# Variations in $H_2$ thresholds and growth yields reveal bioenergetic diversity among hydrogenotrophic methanogens

**Timothé Philippon [(1)], Jo Philips [(1,2)*]**

[(1)] Department of Biological and Chemical Engineering, University of Aarhus, Gustav Wieds Vej 10, 8000 Aarhus C, Denmark

[(2)] Novo Nordisk Foundation $CO_2$ Research Center (CORC), Gustav Wieds Vej 10, 8000 Aarhus C, Denmark

* Corresponding author: jo.philips@bce.au.dk

## Abstract

Hydrogenotrophic methanogens are of high environmental and biotechnological importance, converting $CO_2$ with $H_2$ into $CH_4$. Despite their common metabolism, variations in the energy metabolism among these methanogens exist, likely affecting their $H_2$ thresholds and growth yields. However, a systematic comparison of these traits for a wide range of hydrogenotrophic methanogens has been lacking. Here, we measured the $H_2$ thresholds and growth yields of nine different hydrogenotrophic methanogens. The $H_2$ threshold, i.e. the $H_2$ partial pressure at which $H_2$ consumption halts, ranged over two orders of magnitude from 1.0 ± 0.5 Pa for *Methanobrevibacter arboriphilus* to 120 ± 10 Pa for *Methanosarcina mazei*. Growth yields in our experimental conditions ranged from 0.51 ± 0.28 $g_{DCW}\times(\text{mol } CH_4)^{-1}$ for *Methanococcus maripaludis* to 5.28 ± 1.25 $g_{DCW}\times(\text{mol } CH_4)^{-1}$ for *Methanosarcina mazei*. The ATP gains, estimated from both $H_2$ thresholds and growth yields, correlated reasonably well, confirming that these variations are due to differences in energy conservation strategies. Our results strongly differentiated the two previously proposed groups of hydrogenotrophic methanogens: methanogens with cytochromes had a high $H_2$ threshold (≥ 21 Pa) and high growth yield (> 4.0 $g_{DCW}\times(\text{mol } CH_4)^{-1}$), whereas methanogens without cytochromes had lower $H_2$ threshold (≤ 7 Pa) and low growth yield (< 1.7 $g_{DCW}\times(\text{mol } CH_4)^{-1}$). Moreover, our $H_2$ thresholds indicated that additional variations in energy metabolism exist within both groups. Overall, this study found strong variations between hydrogenotrophic methanogens, which are important to understand their environmental prevalence and biotechnological applicability.


## Importance

Hydrogenotrophic methanogens play key roles in natural ecosystems and biotechnological processes. Even though all hydrogenotrophic methanogens convert $CO_2$ with $H_2$ into $CH_4$, they can differ in their $H_2$ threshold and growth yield, due to variations in their energy metabolism. Here, we found that $H_2$ thresholds of hydrogenotrophic methanogens range over two orders of magnitude, while a ten-fold difference was observed in their growth yields. These strong variations in $H_2$ thresholds and growth yields demonstrate that hydrogenotrophic methanogens are confronted with

a trade-off between the capacity to grow at low $H_2$ levels (low $H_2$ threshold) and efficient growth (high growth yield). Curiously, the observed $H_2$ thresholds also correlate with the electroactivity of different hydrogenotrophic methanogens. Overall, by reporting $H_2$ thresholds and growth yields for a wide range of hydrogenotrophic methanogens, this work provides new insights into the bioenergetic diversity of these microbes, as well as their environmental prevalence and biotechnological applicability.

**Keywords**

Methanogenic archaea, $H_2$ threshold, growth yield, ATP gain, energy conservation, extracellular electron uptake, ecological trade-off

53 # Introduction

54 Hydrogenotrophic methanogenesis is a metabolic pathway used by specific Archaea to
55 produce methane ($CH_4$) from carbon dioxide ($CO_2$) and dihydrogen ($H_2$) for the purpose of
56 energy conservation:

57 $$CO_2 + 4\ H_2 + n\ ADP + n\ Pi \rightarrow CH_4 + 2\ H_2O + n\ ATP \quad (1)$$

58 This metabolism is widely spread in the environment and plays an important role in the
59 global carbon cycle, as methanogens convert yearly about one gigaton of carbon into
60 methane [1]. This production of methane is a major issue in the context of climate
61 change, as methane is a potent greenhouse gas. Methanogenesis can also negatively
62 impact steel infrastructure, as certain hydrogenotrophic methanogens induce strong
63 corrosion [2]. Nevertheless, hydrogenotrophic methanogenesis is of huge interest for the
64 development of energy conversion (e.g. power-to-gas) biotechnologies, as methane is a
65 valuable fuel. Bio-methanation is already an advanced technology, converting $CO_2$-
66 containing gases into methane with the addition of $H_2$ gas (Reaction 1) [3]. Another
67 promising biotechnology is electro-methanogenesis, in which an electrode is used to
68 generate $H_2$ *in situ* of a bioreactor [4, 5]. Because of the technological and environmental
69 importance of hydrogenotrophic methanogens, variations between different
70 methanogens need to be well understood.

71 All hydrogenotrophic methanogens use a common pathway for their energy metabolism,
72 consisting of a succession of steps reducing $CO_2$ to $CH_4$, using electrons obtained from
73 the oxidation of $H_2$ and generating a sodium ($Na^+$) gradient (Figure 1) [6]. Besides this
74 common pathway, hydrogenotrophic methanogens vary in the involvement of
75 cytochromes [1, 6]. Hydrogenotrophic methanogens without cytochromes (Figure 1A)
76 generate ATP only through the $Na^+$ gradient [7]. In contrast, *M. barkeri* creates an
77 additional $H^+$ gradient through two protein complexes (*Vht* and *hrdDE*), each containing
78 cytochromes (Figure 1B) [6]. Methanogens with cytochromes thus have a higher ATP gain
79 (amount of ATP generated (*n*) in Reaction 1) than methanogens without cytochromes
80 (Table 1, Figure 1). Moreover, further variations in the energy conservation components
81 of both groups of methanogens have been reported [6, 8, 9], suggesting that additional
82 differences in ATP gains may exist.

83 Different ATP gains in turn affect various metabolic characteristics of methanogens,
84 including their $H_2$ threshold and growth yield (Table 1). The $H_2$ threshold quantifies the $H_2$
85 concentration at which $H_2$ oxidation and therefore methanogenesis halts due to
86 thermodynamic constraints. This $H_2$ threshold is generally lower for methanogens
87 without cytochromes than for methanogens with cytochromes (Table 1 and S1).
88 Accordingly, the growth yield, i.e. the amount of biomass created per amount of $CH_4$
89 produced, is higher for methanogens with cytochromes than for methanogens without
90 cytochromes (Table 1, Table S2). Additional variations in these metabolic characteristics
91 likely exist, but a coherent comparison of an extensive number of methanogens has not
92 yet been performed.

93 Most studies measuring growth yields for hydrogenotrophic methanogens included just
94 one to two methanogenic species, while each using different media and experimental
95 setups (Table S2). However, growth yields strongly depend on the medium composition
96 [10, 11], the gas supply [12], the dissolved $H_2$ concentration [13] and the growth rate [14]
97 (Table S2). Growth yields determined in different studies are thus not directly
98 comparable. Similarly, most studies measuring $H_2$ thresholds included only few
99 methanogens, each using different conditions (Table S1). $H_2$ thresholds depend on the
100 experimental conditions (e.g. temperature, total pressure, $CO_2$ and $CH_4$ levels and even
101 trace element concentrations [15-17]), which likely explains why reported $H_2$ thresholds
102 for e.g. *Methanosarcina barkeri* differ widely (from 13 to 150 Pa, Table S1). $H_2$ thresholds
103 determined in different studies are thus neither directly comparable. Recently, strongly
104 different $H_2$ thresholds were unexpectedly found for acetogens [18]. It seems thus
105 worthwhile to revisit the $H_2$ thresholds and growth yields of different methanogens.

106 Insights into the $H_2$ thresholds of different methanogens are also crucial to assess their
107 potential for electromethanogenesis and role in microbial-induced corrosion. In both
108 cases, the cathode or steel surface acts as extracellular electron donor, from which
109 methanogens withdraw electrons, using $H_2$ as main intermediate [5]. Not all
110 hydrogenotrophic methanogens are as effective in this extracellular electron uptake [19].
111 Besides the involvement of extracellular hydrogenases [20], the $H_2$ partial pressure is
112 proposed to play a role in electroactivity [15]. Microbes maintaining low $H_2$ partial
113 pressures likely favor $H_2$ evolution from a cathode or steel surface. As the $H_2$ threshold

114 reflects the lowest $H_2$ partial pressure a strain can sustain, this parameter is thus likely
115 crucial to understand the electroactivity of methanogens.

116 This study aimed to compare $H_2$ thresholds and growth yields of nine strains of
117 mesophilic hydrogenotrophic methanogens in similar conditions (Table 2). The selected
118 methanogens include species with and without cytochromes, originating from different
119 environments and spanning various methanogenic genera. In addition, two
120 methanogenic strains known for their strong corrosive behavior were included. For all
121 these strains, the $H_2$ threshold and the growth yield were experimentally measured.
122 Moreover, ATP gains were estimated from either the $H_2$ threshold or the growth yield. All
123 these variables are relevant to assess variations among methanogens.

124

# Material and methods

## Methanogenic strains and culture conditions

This study included nine different methanogens (Table 2). Seven methanogenic strains were ordered from the Leibniz Institute DSMZ, including *Methanococcus maripaludis* (DSMZ 2067 designated as type strain in this study), *Methanolacinia petrolearia* (DSMZ 11571), *Methanobacterium palustre* (DSMZ 3108), *Methanobacterium formicicum* (DSMZ 1535), *Methanosarcina mazei* (DSMZ 2053), *Methanosarcina barkeri* (DSMZ 800) and *Methanobrevibacter arboriphilus* (DSMZ 744). Once received, the strains were transferred to their recommended DSMZ medium (exact composition but without $Na_2S$, sludge fluid or fatty acid mixture) and stored frozen at – 80 °C in a 2 mL tube with 25% glycerol.

In addition, a culture containing *Methanobacterium* strain IM1 [21, 22] was obtained from Florin Musat (Aarhus University). The culture was inoculated in DSMZ medium 141 (without $Na_2S$) and was stored frozen at – 80 °C. Microscope imaging (Leica ICC50 W, Germany) showed that an additional coccus shaped microbe was present in this culture. Nevertheless, when cultivated in a medium with only $H_2$ as electron donor (no acetate), the IM1 strain (bacille shape [22]) became dominant. Acetate was thus further omitted from the medium. Therefore, we assume that the measured $H_2$ threshold was for *Methanobacterium* strain IM1.

*Methanococcus maripaludis* Mic1c10, was provided as living culture by Amelia-Elena Rotaru (Southern University of Denmark). This strain was cultured in DSMZ medium 141 (recommended for *Methanococcus maripaludis*, but without $Na_2S$) and stored at – 80 °C. Both *Methanococcus maripaludis* Mic1c10 and *Methanobacterium* strain IM1 are known corrosive methanogens, as previously documented [20, 21, 23, 24].

To start experiments, frozen stocks were revived in their respective DSMZ medium and transferred to the same medium once. When the late exponential growth phase was reached, 10 mL of culture was transferred to a sterile glass tube with conical bottom, which had first been hermetically sealed using a rubber stopper and flushed with $N_2$. These tubes were then centrifuged at 2500 rpm for 15 minutes. The cell pellet was washed two times using limited medium (described below), before being resuspended in

155 the same limited medium. For the $H_2$ threshold experiment, this cell suspension was
156 diluted with limited medium until an optical density (OD) (method described below)
157 between 0.10 and 0.15 was reached. This procedure was used both for strains that grow
158 planktonically or as aggregates (notably *M. barkeri* and *M. mazei*), even though similar OD
159 values in this case do not correspond to comparable biomass densities.

160 All experiments were performed in limited medium. This limited medium was derived
161 from the DSMZ medium 141 and included 0.34 g of KCl, 3.45 g of $MgSO_4$ x 7 $H_2O$, 4 g of
162 $MgCl_2$, 0.25 g of $NH_4Cl$, 0.14 g of $CaCl_2$, 0.14 g of $K_2HPO_4$, 0.2 mg of $Fe(NH_4)_2(SO_4)_2$, 0.2 g
163 yeast extract powder (Y1625), 10 mL of Wolin mineral solution and 10 mL of Wolin
164 vitamins solution for a final volume of one liter. In addition, NaCl was added for a final
165 concentration of 0.4 g per L or 18 g per L, respectively for freshwater methanogens (*M.*
166 *palustre, M. formicicum*, *M. barkeri*, *M. arboriphilus* and *M. mazei*) and marine
167 methanogens (*strain IM1*, *M. maripaludis* DSMZ 2067 and Mic1c10 strain and *M.*
168 *petrolearia*) (Table 2). In contrast to regular DSMZ medium 141, this limited medium did
169 not contain acetate and had a ten-times lower yeast extract concentration. After
170 autoclaving this basal medium, filter-sterile stocks solutions were added for a final
171 concentration of 5 g of $NaHCO_3$ and 0.5 $g.L^{-1}$ of L-cysteine.

## 172 Measurement of the $H_2$ threshold

173 A volume of 80 mL of the cell suspensions obtained after washing was brought into 250
174 mL serum bottles. For each strain, four replicates were prepared, while three additional
175 bottles were filled with 80 mL of limited medium as abiotic controls. The $H_2$ threshold
176 experiment was initiated by flushing the headspace of the bottles with a gas mix
177 composed of 2 % $H_2$, 78 % $N_2$ and 20 % $CO_2$. This mix was prepared using gas flow meters
178 (Alicat®, MCW Series, USA) with two gas tanks, one containing 80% $H_2$ and 20% $CO_2$ and
179 the other containing 80% $N_2$ and 20 % $CO_2$. Each bottle was pressurized at 2 bars (1 bar
180 overpressure). The bottles were incubated upright at 30 °C with horizontal orbital shaking
181 at 100 rpm. The total pressure was measured with a digital manometer (OMEGA DPG108,
182 UK) before each sampling event. The headspace of each bottle was frequently sampled
183 with a syringe for $H_2$ analysis (1.5 mL). The $H_2$ threshold was considered reached when
184 the $H_2$ partial pressure did not significantly decrease for at least four days with
185 measurement intervals of maximally two days. The value of the $H_2$ threshold was

186 calculated from the average of two to four measurements performed during those last
187 four days. After the threshold was reached, the headspace was flushed and pressurized
188 again with the gas mix as described above. The headspace was sampled again until the
189 $H_2$ threshold was reached for a second time. The OD and the methane content in the
190 headspace were measured at the end of the experiment. For all methanogens, the
191 amount of $CH_4$ at the end of the $H_2$ threshold experiment was too low to be accurately
192 quantified, even though detectable by GC.

193 **Growth yield measurements**

194 For each methanogen, the inoculum was pre-grown and washed as described above.
195 Four replicated bottles (250 mL) with 80 mL of limited medium were inoculated by
196 injecting 0.5 mL of washed culture. These bottles were pressurized at 2 bars
197 (overpressure of 1 bar) using a gas mix with 80% $H_2$ and 20 % $CO_2$. The bottles were
198 incubated upright at 30 °C under horizontal orbital shaking at 100 rpm. The pressure was
199 measured daily until at least one of the four replicates reached a negative pressure, after
200 which the incubation was ended for all replicates. Before biomass was harvested, the
201 headspace composition was analyzed. Next, the bottles were opened and 10 mL of each
202 culture was transferred into a 20 mL glass tube with conical bottom and centrifuged at
203 3000 rpm for 15 minutes. The clear supernatant was discarded and an additional 10 mL
204 of culture was placed in the same tube, while keeping the cell pellet. This centrifugation
205 procedure was repeated until all 80 mL of each bottle was harvested. Each of the tubes
206 containing the stacked cell pellets were then dried at 100 °C for 48 hours, cooled down
207 for one hour in a desiccator and the dry weight was measured using an analytical balance
208 (± 0.0001 g precision) (VWR, LD17 4XN). The growth yield was calculated by dividing the
209 dried cell weight by the quantity of methane measured in the headspace at the end of the
210 experiment.

211 The effect of the medium composition on the growth yield was further investigated by
212 measuring the growth yield of one strain (*M. palustre*) in limited medium (described
213 above) and medium with additional yeast extract and sodium acetate (final
214 concentration of 1 $g \cdot L^{-1}$ each).

215

## Analytical methods

Gaseous samples were analyzed for $H_2$ and $CH_4$ using a Compact GC 4.0 (Interscience, Netherlands) with two different columns: one Molsieve 5A 30 m x 0.32 mm and one Rt-QBond 3 m x 0.32 mm, using helium as gas carrier and a TCD detector [18]. Calibration was performed using calibrating gases mixed with $N_2$. The quantification limit was 1 Pa or 10 ppm for $H_2$ and10 000 ppm for $CH_4$. For $H_2$ partial pressures below 50 ppm, a Peak Performer 1 (PP1) (Peak Laboratories, USA) GC unit based on HgO–Hg conversion with a reducing compound photometer (RCP) was also used. This GC measures trace $H_2$ levels (0.1–50 ppm of $H_2$) and has quantification limit well below that of the CompactGC. Partial pressures were calculated by multiplying the mol fraction, as measured by GC, with the total pressure. Dissolved concentrations were calculated from the partial pressures using the Henry constants for $H_2$ at 30°C (7.8 x $10^{-4}$ mol.$l^{-1}$.$bar^{-1}$) [25]. Optical densities (OD) were measured at 600 nm using a Denovix DS-11+ spectrophotometer.

## Calculations

### Estimation of the ATP gain from the $H_2$ threshold

As explained previously [18], $H_2$ thresholds can be used to calculate an estimate for the ATP gain through calculating the critical Gibbs free energy, i.e. the Gibbs free energy change when the $H_2$ threshold is reached [1].

Considering hydrogenotrophic methanogenesis:

$$CO_2 + 4\,H_2 \rightarrow CH_4 + 2\,H_2O \qquad (2)$$

The critical Gibbs free energy ($\Delta G_c$) becomes:

$$\Delta G_c = \Delta G^{0'}_{\text{methanogenesis}} + R \cdot T \cdot \ln \frac{p_{\text{CH}_4}}{p_{\text{CO}_2} * \theta_{\text{H}_2}{}^4} \qquad (3)$$

With $\Delta G^{0'}_{\text{methanogenesis}}$ the Gibbs free energy of methanogenesis (Reaction 2) in physiological standard conditions (-131 kJ.$mol^{-1}$ [1]), $\theta_{H_2}$ the $H_2$ threshold (in atm), $p_{\text{CO}_2}$ the partial pressure of $CO_2$ (in atm) and $p_{\text{CH}_4}$ the partial pressure of methane (in atm) at the $H_2$ threshold. As the methane partial pressures in the $H_2$ threshold experiment were

too low to be quantified, they were calculated from the amount of $H_2$ consumed, according to the stoichiometric ratios of Reaction 2.

The critical Gibbs free energy at the $H_2$ threshold is not zero, because methanogenesis should be considered as coupled to ADP to ATP phosphorylation (Reaction 1). At the $H_2$ threshold, it can be assumed that the total Gibbs free energy change of Reaction 1 considering both methanogenesis and ADP phosphorylation, equals zero [1]:

$$\Delta G_c + n \,.\, \Delta G_{ATP} = 0 \qquad (4)$$

Where $\Delta G_{ATP}$ is the Gibbs free energy change for the phosphorylation of ADP into ATP. The value of this $\Delta G_{ATP}$ can vary between microorganisms and different values are used in literature [1, 18], but here we used the value of -75 kJ.mol$^{-1}$ [26]. The ATP gain *n, i.e.* the number of ADP molecules phosphorylated for each completed methanogenesis reaction, can finally be calculated using:

$$n = -\frac{\Delta G_c}{\Delta G_{ATP}} \qquad (5)$$

**Estimation the ATP gain from the growth yield**

The ATP gain *n* can also be estimated from experimental growth yields ($Y^{CH_4}$, g dry cell weight (DCW) per mol methane), using the biomass yield per mol ATP ($Y^{ATP}$, g dry cell weight per mol ATP):

$$n = \frac{Y^{CH_4}}{Y^{\mathrm{ATP}}} \qquad (6)$$

Different $Y^{ATP}$ values reported for methanogenesis are summarized in Table S3. Most of these values are maximum values, meaning that the actual $Y_{ATP}$ must be lower. As our experimental growth yields are observed (net) growth yields and not maximum growth yields (requiring correction for maintenance energy), we selected a value lower than the reported maximum and used 5 g dry cell weight per mole of ATP as $Y^{ATP}$ [27].

## Statistical Analysis

### Welch ANOVA test

Welch-Anova tests were performed to differentiate statistically significant groups using both GraphPad Prism® and RStudio (ver. 4.4.3). The Welch test was chosen because the variances were considered too different to perform a regular ANOVA test. All the tests were performed with $\alpha = 0.05$ and the p-value was set at 0.05 for all tests.

### Error propagation

Standard deviations on the experimental results were calculated using Excel and were further propagated through the different calculations using available calculators [28, 29].

# Results and Discussion

## $H_2$ threshold measurements

In this study, the $H_2$ threshold of nine methanogenic strains was measured, as the $H_2$ partial pressure at which their $H_2$ consumption halts. In all abiotic controls, the $H_2$ partial pressure remained almost stable for the entire duration of the experiment (Figure 2, Figure S1), as only a small drop of the total pressure was observed, as the result of headspace sampling (Figure S1).

For all inoculated bottles, a clear drop in the $H_2$ partial pressure was observed (Figure 2), confirming $H_2$ consumption by the different methanogens. Moreover, $H_2$ consumption halted at a certain $H_2$ partial pressure for each strain. To confirm these $H_2$ thresholds, $H_2$ was reinjected, once stable $H_2$ partial pressures were reached. For most methanogenic strains, the $H_2$ thresholds reached after the re-addition of $H_2$ were comparable to those before the re-addition, except for *Methanosarcina mazei* and *Methanosarcina barkeri* (Figure S2). *M. mazei* consumed $H_2$ after the $H_2$ re-addition, but its $H_2$ consumption halted at a higher $H_2$ partial pressure (260 ± 49 Pa versus 120 ± 10 Pa before the re-addition). In contrast, *M. barkeri* no longer consumed $H_2$ after the re-addition. Possibly, this is due to the loss of activity of these strains over the long incubation times (*M. mazei* reached its $H_2$ threshold after 12 days, while *M. barkeri* required 28 days). All other methanogens required only five days or less to reach their $H_2$ threshold.

The three *Methanobacterium* species displayed similar low $H_2$ thresholds around 2 Pa (Figure 2, Figure 3, Table 2). Also, *Methanosarcina petrolearia* and *Methanobrevibacter arboriphilus* reached $H_2$ thresholds in this range. *M. arboriphilus* was the only species that reached a $H_2$ partial pressure of 1 Pa (Figure 2, Figure 3, Table 2). The Welch-ANOVA test gathered these five strains with the lowest $H_2$ thresholds in one statistical group (Figure 3). In contrast, all the other strains had higher $H_2$ thresholds and were classified each as a significantly different group (Figure 3). The two strains of *Methanococcus maripaludis* displayed $H_2$ thresholds slightly higher than the five strains with the lowest $H_2$ thresholds (Figure 2, Figure 3, Table 2). Interestingly, the corrosive strain *Mic1c10* had a significantly lower $H_2$ threshold (around 4 ± 1 Pa) than the type strain (7 ± 1 Pa).

The *Methanosarcina* species differed strongly in their $H_2$ thresholds, as *M. barkeri* had a $H_2$ threshold of 21 Pa ± 2 Pa, while the highest $H_2$ threshold was recorded for *M. mazei* (120 ± 10 Pa). Previously, widely varying $H_2$ thresholds were reported for *M. barkeri* (Table S1). These variations are possibly due to different experimental conditions (Table S1) or the slow $H_2$ consumption by this strain. Our $H_2$ threshold for *M. barkeri* could be somewhat overestimated, as this strain could not be reactivated after the halt of $H_2$ consumption. In contrast, *M. mazei* was still active after the $H_2$ re-addition, so its six-times higher $H_2$ threshold in comparison to *M. barkeri* (Table 2) cannot be due to an experimental artefact.

Overall, we obtained experimental $H_2$ thresholds ranging over two orders of magnitude (Figure 3, Table 2), which is a similar range as previously recorded (Table S1). The $H_2$ thresholds also agree with the classification based on the presence of cytochromes (Table 1). We found $H_2$ thresholds below 7 Pa for all methanogens without cytochromes, while methanogens with cytochromes had $H_2$ thresholds above 20 Pa (Table 2). Our results thus differentiate both groups more strongly than previous recordings (Table 1). Moreover, in both groups, we found methanogens with statistically different $H_2$ thresholds (Figure 3), showing that additional variations in $H_2$ thresholds exist.

326 **Growth yield measurements**

327 In parallel of the $H_2$ threshold study, all methanogenic strains were grown in bottles on
328 80% $H_2$ and 20% $CO_2$ to determine their growth yields. In all these bottles methane levels
329 significantly increased over the incubation period, while $H_2$ was consumed and cell
330 growth occurred (Table S4). These results were used to calculate the growth yield, i.e. the
331 increase of biomass expressed as gram dry cell weight ($g_{DCW}$) versus the amount of
332 methane produced (Figure 4, Table 2). For one strain, *M. palustre*, the growth yield was
333 also determined with additional acetate and yeast extract in the medium (1 $g \cdot L^{-1}$ each). A
334 significantly higher growth yield was found with the addition of extra organic carbon
335 sources (Figure S3), demonstrating the importance of using the same medium conditions
336 when comparing growth yields.

337 Relatively high variabilities between the replicates were obtained for the growth yields
338 (large error bars in Figure 4). This is likely due to the low total weight of dried biomass (in
339 mg) obtained during the growth yield experiment (Table S4), limiting the accuracy of this
340 measurement. In addition, the centrifugation steps were possibly not always as effective
341 in removing cells from the culture suspension, which could also explain the high
342 variability between the replicates and could have underestimated some of the growth
343 yields. A high variability is fairly common for growth yield measurements, as this was also
344 observed in other studies determining methanogenic growth yields [11, 30, 31].

345 Despite the high variability in the dry cell weight, Welch-ANOVA tests identified two
346 significantly different groups in the growth yields (Figure 4, right). The two
347 *Methanosarcina* strains form one group with high growth yields of 4.09 ± 1.16 and 5.28 ±
348 1.25 $g_{DCW}$/mole of $CH_4$ for *M. barkeri* and *M. mazei* respectively. All other methanogens
349 had significantly lower growth yields, ranging from 0.51 ± 0.28 $g_{DCW}$/mole of $CH_4$ (for *M.*
350 *maripaludis* type strain) to 1.69 ± 0.58 $g_{DCW}$/mole of $CH_4$ (for *M. formicicum*). Growth
351 yields were also expressed as $g_{DCW}$ per mole $H_2$ consumed (Figure S4), leading to the same
352 statistical groups.

353 Overall, our growth yields are consistent, but in the lower range, of previously reported
354 growth yields (Table 2 and S2), likely because our medium did not contain organic carbon
355 sources (Figure S3). Moreover, the two significantly different groups identified in the

growth yields agree with the classification based on the presence of cytochromes, while our results differentiate both groups more strongly than previous findings (Table 1). In both groups, no further significant differences in growth yields were identified. Even for *M. mazei* and *M. barkeri*, which differ six times in their $H_2$ threshold, no significant different growth yields were found (Figure 4). It is likely that this incoherence is due to the high variability of the growth yield measurement (high error bars in Figure 4).

363 ## Comparison of ATP gain estimates

364 Both the $H_2$ threshold and the growth yield allow to estimate the ATP gain. Using the $H_2$
365 threshold, the ATP gain estimates ranged from 0.32 ± 0.07 for *M. arboriphilus* to 0.97 ±
366 0.01 for *M. mazei* (Table 2). Estimating ATP gains from the growth yields gave a slightly
367 wider range, starting at 0.10 ± 0.03 for *M. maripaludis* up to 1.06 ± 0.12 for *M. mazei*.
368 Despite the totally different experimental procedures and the various assumptions
369 underlying these calculations (Equations 5 and 6), both estimates led to a largely
370 overlapping range, validating our approach. These ATP gain estimates were further
371 correlated to each other, leading to a clear but not perfect correlation (Figure 5, $R^2$ of
372 0.84). Only the two *Methanosarcina* species, *M. mazei* and *M. barkeri,* fall on the 1:1
373 correlation line. For all other strains, higher ATP gains were calculated from the $H_2$
374 thresholds than from the growth yield, demonstrating that there were discrepancies
375 between our methods, possibly because the growth yields were somewhat
376 underestimated. This illustrates that our ATP gains should be considered as estimates,
377 rather than absolute values. Nevertheless, the clear correlation (Figure 5) does suggest
378 that the ATP gains differed among the tested strains.

379 These ATP gain estimates also agree with the cytochrome-dependent classification of
380 methanogens (Table 1 and 2). Kaster et al. [7] estimated an ATP gain higher than 1 for *M.*
381 *barkeri*, while we calculated this to be 0.82 ± 0.11 ATP per mol $CH_4$, when derived from
382 the growth yield (Table 2). For methanogens without cytochromes, Kaster et al. [7]
383 proposed an ATP gain lower than 1, while Goyal et al. [32] estimated an ATP gain of 0.35
384 for *M. maripaludis*. Both correspond well with the range of ATP gain estimates (between
385 0.10 and 0.56 mol ATP per mol $CH_4$) determined here for methanogens without
386 cytochromes (Table 1 and 2).

387 In addition, our ATP gain estimates calculated from the $H_2$ thresholds suggest that
388 additional variations in energy conservation exist, as additional significant differences in
389 $H_2$ thresholds were recorded in both groups of methanogens (Figure 3). Unfortunately,
390 this was not confirmed by the ATP gains derived from the growth yields, likely again due
391 to the high variability on the growth yields. Besides the two different mechanisms for
392 energy conservation in methanogens (Figure 1), various additional variations in energy
393 conservation components are currently being unraveled [6, 8, 9, 33]. Moreover,

*Methanothermobacter marburgensis* was recently shown to have a flexible energy conservation system, adapting to the nickel concentration in the medium [34]. We therefore conclude that the measurement of $H_2$ thresholds could offer a valuable tool to assess how variations in energy conservation affect the ATP gain.

## Link between the $H_2$ threshold and electroactivity of methanogens

The experimental $H_2$ thresholds obtained in this study allow to reevaluate previously reported differences in electroactivity among methanogens. Mayer et al [19] studied the electromethanogenesis performance of several methanogens. *M. maripaludis* and *M. petrolearia*, for which a low $H_2$ threshold was found here (Figure 3), increased cathodic currents (i.e. key signature of electroactivity), just as several other methanogens without cytochromes. In contrast, *M. mazei*, for which the highest $H_2$ threshold was recorded in this study (Figure 3), was incapable of electromethanogenesis [19]. Similarly, Jaramillo *et al*. investigate the corrosion potential of different methanogens [35]. Non-hydrogenotrophic methanogens were reported as non-corrosive, confirming the important role of $H_2$ as intermediate in microbial corrosion. *M. mazei* and *M. barkeri* (high $H_2$ threshold) were mild corrosive, whereas *Methanobacterium* spp. (low $H_2$ threshold) were highly corrosive. Overall, these correlations thus suggest that methanogens with a low $H_2$ threshold are more electroactive than methanogens with a high $H_2$ threshold.

Nevertheless, several other variables likely affect the electroactivity of methanogens, in addition to the $H_2$ threshold. The $H_2$ pressure maintained at the cathode or steel surface also depends on the rate of $H_2$ consumption. Moreover, the $H_2$ evolution reaction on a cathode or steel surface is stimulated by extracellular enzymes, such as hydrogenases. *M. maripaludis* Mic1c10 has a unique extracellular hydrogenase anchored to its cell surface [23], causing stronger corrosion than other *M. maripaludis* strains [23]. Moreover, its cathodic current increase was double as high than for a non-corrosive *M. maripaludis* strain [19, 36]. Here, we found that strain Mic1c10 had a significantly lower $H_2$ threshold than the *M. maripaludis* wild type (Figure 3, Table 2). Kawaichi et al [23] also recorded that strain Mic1c10 maintained slightly lower $H_2$ levels during steel corrosion than by the *M. maripaludis* type strain. These differences, however, are possibly too small to explain the strong corrosion rates caused by Mic1c10, which is thus likely mostly due to its extracellular hydrogenases. Similarly, *Methanobacterium* -IM1 was more corrosive than other tested methanogens [24], but here we did not find that strain IM1 had a lower $H_2$ threshold than other tested *Methanobacterium* strains (Figure 2, Table 3). *Methanobacterium* IM1 thus likely has an additional mechanism to enhance extracellular

429 electron uptake rate, which is possibly similar to the one described for *M. maripaludis*
430 Mic1c10.

431 Overall, we conclude that the electroactivity of methanogens is inversely proportional to
432 their $H_2$ threshold, whereas few methanogens (e.g. *M. maripaludis* Mic1c10,
433 *Methanobacterium* IM1) have additional mechanisms to increase their electroactivity.

## Ecological and biotechnological implications of variations among methanogens

We systematically found that methanogens with a low $H_2$ threshold have a low growth yield and a low ATP gain (Table 2). In contrast, methanogens with a high $H_2$ threshold have a high growth yield and have a higher ATP gain (Table 2). Hydrogenotrophic methanogens are thus confronted with a trade-off between the capacity to consume $H_2$ at trace levels or the ability to grow strongly (high growth yield) with a high energetic efficiency (high ATP gain). This trade-off allows to understand the ecological niche and biotechnological relevance of the different types of methanogens. For instance, Mills *et al.* [37] found that the presence of the *Methanosarcina* genus in electro-methanogenesis systems was correlated to high $H_2$ levels (reactors with low coulombic efficiency), while the *Methanobacterium* genus was rather correlated to low $H_2$ levels (high coulombic efficiency).

In addition, a correlation between the growth yield (high ATP gain) and the growth rate of methanogens would be expected [18]. However, Thauer [1] described that methanogens with cytochromes (high ATP gain) have a lower growth rate (high doubling time) than methanogens without cytochromes (low ATP gain, but low doubling times). Comparing doubling times is though not adequate, since methanogens strongly vary in cell size (large clustered cells for *Methanosarcina* species versus small different shaped cells for other methanogens) [38, 39]. Nevertheless, also in the $H_2$ threshold experiment of this study, $H_2$ was faster consumed by methanogens without cytochromes, than by methanogens with cytochromes (Figure 2). This observation possibly reflects that *Methanosarcina* species are not well adapted to consume $H_2$ at lower levels. These methanogens are also capable of acetoclastic and methylotrophic methanogenesis, in contrast to the other tested methanogens. Methanogens with cytochromes are thus possibly mainly adapted to use other substrates than $H_2$ and only shift to $H_2$ consumption at high $H_2$ partial pressures.

In this study, we found that $H_2$ thresholds of methanogens range over two orders of magnitude (Figure 3). In comparison, we previously found three orders of magnitude difference in the $H_2$ thresholds of acetogenic bacteria (6 Pa to 1990 Pa) [18]. It is often

assumed that acetogens cannot compete with methanogens for low $H_2$ levels, as the latter have lower $H_2$ thresholds [40]. Our results, however, demonstrate that such a competition rather should be evaluated on species level. For instance, *Methanobacterium* strains indeed have lower $H_2$ thresholds than *Acetobacterium* spp, but acetogenic *Sporomusa* spp. have $H_2$ thresholds in the range of those of methanogens without cytochromes and well below those with cytochromes. The outcome of competition for trace $H_2$ levels between methanogens and acetogens likely also depends on the $H_2$ consumption rates. Recently, $H_2$ consumption by acetogens was described by first order kinetics (increasing rates at increasing $H_2$ levels) [41], while the kinetics of $H_2$ consumption by methanogens rather follows Monod kinetics with low half-saturation constants [42] or even zero-order kinetics [43]. Based on these kinetic differences, methanogens (without cytochromes) consume $H_2$ likely faster at low $H_2$ levels, while acetogens have a faster $H_2$ consumption at higher $H_2$ levels, even though $H_2$ consumption rates can still widely vary within these groups [41].

Overall, we conclude that the trade-off between a low $H_2$ threshold and high growth yield of hydrogenotrophic methanogens explains their ecological prevalence and affects their biotechnological applicability.

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**Data availability statement**

The data that supports the findings of this study are available in the supporting information file.

**Conflict of interest statement**

The authors declare no conflict of interest.

# Figures

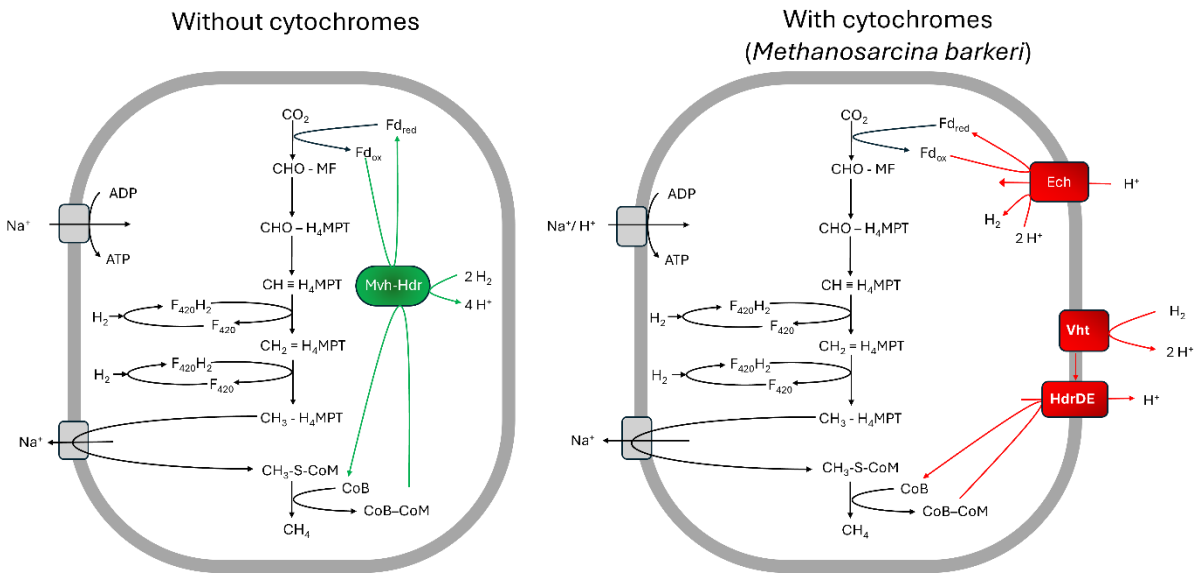
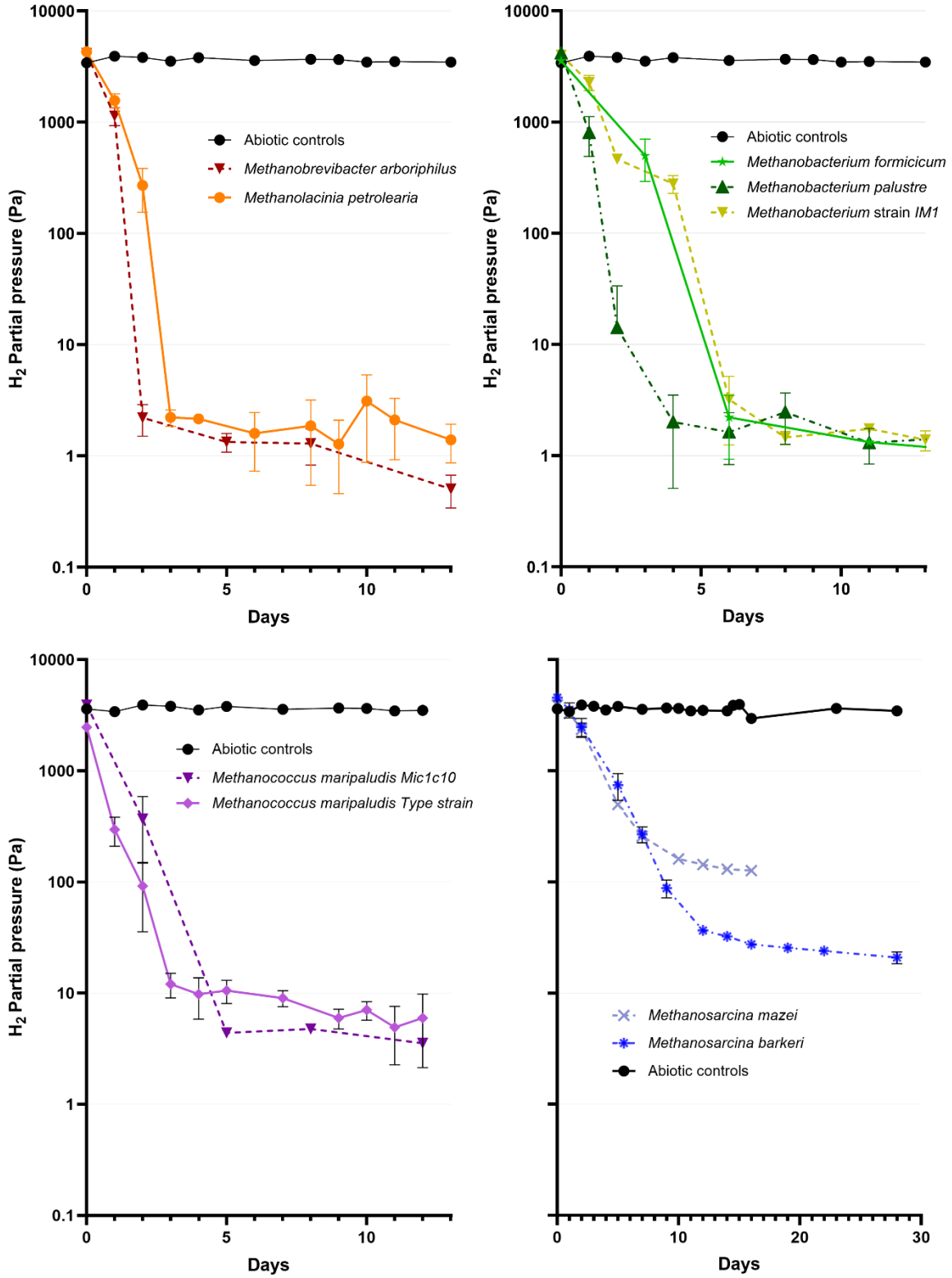



Figure 1: Energy metabolism for hydrogenotrophic methanogenesis without cytochromes (left) and with cytochromes (for *M. barkeri*) (right). Black colored components and reactions are common for all hydrogenotrophic methanogens, including the generation of a sodium ($Na^+$) gradient through the transfer of the methyl group from tetrahydrometanopterin ($H_4PMT$) to Coenzyme M (CoM). Hydrogenotrophic methanogens without cytochromes generate ATP only through this $Na^+$ gradient. They use flavin-based electron bifurcation to couple the endergonic reduction of Ferredoxin (Fd) with $H_2$ as electron donor to the exergonic reduction of CoM-CoB, through the action of the *Mvh-Hdr* ([NiFe]-hydrogenase - heterodisulfide reductase) complex. In contrast, *M. barkeri* (with cytochromes) generates ATP through both a $Na^+$ and $H^+$ gradient. In addition to the $Na^+$ gradient, *M. barkeri* creates a $H^+$ gradient through two protein complexes *Vht* (methanophenazine-reducing hydrogenase) and *hrdDE* (methanophazine-dependent heterodisulfide reductase), each containing cytochromes and together forming an electron transport chain transporting electrons from $H_2$ to CoM-CoB and pumping $H^+$ out of the cell. In addition, *M. barkeri* reduces Fd with the electron donor $H_2$ through consumption of the $H^+$ gradient using an *Ech* (energy converting hydrogenase) complex. Other abbreviations: methanofuran (MF), Coenzyme B (CoB)[6, 8].

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517 Figure 2: Change of the $H_2$ partial pressure (Pa) over time in the $H_2$ threshold experiment for the nine
518 methanogenic species and compared to the abiotic controls. The data points represent the average of four
519 replicates; the error bars show the standard deviations. Only datapoints before the re-addition of $H_2$ are
520 shown, later datapoints are shown in Figures S1 and S2.

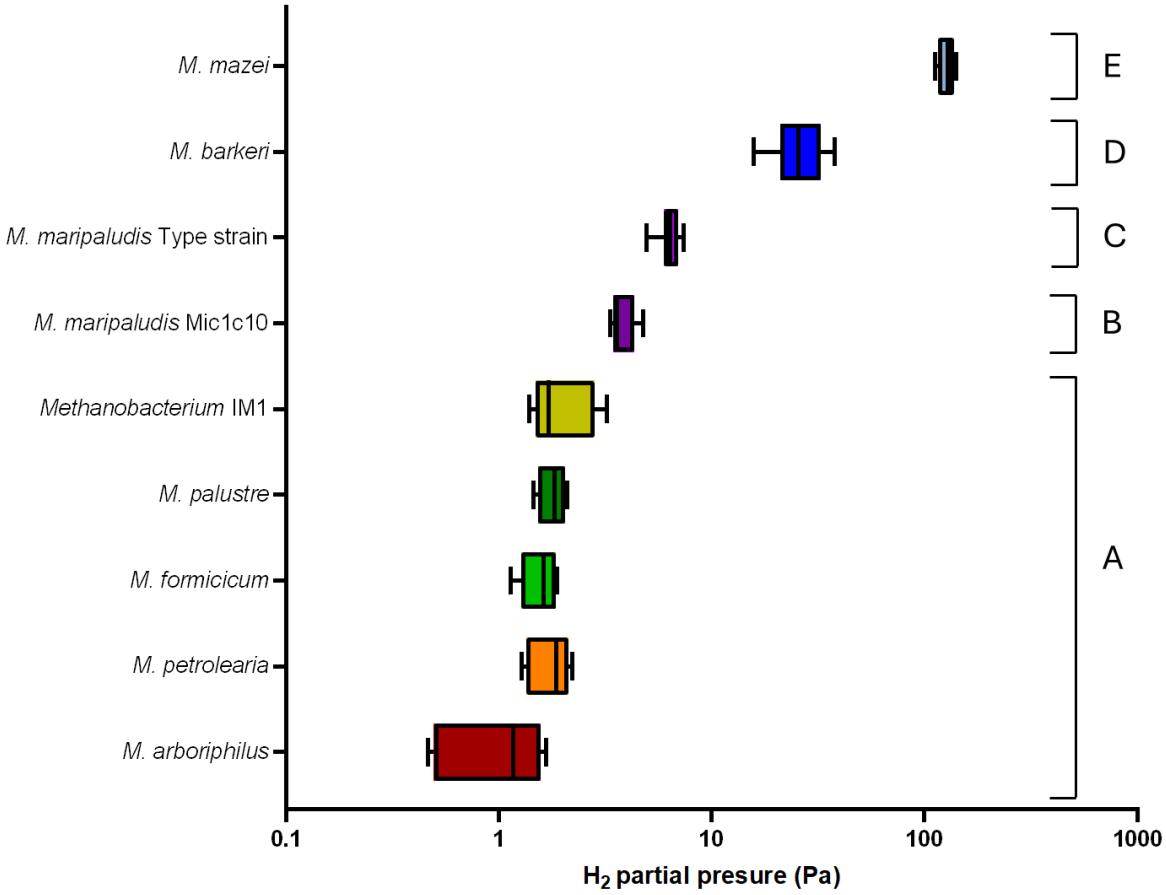


Figure 3: Box plots summarizing the experimental $H_2$ threshold values for the nine methanogenic strains. The letters indicate statistically different groups, as determined using a Welch-ANOVA test.

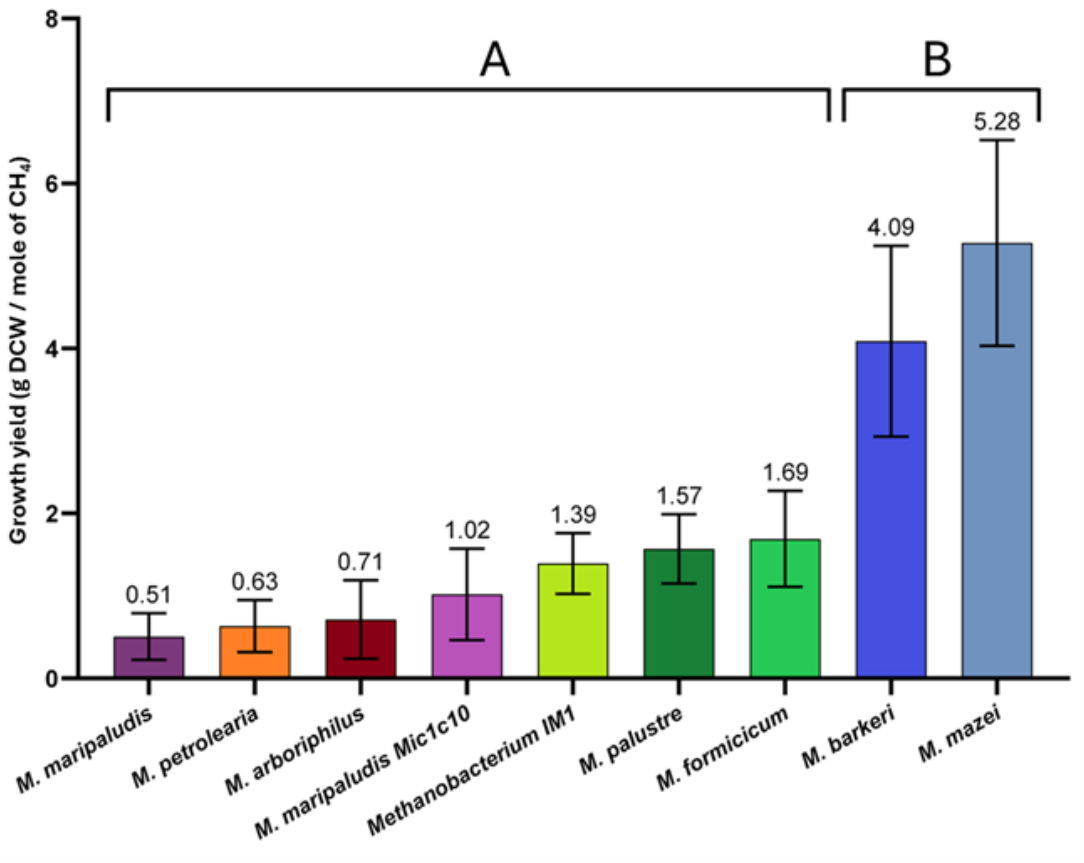


Figure 4: Comparison of the growth yields expressed as the increase of gram Dry Cell Weight ($g_{DCW}$) per mole of methane produced for the different methanogenic strains. The letters indicate statistically different groups, as determined using a Welch-ANOVA test.

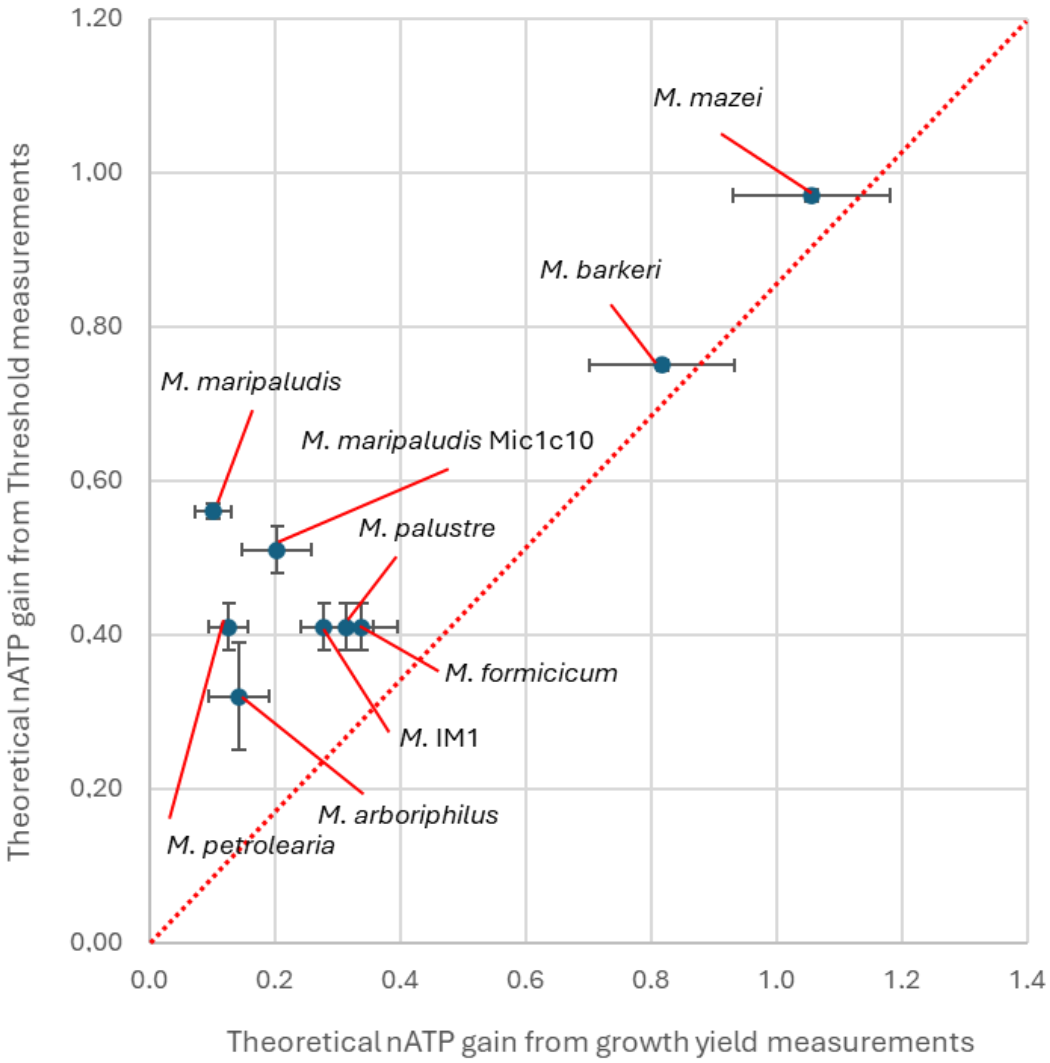


Figure 5: Correlation of the ATP gains estimations from the experimental $H_2$ thresholds versus the ATP gains estimated from the experimental growth yields. The vertical and horizontal error bars reflect the standard deviation on these estimates and were propagated from the experimental errors. The red line indicates a 1:1 correlation. The correlation coefficient (R2) was 0.84.

537 Table 1: Comparison of metabolic characteristics of hydrogenotrophic methanogens depending on the
538 presence or absence of cytochromes according to previous literature, as well as this study.

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| | Methanogens without cytochromes<br><br>Including<br>*Methanococcus*<br>*Methanobrevibacter*<br>*Methanobacterium*<br>*Methanolacinia* | Methanogens with cytochromes<br><br>Including<br>*Methanosarcina* |
|---|---|---|
| $H_2$ threshold (Pa) | | |
| Previous literature (Table S1) | ≤20 | ≥13 |
| This study (Table 2) | ≤7.0 | ≥21 |
| Growth yield ($g_{DCW} \cdot mol^{-1}$ $CH_4$) | | |
| Previous literature (Table S2) | 0.8 - 3.5 | 5.4 - 8.4 |
| This study (Table 2) | 0.5 - 1.7 | 4.1 - 5.3 |
| ATP gain (mol ATP·$mol^{-1}$ $CH_4$) | | |
| [7] | <1 | >1 |
| This study, based on $H_2$ threshold (Table 2) | 0.32-0.56 | 0.75-0.95 |
| This study, based on growth yield (Table 2) | 0.10-0.34 | 0.82-1.06 |

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547 Table 2: Overview of the methanogenic strains examined in this study, as well as their experimentally determined $H_2$ thresholds andgrowth yields. The table also
548 includes the ATP gains estimated either from the experimental $H_2$ thresholds or the experimental growth yields. The $H_2$ thresholds measured are expressed as partial
549 pressures in the headspace (both in ppm and Pa) and as concentration dissolved in the liquid phase (nM).

| Methanogenic strain | Cytochromes | Salinity level | $H_2$ Threshold | | | Growth yield | ATP gain estimated from $H_2$ threshold | ATP gain estimated from growth yield |
|---|---|---|---|---|---|---|---|---|
| | | | ppm | Pa | nM | $g_{DCw}\cdot(mol\ CH_4)^{-1}$ | mol ATP $(mol\ CH_4)^{-1}$ | mol ATP $(mol\ CH_4)^{-1}$ |
| *Methanobrevibacter arboriphilus* | No [33, 44] | Fresh water | 7 ± 1 | 1.0 ± 0.5 | 8 ± 3 | 0.71 ± 0.47 | 0.32 ± 0.07 | 0.14 ± 0.05 |
| *Methanolacinia petrolearia* | No [33] | Marine water | 10 ± 3 | 2.0 ± 0.5 | 16 ± 4 | 0.63 ± 0.31 | 0.41 ± 0.03 | 0.13 ± 0.03 |
| *Methanobacterium palustre* | No [33] | Fresh water | 11 ± 2 | 2.0 ± 0.5 | 16 ± 4 | 1.57 ± 0.41 | 0.41 ± 0.03 | 0.31 ± 0.04 |
| *Methanobacterium* IM1 | No [33]* | Marine water | 9 ± 1 | 2.0 ± 0.5 | 16 ± 4 | 1.39 ± 0.36 | 0.41 ± 0.03 | 0.28 ± 0.04 |
| *Methanobacterium formicicum* | No [33] | Fresh water | 10 ± 1 | 2.0 ± 0.5 | 16 ± 4 | 1.69 ± 0.58 | 0.41 ± 0.03 | 0.34 ± 0.06 |
| *Methanococcus maripaludis* Mutant strain Mic1c10 | No [32, 33] | Marine water | 17 ± 3 | 4.0 ± 1.0 | 31 ± 8 | 1.02 ± 0.55 | 0.51 ± 0.03 | 0.20 ± 0.05 |
| *Methanococcus maripaludis* Type strain | No [32, 33] | Marine water | 33 ± 4 | 7.0 ± 1.0 | 47 ± 12 | 0.51 ± 0.28 | 0.56 ± 0.01 | 0.10 ± 0.03 |
| *Methanosarcina barkeri* | Yes [6, 33] | Fresh water | 158 ± 18 | 21 ± 2 | 195 ± 31 | 4.09 ± 1.16 | 0.75 ± 0.01 | 0.82 ± 0.11 |
| *Methanosarcina mazei* | Yes [6, 33, 44] | Fresh water | 720 ± 20 | 120 ± 10 | 936 ± 78 | 5.28 ± 1.25 | 0.97 ± 0.01 | 1.06 ± 0.12 |

550 * The assertion that *Methanobacterium* IM1 does not possess cytochrome is based on the fact that no other *Methanobacterium* was found to have cytochromes.

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