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Pierre Buffière[1], Diana Amaya Ramirez[2], Ruben Teixeira Franco[2], Julie Figueras[1], Stéphane Hattou[2], Hassen Benbelkacem[1]

[1]INSA Lyon, DEEP, UR7429, 69621 Villeurbanne Cedex, France

[2]Arkolia Energies, 16 Rue des Vergers, 34130 Mudaison, France

Corresponding author: Pierre BUFFIERE, Professor, INSA Lyon, DEEP, UR7429, 69621 Villeurbanne Cedex, France. pierre.buffiere@insa-lyon.fr; +33 (0)4 72 43 84 78

Abstract

Biomethane production from biological methanation of CO_2 is promising both for biogas upgrading and surplus renewable energy storage. One of the questions for process upscaling is the impact of oxygen (in the biogas or in the purified CO_2-rich off-gas) on the biological process. An adapted anaerobic thermophilic consortium was submitted to increasing amounts of oxygen in batch and continuous tests at partial pressures ranging from 0 to 50 mbar. Oxygen was quickly consumed and hydrogen uptake remained similar. In the same time, methane production dropped (-4% in continuous tests). Part of the oxygen introduced was reduced biologically by hydrogen. The amount of hydrogen diverted to oxygen reduction (up to 15% at 50 mbar O_2) was proportional to the oxygen partial pressure. These results suggest that biological methanation systems tolerate the presence of oxygen. However, additional hydrogen should be added to maintain the conversion of CO_2 into methane.

Keywords: biomethane, biological methanation, oxygen, hydrogen conversion

1. Introduction

Biomethane plays a strategic role in European energy policy, particularly in the context of the transition towards more sustainable energy sources and the reduction of reliance on fossil fuels. It is commonly produced through the anaerobic digestion of organic waste (agricultural, industrial, household). It is particularly important in achieving the European Union's climate goals, including reaching carbon neutrality by 2050 as claimed by the European Green Deal and related EU directive 2023/2413. Biomethane can be injected directly into existing natural gas networks, allowing for the gradual replacement of fossil gas with a renewable alternative, without requiring any modifications of the infrastructure. Biomethane plays a key role in achieving these targets, particularly through the REPowerEU plan, with an objective of 35 bcm (billions of cubic meter) by 2030(European Commission, 2022).

The biogas produced through anaerobic digestion contains methane (CH_4), carbon dioxide (CO_2) and other trace gases like hydrogen sulfide (H_2S). Conventional processes for biogas upgrading are generally based on a process unit aimed at separating methane from the other gases in order to meet the requirements for biomethane injection in the gas grid (Angelidaki et al., 2018). In this context, the methanation reaction (ie, the conversion of carbon dioxide CO_2 and hydrogen H_2 into methane CH_4) is an emerging pathway for upgrading biogas since it can convert the CO_2 present in the biogas (or CO_2-rich off-gas from the separation unit) into CH_4 instead of losing it. The methanation process can be advantageously combined with H_2 production from surplus renewable electricity in a Power to Gas mode (Götz et al., 2016; Thema et al., 2019).

Recently, the biological methanation process has been extensively investigated in the context of CO_2 or biogas upgrading (Chatzis et al., 2024; Lecker et al., 2017; Rafrafi et al., 2021). One of the main advantage over the catalytic route is that the biological process can be operated in milder conditions of temperature, and exhibits a higher tolerance to biogas impurities (Figueras et al., 2023). The biological methanation process has proven its efficiency in terms of performance and flexibility at the

demonstration scale (Jønson et al., 2022, Feickert Fenske et al., 2023). It is also a valuable technique in terms of environmental impact and economy, even if the scalability of the process is however limited by the cost of energy for hydrogen production (Vinardell et al., 2024).

Nevertheless, many biological methanation projects are facing an issue that has not been addressed so far, which is the question of the presence of oxygen traces in the different gas streams. In biogas plants for instance, many operators are using micro-aeration to mitigate the production of hydrogen sulfide (Chen et al., 2020). Alternatively, when methane is separated from the other gases upstream, the CO_2-rich off-gas also contains oxygen (Rodin et al., 2020). The data from an industrial biological methanation project developed by Arkolia Energy show that oxygen is present whith both gas type compositions (0.2% in CO_2-rich off-gas after methane separation and 0.1% in raw biogas, see supplementary material). This means that in both cases (direct biomethanation of raw biogas or biomethanation of CO_2-rich off-gas), oxygen is present in the gas stream. Additionally, the hydrogen produced by the electrolysis unit may also content some oxygen traces due to oxygen transfer in the membrane during electrolysis, and a higher purity of the hydrogen stream means a higher cost for H_2 production (Martin et al., 2021; Trinke et al., 2017). Even if the gas compositions are relatively poor in terms of oxygen content, it is important to note that the operating pressure of biological methanation units are elevated (frequently over 10 bars). For instance, if the O_2 gas composition is 0.1%, this means that the O_2 partial pressure in the biomethanation unit operating at 10 bars would be 10 mbar.

Biological methanation is performed by anaerobic methanogenic archaea in pure culture or in consortia. In the latter case, the inoculum source generally comes from an anaerobic digester. Methanogenic organisms are traditionally considered strict anaerobes. However, anaerobic consortia are known to tolerate the presence of oxygen to some extent (Lu and Imlay, 2021). For example, in thermophilic anaerobic reactors, low oxygen levels have minimal impact, but at higher concentrations (4.3–8.8 mg/L), methane production decreases by 40%, and the methanogenic

community's development is slowed, though this inhibition is reversible (Pedizzi et al., 2016). Indeed, in anaerobic digestion consortia, the presence of facultative aerobic organisms helps to eliminate oxygen to secure strict anaerobic conditions for methane production (Magdalena et al., 2022). In addition, some aceticlastic methanogens have also shown some skills in adapting to oxidative stresses (Horne and Lessner, 2013). In pure cultures, exposing hydrogenotrophic archaea to oxygen may lead to lower viability of the cells, even if some of them able to grow in the presence of oxygen, especially *M. thermoautotrophicum*, which is commonly encountered in biomethanation reactors (Kiener and Leisinger, 1983). Some of them also have the ability not only to grow, but also to use oxygen by developing a specific metabolism able to reduce oxygen (Tholen et al., 2007) and, in the presence of hydrogen, to produce water (Seedorf et al., 2004), in the detriment however to their methanogenic metabolism.

All collected data so far come from pure culture of dedicated archaea, or from anaerobic digestion of organic biomass. To our knowledge, there have been no investigation on biological methanation reaction on short-term or long term (continuous) operation. The present study focused on the implementation of a thermophilic microbial consortium for biological methanation, which was exposed to increasing amounts of oxygen. The experiments were carried out in batch and continuous mode and monitored for the conversion balances of the gases introduced (O_2, CO_2 and H_2) and the methane produced. The aim was to assess the oxygen tolerance of the biomethanation process up to a partial pressure of 50 mbar, which is well beyond the upper limit that can be encountered industrially.

2. Materials and Methods

The experimental strategy relies on batch and continuous tests. Batch tests (see section 2.1) enables to understand the dynamics of the evolution of the different gases. The tests were performed with 4 levels of oxygen partial pressure ranging from 0 (control) to 50 mbar (Table 1). The continuous tests were performed on a 10 L reactor. Three different oxygen levels were tested (2, 5 and 10 mbar) over operating periods ranging from 6 to 18 days (see section 2.2). The continuous reactor medium was used as the inoculum source for the batch tests.

2.1 Batch tests setup

The methodology used for the batch test was adapted from activity measurements with gaseous substrates (Figueras et al., 2023; Laguillaumie et al., 2023). The tests were carried out in triplicate with 500 mL bottles (Schott) filled with 180 g of inoculum. The bottles were first bubbled with nitrogen for 10 minutes and with a standard gas bottle (Linde, France) containing N_2: 50%, H_2: 40%, CO_2: 10%, for 8 minutes. The presence of nitrogen was necessary to prevent the pressure to drop below the ambient pressure, as the gas mole number decreases during the methanation reaction. The initial pressure in the bottles was set at 1900 mbar. Butyl rubber septa and screw tops were used to seal the bottles. Then, a known volume of air was injected into the bottles with a needle to adjust the desired initial oxygen partial pressure. All bottles were placed at 55°C in an orbital shaker incubator (110 rpm). The gas headspace pressure and composition were monitored for 3 days. The initial partial pressure in the bottles headspace for each condition is shown in Table 1. For the calculations, the total pressure measured was corrected for the water partial pressure.

The inoculum used for came from the continuous 10L reactor (see next section). The initial TS content was 3.8 g/L and VS content 3.1 g/L, initial pH was 7.12 and N-NH_4^+ initial concentration was 0.33 g_N/L. The soluble phase of the inoculum (after filtration at 0.45µ) provided all the necessary elements (sulfur and trace metals) as revealed by ICP-OES measurement (see supplementary material).

2.2 Continuous Reactor setup and operating conditions

The continuous reactor is a stirred column as schematized in Figure 1. This technology was preferred to the trickled bed reactor (TBR) system by Arkolia Energies based on its economical balance, even if TBR have been intensively investigated as efficient systems for this application, notably for mass transfer performances (Jensen et al., 2021b; Orgill et al., 2013; Strübing et al., 2017). In addition, using free-suspended cell systems in both batch and continuous experiments was found more appropriate for the present study. Indeed, in biofilm systems like TBR, the impact of oxygen may not be as important due to the protection effect and diffusion limitations in the biofilm (Dupnock and Deshusses, 2019).

The reactor consists of a glass jacketed column with 600 mm height and 150 mm inner diameter (total volume 10.6 L). It was operated at atmospheric pressure and the liquid height was regulated at 500 mm by hydrostatic pressure using a U-shaped plastic tube filled with liquid and open to external atmosphere. This allowed the working volume to be fixed at 8.8 L and at the same time to recover the excess liquid obtained from biological water production and nutrient solution addition. The reactor was equipped with four standard baffles (12 mm diameter and 4 mm wall clearance) and two 800 mm diameter radial impellers (Ekato, Germany) mounted on a vertical stirrer shaft (500 mm working length and 120 mm diameter). Stirring was controlled by an electric engine (Heidolph Instruments, Germany) connected to a magnetic coupling (Büchi AG, Switzerland). Temperature in the reactor was controlled at 55.0 ± 0.3 °C with a thermostatic water bath circulator (Julabo, Germany). A peristaltic pump (MasterFlex, USA) was used to control the flowrate of the nutrient solution added to the reactor, macro and micronutrient solution (S1) and sulfur solution (S2) are added separately (Figure 1), 150 mL per day of each solution were injected in the reactor (see supplementary materials for the composition of S1 and S2).

Process gases were injected at the bottom of the reactor through a sintered gas diffuser. H_2 (> 99.9995%) was produced in-situ through a H_2 generator (F.DGSi, France) and CO_2 (> 99.9%) was

144 provided from gas bottle (Air Liquide, France). For H_2, CO_2 and O_2 inlet gas flow were regulated by an
145 independent mass flow controller (Brooks Instrument, USA). Airflow was injected with an air
146 compressor MXF08-10T/20L (Mauguière, Italy) and pure nitrogen was injected with an on-site
147 nitrogen generator NG4-1DC (Noblegen, United Kingdom). Outlet gas (top of the column) passed
148 through a foam trap bottle, and it was dried in a cold-water bath, model F12, at 10 ± 1 °C (Julabo,
149 Germany). The flow rate of dried outlet gas was measured by a drum-type gas meter (Ritter,
150 Germany).

151 The reactor was initially inoculated with a mesophilic digested sludge provided by a nearby
152 wastewater treatment plant (La Feyssine WWTP, Villeurbanne, France). Prior to this study, it had been
153 operated for 690 days with an input gas (80% H_2 / 20% CO_2) ranging from 5 to 15 NL/h in thermophilic
154 conditions. The operational conditions are presented in Table 2. They were selected according to the
155 nominal operating conditions (10 NL/h, ie., 8 NL/h of $H_{2,\,in}$ and 2 NL/h of $CO_{2,in}$). The first period
156 (control period) lasted 28 days in stable conditions (condition 1). Then, for 14 days air was injected
157 with an oxygen partial pressure of 10 mbar (condition 2). Then, oxygen partial pressure was switched
158 to condition 3 (5 mbar) and 4 (2 mbar) successively. Two additional conditions with nitrogen alone
159 were used (conditions 5 and 6). The total gas flow rate was thus comprised 10 and 10.5 L/h, which
160 correspond to a gas retention time between 3000 and 3170 seconds on empty reactor volume (450
161 to 475 seconds gas residence time with an average gas holdup of 1.5%). The idea behind conditions 5
162 and 6 tests was to operate the reactor with a N_2 partial pressure similar to that used with oxygen in
163 order to check the influence of dilution effects on the performances with an inert gas. The duration of
164 each operation can be different based on several practical issues, but the criteria used was to get
165 several successive stable days of operation with all measurements available. The data used for mass
166 balance calculations (detailed in section 2.4) were taken during stable operation periods: constant
167 outlet gas flow rate and composition (usually 5 days, except for condition 5 where only 2 days data
168 have been used).

2.3 Analytical methods

For the batch tests, the gas composition was analyzed by gas chromatography (Global Analyser Solutions G.A.S, Netherlands) with thermal conductivity detector (GC-TCD). Molsieve 5A 0.32 mm ID (10 m length) and Rt-U-Bond (10 m length; 0.32 mm ID) columns were used as stationary phases for GC-TCD, with Argon as carrier gas, for both columns. Headspace pressure was measured with a precision electronic manometer (Digitron 2025P7).

For continuous tests, the composition of outlet gas was measured every 15 minutes by a micro gas chromatography (Inficon, Switzerland) with thermal conductivity detector (GC-TCD). Rt-Molsieve 5A 0.25 (12 m length) and RT-Q-Bond (12 m length; 0.25 mm ID) columns were used as stationary phases for GC-TCD, with Argon and Helium as carrier gases, respectively. Temperature and pH of liquid inside the reactor were measured on-line with a thermocouple type J (TC direct, France) and an easySense 31 pH probe (Mettler Toledo, USA), respectively. Redox potential was measured with a redox probe HI3210B/5 (Hanna Instruments, USA). The liquid output from biological water production and nutrient management was collected in a 2 L glass bottle and weighed on average twice a week.

2.4. Calculations and hypothesis

All calculations are performed on a molar basis. In the batch tests, the number of moles for each gas component is calculated from the ideal gas state equation:

$$n_i = \frac{p_i V_h}{RT} \quad (1)$$

Where p_i is the gas partial pressure in Pa (calculated from total pressure and gas composition measurement), V_h is the headspace volume in m^3 and T the temperature (K). For the continuous tests, a similar approach is used: when the reactor operation is stable, the cumulated inlet and outlet mole number of each gas was calculated through the cumulated volume, temperature, operating pressure and gas composition. Since the water composition of the gas is not measured, the total pressure used for the calculations is the dry pressure (corrected for water pressure at the operating temperature).

The global conversion efficiencies $X_{i,conv}$ of carbon dioxide and hydrogen are then calculated as follow:

$$X_{i,conv} = \frac{n_{i,in} - n_{i,out}}{n_{i,in}} \quad (2)$$

In this equation, $n_{i,in}$ is the initial (in batch mode) or inlet (in continuous mode) mole number of each gas, and $n_{i,out}$ is the final (in batch mode) or outlet (in continuous mode) mole number.

Similarly, the product (methane) yield (in $mol_{CH4}.mol_{H2}^{-1}$) was calculated as the ratio between the amount of methane produced divided by the amount of hydrogen consumed:

$$Y_{CH4} = \frac{n_{CH4,out}}{n_{H2,in} - n_{H2,out}} \quad (3)$$

In this expression, $n_{CH4,out}$ is the molar amount of methane produced and $n_{H2,in} - n_{H2,out}$ represent the amount of hydrogen converted.

Based on the results obtained, it appeared that the uptake of hydrogen could be associated not only to the methanogenic reaction, but also to the oxygen reduction reaction. In order to evaluate how hydrogen was used, we assumed that the following mechanisms took place:

- The catabolic reaction of hydrogenotrophic methanogenesis (equation 4);
- The catabolic reaction of oxygen reduction (equation 5);
- Other metabolic reactions (growth, maintenance).

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

$$2H_2 + O_2 \rightarrow 2H_2O \quad (5)$$

The production of methane during the tests enables to calculate the fraction of hydrogen used for methane production through reaction 1 ($f_{H2,CH4}$):

$$f_{H2,CH4} = \frac{4 \times n_{CH4,out}}{n_{H2,in} - n_{H2,out}} \quad (6)$$

Similarly, the uptake of oxygen enables to estimate the fraction of hydrogen used for oxygen reduction through reaction 2 ($f_{H2,O2}$), where $n_{O2,in} - n_{O2,out}$ is the molar amount of oxygen removed.

$$f_{H2,O2} = \frac{2\times(n_{O2,in}-n_{O2,out})}{n_{H2,in}-n_{H2,out}} \quad (7)$$

The remaining fraction of hydrogen removed $f_{H2,other}$ is thus considered as what is used for other metabolic routes:

$$f_{H2,other} = 1 - f_{H2,CH4} - f_{H2,O2} \quad (8)$$

If some of the hydrogen is used for oxygen reduction, then the observed methane yield defined in equation 3 should be lower than without oxygen. In order to take this effect into account, a correction was used to calculate the actual methane yield of the methanogenic reaction. With the assumption that 1 mole of oxygen consumed 2 moles of H_2, the corresponding amount of hydrogen available for methanogenesis is reduced (equation 9).

$$Y_{CH4,corr} = \frac{n_{CH4,out}}{(n_{H2,in}-n_{H2,out})-2\times(n_{O2,in}-n_{O2,out})} \quad (9)$$

For batch test, uncertainties were determined from the standard deviation between the replicates. For continuous operations, uncertainties were determined from the standard deviation of the measured quantities during stable operation periods.

3. Results and discussion

3.1 Batch test

Figure 2 presents the evolution of the headspace composition (amount of oxygen, methane, carbon dioxide and hydrogen conversion). Oxygen uptake began in the first 0.25 days of the experiment. The reactor's headspace was then fully depleted within 0.25 and 1 day for initial partial pressure of 3.8 mbar and between 1 and 2 days for initial oxygen partial pressure of 23 and 50 mbar. Methane production (Figure 2B) started after 0.25 days (at 0 mbar O_2), but seemed to be all the more delayed when the initial amount of oxygen increased. In the same time, H_2 uptake began earlier as shown in Figure 2C. It has to be mentioned that due to GC calibration issues associated to H_2 composition, there was an important uncertainty on the calculation of H_2 conversion efficiencies when the H_2 content in the headspace was high. It can also be noticed that the CO_2 uptake was in line with the

production of methane. The maximal methane production was reached after 2 days for the tests operated at 0 and 3.8 mbar initial P_{O2}, and after 3 days P_{O2} of 23 mbar (all the hydrogen being converted). The methane production was not maximal for the tests done at 50 mbar oxygen (only 90 % of the hydrogen was converted for this test after 3 days). Another interesting feature is the comparison between the amount of hydrogen consumed and the amount of methane produced. In spite of the calibration issues it was possible to calculate the methane yield Y_{CH4}, since the initial amount of hydrogen was known and the final amount was accurately measured. The amount of hydrogen consumed, methane produced and the calculated methane yield are presented in Table 3. The results show that the methane yield was directly affected by the presence of oxygen. It is dropping from 0.248 (without oxygen) to 0.209 (at 50 mbar initial P_{O2}). The values are consistent with the expected yield, since the stoichiometry of the catabolic methanogenesis reaction (equation 4) predicts a maximal yield of 0.25. This result shows that, in the presence of oxygen, some of the hydrogen was not used for methanogenesis. The decrease of the methane yield, together with the fact that oxygen was consumed, leads to the hypothesis that some of the hydrogen could be derived towards oxygen reduction (equation 5). In order to check this hypothesis, it was possible to recalculate the methane yield of the methanogenic reaction by correcting the amount of H_2 used for oxygen reduction, on order to get a corrected methane yield (equation 9). All values of the corrected metabolic methane yield of the methanogenesis reaction (last column in Table 3) fall in the same range, independently of the initial P_{O2} (between 0.239 and 0.248, with uncertainties ranging from 0.010 to 0.030). This means that in spite of the presence of oxygen, there were no specific disturbance of the methanogenic metabolism concerning methane production: only the observed methane yield (column 4 in Table 3) was affected due to use of hydrogen for oxygen reduction. The corrected methane yield was very similar to the methane yield of 0.24 estimated with pure culture of *Methanobacterium Thermoautotrophicum* (Schill et al., 1996), and with the value of 0.23 used in the model developed for thermophilic methanation in mixed culture (Grimalt-Alemany et al., 2020).

These results reveal two important features. First, oxygen was consumed quickly by the micro-organisms of the thermophilic mixed culture used, even after a long time of acclimatization under anaerobic conditions. Second, methane production was delayed in the presence of oxygen. In addition, it is highly probable that some of the microorganisms present in the culture were able to oxidize hydrogen in the presence of oxygen. As mentioned in section 2.2, the inoculum used for the batch tests came from the continuous reactor that has been operated for 600 days with H_2 and CO_2 (and no other energy source than hydrogen). It was thus expected that the microbial consortium was mostly enriched with thermophilic hydrogenotrophic archaea. It has been shown that some methanogenic species can survive in the presence of oxygen (Kiener and Leisinger, 1983), and others are even able to oxidize hydrogen (Tholen et al., 2007). In the present case however, it was difficult to tell if the aerobic oxidation of hydrogen was done by the dominant archaea or by other bacterial species. Indeed, some hydrolytic bacterial species present in the mixed culture might have the ability to use hydrogen aerobically. Nevertheless, it was clear that the onset of methane production was delayed. This shows either that hydrogen would preferentially be used through an aerobic metabolism, or that oxygen actually inhibits reversibly the methanogenesis reaction. Some authors have already noted that the rapid growth of aerobic facultative organisms would probably outcompete the methanogens for hydrogen uptake due to their higher specific activity and growth yield (Botheju and Bake, 2011; Pedizzi et al., 2016). Understanding the metabolism of the microbial community would require further investigations and additional metabolic tools. However, the question of the resilience of the methanogenic activity is addressed in the next section, where continuous tests results are presented.

3.2 Continuous test

The results of the continuous tests are summarized in Table 4. Condition 1 corresponds to the reference (no gas injected other than H_2 and CO_2). Conditions 2, 3 and 4 correspond to an additional

air sparging at oxygen partial pressures of 10, 5 and 2 mbar, respectively (at the working pressure of 1 bar, these values correspond to oxygen compositions of 1%, 0.5% and 0.2%, respectively). Conditions 5 and 6 are performed with nitrogen alone instead of air, to reproduce the same gas dilution effect as for conditions 2 and 3.

Hydrogen conversion was high in all tests (over 99%). However, H_2 conversion is slightly lower (98.8 ± 0.2%) for condition 2 (P_{O2}= 10 mbar). This slight difference does not necessarily mean that conversion has decreased due to the presence of oxygen. Indeed, this value is close to that obtained under the same conditions with nitrogen instead of air (condition 5, conversion 99.0 ± 0.3%) and could therefore be attributed to the dilution effect that could impact the hydrogen mass transfer efficiency by reducing its partial pressure (Jensen et al., 2021a).

The conversion efficiency of CO_2 and hydrogen, together with the methane composition in the outlet gas are plotted in Figure 3. The effect of the presence of oxygen on CO_2 conversion is obvious here. The rate of CO_2 conversion decreased linearly with the partial pressure of oxygen applied (from 98.8% for reference, to 93.7% for condition 2). Even if the pH conditions are not strictly identical (7.6 for reference and 7.3 for condition 2), they alone do not explain the difference observed.

Similarly, the methane composition of the outlet gas decreased with increasing oxygen partial pressure. In Figure 3, this composition was corrected to eliminate the presence of nitrogen (in variable amounts depending on the conditions). It should be noted that the methanation reaction (equation 4) is accompanied by a decrease in flow rate (5 moles of gas disappear for a single mole of gas produced). Consequently, the presence of nitrogen from the air leads to a variable nitrogen composition at the outlet (2.8, 12.1 and 20.8% for conditions 4, 3 and 2, respectively). This correction enables the results to be compared with each other, and shows that the reduction in methane content is noticeable for conditions 3 and 2 (5 and 10 mbar O_2). It should also be noted that in all tests, almost all the oxygen was consumed (Table 4). The presence of traces of oxygen (up to 241

315 ppm, condition 2) in the outlet gas is rather a residue due to the amount that has not transferred
316 into the liquid phase.

317 Another consequence of the presence of oxygen is the slight decrease in methane productivity, from
318 5.20 $NL.L^{-1}.d^{-1}$ for the control, to 5.02 for condition 2 (10 mbar O_2). Finally, it can be noted that, quite
319 logically, the oxido-reduction potential (ORP) rose in the presence of oxygen, which is a common
320 feature of micro-aerated anaerobic systems (Magdalena et al., 2022; Yu et al., 2020).
321 The intermediate conclusions that can be drawn from the continuous tests are as follows. First,
322 almost all the oxygen was consumed. Second, it appeared that almost all hydrogen was consumed,
323 while CO_2 conversion decreased concomitantly with the increase in oxygen partial pressure. Finally,
324 methane production decreased slightly. These findings confirm the results obtained in batch
325 operation. They also suggest that a metabolism other than methane production is associated with
326 hydrogen consumption.
327

328 **3.3 The fate of hydrogen in the presence of oxygen**

329 From both batch and continuous tests results, there was a correlation between the amount of
330 oxygen added (and consumed) and the amount of methane produced, while the hydrogen uptake did
331 not vary. It is thus likely that some of the hydrogen was used to reduce oxygen into water (reaction
332 5). Indeed, hydrogen is the only available energy source since the reactors are only fed with H_2, CO_2
333 and air. In addition, some methanogens are known to be able to reduce oxygen through the
334 modification of the F420 cofactor (involved in the methane production) into a F420H2 oxidase
335 (Seedorf et al., 2004). This protection mechanism seems to be associated with a stop of the
336 methanogenic activity, even if it is reversible (Tholen et al., 2007). In the present case, mixed culture
337 were used. It is thus difficult to tell if the thermophilic methanogens actually changed their
338 metabolism to reduce oxygen, or if other species present in the microbial consortium are responsible
339 for this O_2 conversion. A thorough study of the microbial population, focusing on potential shifts
340 within the communities, could provide further insights. However, it was noticed (Figure 2A) that the

341 oxygen uptake started immediately without any observable lag-phase in batch tests. In continuous
342 test (Table 4), the oxygen level was very low, suggesting also the ability of the consortium to use
343 oxygen.
344 Based on this hypothesis, hydrogen and oxygen uptake (on the one hand), and methane production
345 (on the other hand), were used to calculate how hydrogen was used among methane production,
346 oxygen reduction, or other anabolic activity (equations 6, 7 and 8). The results are presented on
347 Figure 4 in batch mode (left) and continuous mode (right). The common feature is that the fraction of
348 hydrogen used for methane production decreased with increasing oxygen partial pressure. At the
349 same time, the amount of hydrogen used for other anabolic activities remained stable (given
350 measurement uncertainties). Finally, the proportion of hydrogen used for oxygen reduction also
351 increased significantly to reach 15% at 50 mbar P_{O2} (batch tests). This fraction was plotted against the
352 applied oxygen partial pressure (Figure 5). Interestingly, it did not depend on the reactor operation
353 mode (batch or continuous). In addition, the fraction of hydrogen used for oxygen reduction varied
354 linearly with the oxygen partial pressure. This result strongly supports the hypothesis of H_2 utilization
355 for oxygen reduction.
356 In the two sets of experiments, the presence of oxygen at low amounts (below 50 mbar of partial
357 pressure) did not affect the anaerobic metabolism of hydrogenotrophic methanogens in thermophilic
358 methanation mixed culture. However, based on mass balance calculations, it was demonstrated that
359 oxygen was consumed. In the same time, there were evidence that some of the hydrogen present in
360 the system was preferentially used for oxygen reduction. The consequence for practical applications
361 is that the applied amount of hydrogen should be increased to make sure that all the CO_2 can be
362 converted into methane. According to the data presented (Figure 5), in a biomethanation reactor
363 operating at 10 bars with 0.2% oxygen in the inlet gas (20 mbar O_2), 6% of the hydrogen would be
364 derived for oxygen reduction. It means that the H_2/CO_2 ratio should be increased from 4/1
365 (stoichiometric value) to 4.24/1 in order to reach the methane yield and productivity that would be

obtained without oxygen. Even if this increase is limited, the impact of the presence of oxygen traces has to be accounted for in the design and operation of biological methanation units.

4. Conclusions

The presence of oxygen (at partial pressures below 50 mbar) did not stop biological methanation. Oxygen was rapidly consumed. The uptake of hydrogen remained unchanged, while methane production and CO_2 uptake decreased at higher oxygen pressure. Molar balance showed that up to 15% of the hydrogen was used to reduce oxygen. This fraction increased linearly with oxygen partial pressure. Low amounts of oxygen in biomethanation system are thus tolerable. However, to counterbalance the amount of hydrogen diverted to reduce oxygen, it is necessary to increase the H_2/CO_2 ratio for maintaining a satisfactory methane productivity and CO_2 conversion.

E-supplementary data for this work can be found in e-version of this paper online.

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Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used DeepL in order to improve the readability and language of the manuscript. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the published article.

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Supplementary data

Table S1: Gas input composition for a biomethanation plant from CO_2-rich off-gas (column 2) or raw biogas (column 3). The data are from a digester producing 91 Nm^3/h biomethane (Ariège Biomethane, Arkolia Energies, France).

Compound	Off-gas	Raw biogas
CO_2 (%)	98.90	44.5
CH_4 (%)	0.80	55.0
O_2 (%)	0.20	0.1
N_2 (%)	0.01	0.4
H_2O (%)	0.18	0.8
H_2S (ppm)	<10	100

Table S2: Inoculum elemental content in the soluble phase measured by ICP-MOES.

Element	Concentration (mg/L)
S	54.3
Na	60
K	78
P	32
Ca	46
Mg	3.88
Fe	0.820
Ni	0.670
Co	0.120
Zn	1.19
Cu	1.370
Se	0.189

501 Table S3: Composition of nutrient solutions S1 and S2 for continuous reactor

Solution	Compound	Concentration (mg/L)
S1	NH_4OH	8045.5
	K_2HPO_4	319.1
	$CaCl_2,2H_2O$	320.0
	$MgCl_2,6H_2O$	63.6
	$FeCl_3$	8.3
	$NiCl_2,6H_2O$	6.7
	$CoCl_2,6H_2O$	1.60
	$Na_2O_3Se,5H_2O$	3.3
	$Na_2O_4W,2H_2O$	5.80
S2	$Na_2S,9H_2O$	360

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