and in the sample used for inoculation.

4. Discussion

4.1. Isotope effects, putative microbial processes and microbial community composition

All biological setups were active and consumed H_2 despite different amendments. Dihydrogen isotope values of H_2 tended towards or approximated isotope equilibrium between H_2 and H_2O, which is about δ^2H = –744 ‰ for water in Leipzig (δ^2H–H_2O = -62 ‰) [30]. Establishing such an isotope equilibrium requires a catalyst. In microbial reactions, the enzyme hydrogenase facilitates the isotope equilibrium exchange between H_2 and water [29,30,46–48]. The isotopic signature of H_2 in abiotic, sterilized controls remained stable over the experimental timeframe, demonstrating that the isotope equilibrium exchange between H_2 and water was not catalysed chemically by components (e.g. minerals) of the sediment, and that no catalytic enzymes or H_2-using microbes survived the sterilization procedure. An isotope exchange reaction between water and gaseous H_2 was not expected [29]. Thus, isotope effects of H_2 were interconnected with microbial activity and potentially consumption of H_2, which was the main electron donor in the system. All biological setups produced acetate and most produced methane, indicating that oxidation of H_2 was coupled to reduction of CO_2 by

methanogenesis and homoacetogenesis and probably carbon fixation; reduction of CO_2 was also indicated by carbon isotope fractionation of CO_2, which was a common electron acceptor for H_2 oxidation across all replicates, and methanogenesis and homoacetogenesis are well known to show high carbon isotope fractionation [49–52]. Methane can stem from different sources, which can be differentiated by two-dimensional isotope ratio analysis [34,53,54]. We observed δ^2H-values for methane in two distinct sets over time. In the beginning, methane was generated with δ^2H-values ranging from –100 ‰ to about –250 ‰; those values are typical for being produced by hydrogenotrophic methanogenesis ([53]; Figure 4). Later on, the produced methane showed δ^2H-values ranging from –350 ‰ to about –470 ‰ (see Figure 4), values which are more typical for being generated by fermentation of organic compounds, indicating that organic acids, such as the detected acetate and formate, produced by the microbial community via homoacetogenesis, might have been used for methanogenesis.

The results of the community composition analysis indicate that, beside methanogenesis and homoacetogenesis, also reduction of ferric iron (Fe(III)) may played a considerable role for H_2 oxidation since phylotypes belonging to *Ferribacterium* sp. and *Geothrix* were dominant in both the origin and all biotic setups. Until now, only one species of the genus *Ferribacterium* has been described (*F. limneticum*), a motile, rod-shaped, gram-negative, obligatory anaerobic ferric iron reducer [55]. Growth was described on organic acids, but not on H_2 [55]. *Geothrix* gen. nov. has been described as strictly anaerobic acetate-oxidizing ferric iron reducer [56]. The dominance of *Ferribacterium* and *Geothrix* in all microcosms and in the origin sample, regardless of nutrient and iron limitations, imply that acetate (produced by homoacetogenesis) and maybe also H_2 were electron donors for iron reduction in the

aquifer sediment during flow-through column operation before setup of the microcosm experiment. Additionally, typical sulphate reducers (*Desulfovibrio* gen. and *Desulfosporosinus* gen.) were observed in higher abundances in all setups. These are usually able to utilize H_2 [57–63]. *Desulfovibrio* is a genus often described for H_2-consumption or -production [58–60,64–67]. *Desulfovibrio* species are also known for assimilation of acetate while using H_2 as electron donor, hence this process might have occurred in the biological samples in addition [68–71]. Correspondingly to the presence of sulphate reducers, sulphate was depleted in all biological samples and we observed the smell of dihydrogen sulfide during samplings. Thus, we assume that sulphate reduction took place in all biological setups. Well-known homoacetogenic organism were however not detected by community analysis, despite of the strong indications for homoacteogenesis as discussed above. Furthermore, the abundant methanogenic genus *Methanobacterium* is rather known for growing on H_2 and CO_2 [72,73] than acetoclastically; typical acetoclastic methanogens, e.g. belonging to the *Methanosaeta*, were not detected. We cannot explain this discrepancy yet.

The possible ecophysiological functions of a few other enriched taxa in the microcosms (up to 11 % in the community) – *Burkholderiaceae*, *Obscuribacterales*, *Bradyrhizobium*, *Xanthobacter* and *Sediminibacterium* – are difficult to derive by only 16S rRNA gene analysis. Most of these phylotypes were detected in two replicates of the GW setup which produced no methane.

4.2. Effect of nutrient limitations

The nutrient-deficient sediment and water was supplied with different nutrients in consecutive microcosm setups and the resulting changes to the metabolic processes were monitored as well as the community structure. For an overview about general

coping mechanisms, see supporting information. Generally, as discussed above, nutrient limitation did not result in inhibition of H_2 oxidation and associated anaerobic processes; the microbial community of the SC setup was adapted to these limiting conditions and produced methane from H_2 and potentially acetate early on without addition of any nutrients. Some effects were however observed and will be briefly discussed in the following. The addition of K, Ca, Na (GW setup) led to changes compared to the SC setup as methane was not produced at all in replicates 1 and 2, and the microbial community was slightly different compared to the other communities (see discussion above).

Trace elements added in the TE setups are essential for metabolism, as they are part of active centres of enzymes. For example, nickel (Ni) is a part of the methyl (alkyl)-coenzyme M reductase essential for methanogenesis [74], and of methanogen-typical hydrogenase [75]. In natural environments, trace element limitation is unlikely [76], but in systems with e.g. an excess of electron donors it can become relevant. In anaerobic digesters, addition of trace elements triggered a dominance of methanogens and deprivation of trace elements yielded elevated levels of sulphides and acetate [77]. In a recent study, deprivation of trace elements initially lead to acetoclastic methanogenesis, whereas supplementation yielded hydrogenotrophic methanogenesis, hypothesised due to Ni-limitation [77]. In our study, C and H isotope signatures of the generated methane indicated that methane was produced fermentatively in the TE setups (see Figure 4), although not supported by the data of the community analysis (see discussion above). Notably, the TE setups showed no initial methanogenesis; hence the addition of trace elements did not speed up methanogenic metabolic pathways due to its requirements for Ni and other metals, e.g. Fe, Co, Mo/W and Zn [78,79]. The fully

nutrient-amended MN setup showed sulphate reduction and methanogenesis early on compared to the groundwater ions and trace element setups (Figure 2), indicating a positive effect of this amendment on the mentioned processes.

5. Conclusions

In this study, we show the consumption of H_2 and CO_2, as well as the production of CH_4 by nutrient-deficient aquifer sediment microbial communities in a microcosm experiment with high partial pressures of H_2. Overall, monitoring of the isotopic signature of molecular H_2 is shown to be a sensitive indicator of activity for the H_2-cleaving enzyme hydrogenase. We could link biological activity to the isotopic exchange reaction of H_2 and H_2O, which was monitored by GC-IRMS. Potential applications of this approach include monitoring storage solutions of gaseous H_2, e.g. in the subsurface. Considering the ubiquity of hydrogenases in the genetic inventory of microorganisms [12], our results carry implications for H_2-driven biogeochemical processes likely occurring during underground H_2-storage and in areas of H_2-leakages: aquifer microbiomes consist of different types of H_2-consuming prokaryotes using different electron acceptors of H_2 oxidation, which will be active at conditions of constant H_2-supply, supporting results of other studies [13,80]. The isotopic signal of methane revealed two distinct sets indicating that hydrogenotrophic and acetoclastic methanogenesis proceeded, respectively, although typical acetoclastic methanogens were not found by community analysis. Even though these microcosms were significantly limited in nutrients and trace elements, they were able to consume H_2. It is therefore reasonable to assume that any gaseous H_2 stored underground can potentially experience loss of H_2 by microbial consumption. The question is whether subsurface conditions exist, which prevent H_2 oxidation and associated growth of indigenous

microbial communities.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

Bundesministerium für Bildung und Forschung
03G0875B
Bundesministerium für Wirtschaft und Klimaschutz
03ET6122B

References

[1] BMWi. Die Nationale Wasserstoffstrategie. Berlin: Bundesministerium für Wirtschaft und Energie; 2020. German. https://www.bundesregierung.de/breg-de/suche/die-nationale-wasserstoffstrategie-1759080

[2] European Commission. A hydrogen strategy for a climate-neutral Europe. Brussels: European Union; 2020.

[3] Tarkowski R. Underground hydrogen storage: Characteristics and prospects. Renew Sustain Energy Rev. 2019;105:86–94.

[4] Cahill AG, Steelman cm, Forde O, et al. Mobility and persistence of methane in groundwater in a controlled-release field experiment. Nat Geosci. 2017;289–294.

[5] Forde ON, Cahill AG, Mayer KU, et al. Hydro-biogeochemical impacts of fugitive methane on a shallow unconfined aquifer. Sci Total Environ. 2019;1342–1354.

[6] Griebler C, Lüders T. Microbial biodiversity in groundwater ecosystems. Freshw Biol. 2009;54:649–677.

[7] Lovley DR, Goodwin S. Hydrogen concentrations as an indicator of the predominant terminal electron-accepting reactions in aquatic sediments. Geochim Cosmochim Acta. 1988;52:2993–3003.

[8] Bryant MP, Campbell LL, Reddy CA, et al. Growth of desulfovibrio in lactate or ethanol media low in sulfate in association with H_2-utilizing methanogenic bacteria. Appl Env Microbiol. 1977;33:1162–1169.

[9] Seitz H-J, Schink B, Conrad R. Thermodynamics of hydrogen metabolism in methanogenic cocultures degrading ethanol or lactate. FEMS Microbiol Lett. 1988;55:119–124.

[10] Gregory SP, Barnett MJ, Field LP, et al. Subsurface microbial hydrogen cycling: Natural occurrence and implications for industry. Microorganisms. 2019;7:53.

[11] Nealson KH, Inagaki F, Takai K. Hydrogen-driven subsurface lithoautotrophic microbial ecosystems (SLiMEs): do they exist and why should we care? Trends Microbiol. 2005;13:405–410.

[12] Greening C, Biswas A, Carere CR, et al. Genomic and metagenomic surveys of hydrogenase distribution indicate H_2 is a widely utilised energy source for microbial growth and survival. ISME J. 2016;10:761.

[13] Dopffel N, Jansen S, Gerritse J. Microbial side effects of underground hydrogen storage – Knowledge gaps, risks and opportunities for successful implementation. Int J Hydrog Energy. 2021;46:8594–8606.

[14] Šmigáň P, Greksak M, Kozánková J, et al. Methanogenic bacteria as a key factor involved in changes of town gas stored in an underground reservoir. FEMS Microbiol Ecol. 1990;6:221–224.

[15] Belay N, Sparling R, Daniels L. Relationship of formate to growth and methanogenesis by *Methanococcus thermolithotrophicus*. Appl Environ Microbiol. 1986;52:1080.

[16] Boone DR, Johnson RL, Liu Y. Diffusion of the interspecies electron carriers H_2 and formate in methanogenic ecosystems and its implications in the measurement of K_m for H_2 or formate uptake. Appl Environ Microbiol. 1989;1735–1741.

[17] Fuchs G. CO_2 fixation in acetogenic bacteria: Variations on a theme. FEMS Microbiol Lett. 1986;181–213.

[18] Rakoczy J, Schleinitz KM, Müller N, et al. Effects of hydrogen and acetate on benzene mineralisation under sulphate-reducing conditions. FEMS Microbiol Ecol. 2011;77:238–247.

[19] Berta M. Experimental investigation of hydrogeochemical consequences of gas leakages into shallow aquifers [Dissertation]. University of Kiel; 2017. https://macau.uni-kiel.de/receive/diss_mods_00020740

[20] Lagmöller L, Dahmke A, Ebert M, et al. Geochemical effects of hydrogen intrusions into shallow groundwater – an incidence scenario from underground gas storage. Conference presentation, IAH Groundwater Quality, Liège, Belgium; 2019. https://www.uee.uliege.be/upload/docs/application/pdf/2019-10/90._vortrag_gq_2019_lagmoller_05.09.2019_fd5_ds_am_me_ad_ll12.pdf

[21] Berta M, Dethlefsen F, Ebert M, et al. Geochemical effects of millimolar hydrogen concentrations in groundwater: An experimental study in the context of subsurface hydrogen storage. Environ Sci Technol. 2018;52:4937–4949.

[22] Bradley AS, Leavitt WD, Schmidt M, et al. Patterns of sulfur isotope fractionation during microbial sulfate reduction. Geobiology. 2016;14:91–101.

[23] Dinkla IJT, Gabor EM, Janssen DB. Effects of iron limitation on the degradation of toluene by *Pseudomonas* strains carrying the TOL (pWWO) plasmid. Appl Environ Microbiol. 2001;67:3406–3412.

[24] Harder W, Dijkhuizen L. Physiological responses to nutrient limitation. Annu Rev Microbiol. 1983;37:1–23.

[25] Sim MS, Ono S, Bosak T. Effects of iron and nitrogen limitation on sulfur isotope fractionation during microbial sulfate reduction. Appl Env Microbiol. 2012;78:8368–8376.

[26] Fry B. Stable isotope ecology. New York: Springer; 2006.

[27] Gat JR, Mook WG, Meijer HAJ, et al. Atmospheric water. Volume II of: Mook WG, editor. Environmental isotopes in the hydrological cycle: Principles and applications. Paris: UNESCO/IAEA; 2001. (IHP-V Technical Documents in Hydrology; No. 39).

[28] Sharp Z. Principles of stable isotope geochemistry. 2nd ed. London: Pearson/Prentice Hall; 2017.

[29] Hoberman HD, Rittenberg D. Biological catalysis of the exchange reaction between water and hydrogen. J Biol Chem. 1943;147:211–227.

[30] Löffler M, Kümmel S, Vogt C, et al. H_2 kinetic isotope fractionation superimposed by equilibrium isotope fractionation during hydrogenase activity of *D. vulgaris* strain Miyazaki. Front Microbiol. 2019;10:01545.

[31] Vignais PM. Hydrogenases and H^+-reduction in primary energy conservation. In: Schäfer G, Penefsky HS, editors. Bioenergetics: Energy conservation and conversion. Berlin; Heidelberg: Springer; 2007. p. 223–252.

[32] Conrad R. Quantification of methanogenic pathways using stable carbon isotopic signatures: a review and a proposal. Org Geochem. 2005;36:739–752.

[33] Hunger S, Schmidt O, Hilgarth M, et al. Competing formate- and carbon dioxide-utilizing prokaryotes in an anoxic methane-emitting fen soil. Appl Environ Microbiol. 2011;77:3773.

[34] Whiticar MJ. Carbon and hydrogen isotope systematics of bacterial formation and oxidation of methane. Chem Geol. 1999;161:291–314.

[35] Whiticar MJ, Faber E, Schoell M. Biogenic methane formation in marine and freshwater environments: CO_2 reduction *vs*. acetate fermentation—Isotope evidence. Geochim Cosmochim Acta. 1986;50:693–709.

[36] Woltemate I, Whiticar MJ, Schoell M. Carbon and hydrogen isotopic composition of bacterial methane in a shallow freshwater lake. Limnol Oceanogr. 1984;29:985–992.

[37] Dethlefsen F, Nolde M, Schäfer D, et al. Basic parameterization of Schleswig–Holstein's shallow geological formations for numerical reactive transport simulations: representative groundwater compositions. Environ Earth Sci. 2017;76:59.

[38] Coplen TB. Guidelines and recommended terms for expression of stable-isotope-ratio and gas-ratio measurement results. Rapid Commun Mass Spectrom. 2011;25:2538–2560.

[39] Klindworth A, Pruesse E, Schweer T, et al. Evaluation of general ^{16}S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res. 2013;41:e1.

[40] Steinberg LM, Regan JM. Phylogenetic comparison of the methanogenic communities from an acidic, oligotrophic fen and an anaerobic digester treating municipal wastewater sludge. Appl Environ Microbiol. 2008;74:6663–6671.

[41] Bolyen E., Rideout JR, Dillon MR, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol. 2019;37:852–857.

[42] Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet J. 2011;17:10–12.

[43] Callahan BJ, McMurdie PJ, Rosen MJ, et al. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods. 2016;13:581–583.

[44] Quast C, Pruesse E, Yilmaz P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 2012;41:D590–D596.

[45] SILVA database [Internet]. Available from: http://www.arb-silva.de.

[46] Bottinga Y. Calculated fractionation factors for carbon and hydrogen isotope exchange in the system calcite–carbon dioxide–graphite–methane–hydrogen–water vapor. Geochim Cosmochim Acta. 1969;33:49–64.

[47] Crist RH, Dalin GA. “Isotopic equilibria” in the hydrogen-hydrogen oxide system. J Chem Phys. 1934;2:735–738.

[48] Löffler M, Schrader M, Lüders K, et al. Stable hydrogen isotope fractionation of hydrogen in a field injection experiment: Simulation of a gaseous H_2 leakage. ACS Earth Space Chem. 2022;6:631–641.

[49] Blaser MB, Dreisbach LK, Conrad R. Carbon isotope fractionation of 11 acetogenic strains grown on H_2 and CO_2. Appl Environ Microbiol. 2013;79:1787.

[50] Fuchs G, Thauer R, Ziegler H, et al. Carbon isotope fractionation by *Methanobacterium thermoautotrophicum*. Arch Microbiol. 1979;120:135–139.

[51] Gelwicks JT, Risatti JB, Hayes JM. Carbon isotope effects associated with autotrophic acetogenesis. Org Geochem. 1989;14:441–446.

[52] Valentine D L, Sessions A L, Tyler S C, et al. Hydrogen isotope fractionation during H_2/CO_2 acetogenesis: hydrogen utilization efficiency and the origin of lipid-bound hydrogen. Geobiology. 2004;2:179–188.

[53] Douglas PM, Stolper DA, Eiler JM, et al. Methane clumped isotopes: Progress and potential for a new isotopic tracer. Org Geochem. 2017;113:262–282.

[54] McIntosh JC, Hendry MJ, Ballentine C, et al. A critical review of state-of-the-art and emerging approaches to identify fracking-derived gases and associated contaminants in aquifers. Environ Sci Technol. 2018;53:1063–1077.

[55] Cummings D, Caccavo F, Springs S, et al. *Ferribacterium limneticum*, gen. nov., sp. nov., an Fe(III)-reducing microorganism isolated from mining-impacted freshwater lake sediments. Arch Microbiol. 1999;171:183–188.

[56] Coates JD, Ellis DJ, Gaw CV, et al. *Geothrix fermentans* gen. nov., sp. nov., a novel Fe(III)-reducing bacterium from a hydrocarbon-contaminated aquifer. Int J Syst Evol Microbiol. 1999;49:1615–1622.

[57] Alazard D, Joseph M, Battaglia-Brunet F, et al. *Desulfosporosinus acidiphilus* sp. nov.: a moderately acidophilic sulfate-reducing bacterium isolated from acid mining drainage sediments: New taxa: Firmicutes (Class Clostridia, Order Clostridiales, Family Peptococcaceae). Extremophiles. 2010;14:305–312.

[58] Dolla A, Pohorelic BKJ, Voordouw JK, et al. Deletion of the hmc operon of *Desulfovibrio vulgaris* subsp. *vulgaris* Hildenborough hampers hydrogen metabolism and low-redox-potential niche establishment. Arch Microbiol. 2000;174:143–151.

[59] Elias DA, Suflita JM, McInerney MJ, et al. periplasmic cytochrome c_3 of *Desulfovibrio vulgaris* is directly involved in H_2-mediated metal but not sulfate reduction. Appl Environ Microbiol. 2004;70:413–420.

[60] Fauque GD, Berlier YM, Czechowski MH, et al. A proton–deuterium exchange study of three types of *Desulfovibrio hydrogenases*. J Ind Microbiol. 1987;2:15–23.

[61] Ramamoorthy S, Sass H, Langner H, et al. *Desulfosporosinus lacus* sp. nov., a sulfate-reducing bacterium isolated from pristine freshwater lake sediments. Int J Syst Evol Microbiol. 2006;56:2729–2736.

[62] Robertson W, Bowman J, Franzmann P, et al. *Desulfosporosinus meridiei* sp. nov., a spore-forming sulfate-reducing bacterium isolated from gasolene-contaminated groundwater. Int J Syst Evol Microbiol. 2001;51:133–140.

[63] Sánchez-Andrea I, Stams AJ, Hedrich S, et al. *Desulfosporosinus acididurans* sp. nov.: an acidophilic sulfate-reducing bacterium isolated from acidic sediments. Extremophiles. 2015;19:39–47.

[64] Deckers HM, Wilson FR, Voordouw G. Cloning and sequencing of a [NiFe]hydrogenase operon from *Desulfovibrio vulgaris* Miyazaki F. Microbiology. 1990;136:2021–2028.

[65] Noguera DR, Brusseau GA, Rittmann BE, et al. A unified model describing the role of hydrogen in the growth of *Desulfovibrio vulgaris* under different environmental conditions. Biotechnol Bioeng. 1998;59:732–746.

[66] Peck HD. The ATP-dependent reduction of sulfate with hydrogen in extracts of *Desulfovibrio desulfuricans*. Proc Natl Acad Sci. 1959;45:701–708.

[67] Tsuji K, Yagi T. Significance of hydrogen burst from growing cultures of *Desulfovibrio vulgaris*, Miyazaki, and the role of hydrogenase and cytochrome c_3 in energy production system. Arch Microbiol. 1980;125:35–42.

[68] Sun B, Cole JR, Sanford RA, et al. Isolation and characterization of *Desulfovibrio dechloracetivorans* sp. nov., a marine dechlorinating bacterium growing by coupling the oxidation of acetate to the reductive dechlorination of 2-chlorophenol. Appl Environ Microbiol. 2000;66:2408–2413.

[69] Phelps T, Conrad R, Zeikus J. Sulfate-dependent interspecies H_2 transfer between *Methanosarcina barkeri* and *Desulfovibrio vulgaris* during coculture metabolism of acetate or methanol. Appl Environ Microbiol. 1985;50:589–594.

[70] Badziong W, Ditter B, Thauer RK. Acetate and carbon dioxide assimilation by *Desulfovibrio vulgaris* (Marburg), growing on hydrogen and sulfate as sole energy source. Arch Microbiol. 1979;123:301–305.

[71] Jansen K, Thauer RK, Widdel F, et al. Carbon assimilation pathways in sulfate reducing bacteria. Formate, carbon dioxide, carbon monoxide, and acetate assimilation by *Desulfovibrio baarsii*. Arch Microbiol. 1984;138:257–262.

[72] Schauer NL, Ferry JG. Metabolism of formate in *Methanobacterium formicicum*. J Bacteriol. 1980;142:800–807.

[73] Zeikus JG, Wolee RS. *Methanobacterium thermoautotrophicus* sp. n., an anaerobic, autotrophic, extreme thermophile. J Bacteriol. 1972;109:707–713.

[74] Thauer RK. Methyl (alkyl)-coenzyme M reductases: nickel F-430-containing enzymes involved in anaerobic methane formation and in anaerobic oxidation of methane or of short chain alkanes. Biochemistry. 2019;58:5198–5220.

[75] Thauer RK, Kaster A-K, Goenrich M, et al. Hydrogenases from methanogenic archaea, nickel, a novel cofactor, and H_2 storage. Annu Rev Biochem. 2010;79:507–536.

[76] Merchant SS, Helmann JD. Elemental economy: microbial strategies for optimizing growth in the face of nutrient limitation. Adv Microb Physiol. 2012;60:91–210.

[77] Wintsche B, Glaser K, Sträuber H, et al. Trace elements induce predominance among methanogenic activity in anaerobic digestion. Front Microbiol. 2016;7:2034.

[78] Glass J, Orphan VJ. Trace metal requirements for microbial enzymes involved in the production and consumption of methane and nitrous oxide. Front Microbiol. 2012;3:61.

[79] Zerkle AL, House CH, Brantley SL. Biogeochemical signatures through time as inferred from whole microbial genomes. Am J Sci. 2005;305:467–502.

[80] Bagnoud A, Chourey K, Hettich RL, et al. Reconstructing a hydrogen-driven microbial metabolic network in Opalinus Clay rock. Nat Commun. 2016;12770.