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Microbial protein production from sulfide-rich biogas through an enrichment of methane- and sulfur-oxidizing bacteria

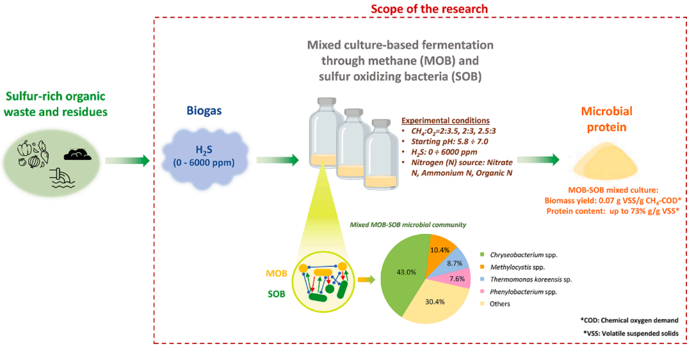
Marica Areniello [a,*], Silvio Matassa [b], Giovanni Esposito [b], Piet N.L. Lens [a]

[a] *University of Galway, University Road, Galway H91 TK33, Ireland*
[b] *Department of Civil, Architectural and Environmental Engineering, University of Naples Federico II, Via Claudio, Naples 80125, Italy*

HIGHLIGHTS

- Microbial protein (MP) production from sulfide-rich biogas was demonstrated.
- Mixed cultures of methane (MOB) and sulfur oxidizing bacteria (SOB) were developed.
- The effect of CH_4:O_2 ratio, pH, sulfide and nitrogen sources was evaluated.
- The MOB-SOB culture could withstand up to 26.9 mg Na_2S/L (4000 ppm of H_2S).
- Good biomass yields and high protein contents were achieved with 1500 ppm of H_2S.

GRAPHICAL ABSTRACT



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ABSTRACT

This study evaluated the possibility of combining methane oxidizing bacteria (MOB) with sulfur oxidizing bacteria (SOB) to enable the utilization of sulfide-rich biogas for microbial protein production. For this purpose, a MOB-SOB mixed-culture enriched by feeding both methane and sulfide was benchmarked against an enrichment of solely MOB. Different CH_4:O_2 ratios, starting pH values, sulfide levels and nitrogen sources were tested and evaluated for the two enrichments. The MOB-SOB culture gave promising results in terms of both biomass yield (up to 0.07 ± 0.01 g VSS/g CH_4-COD) and protein content (up to 73 ± 5% of VSS) at 1500 ppm of equivalent H_2S. The latter enrichment was able to grow also under acidic pH (5.8–7.0), but as inhibited outside the optimal CH_4:O_2 ratio of 2:3. The obtained results show the capability of MOB-SOB mixed-cultures to directly upcycle sulfide-rich biogas into microbial protein potentially suited for feed, food or biobased product applications.

1. Introduction

The pressing need to move away from the exploitation of non-renewable resources is driving the so-called circular economy transition, where wastes and residues are regarded as abundant feedstock to replace conventional raw materials (Venkata Mohan et al., 2019). As a matter of fact, waste streams represent an untapped source of energy and nutrients that can be recovered through closed-loop

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* Corresponding author at: Department of Microbiology and Ryan Institute, School of Natural Sciences, University of Galway, University Road, Galway H91 TK33, Ireland.

E-mail address: m.areniello1@universityofgalway.ie (M. Areniello).

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cascading bioprocesses, thereby enabling the shift towards a more sustainable and low-carbon bioeconomy (Mohan et al., 2016).

Among the various bioeconomy sectors seeking to improve their circularity, food production needs to do so while meeting the increasing food demand of an expanding and richer global population (FAO, 2017), particularly in terms of protein-based products (Aiking, 2011). In this scenario, the conversion of waste-derived biogas into high-value products, such as microbial protein (MP), enables the production of alternative protein with a lower environmental footprint as compared to plant- or animal-based sources, and which can be used to replace the latter in the feed, food or agronomic sectors (Areniello et al., 2022).

Biogas contains 50–70% of methane, which can serve as energy and carbon source for the production of protein-rich microbial biomass (Angelidaki et al., 2018). Aerobic methane oxidizing bacteria (MOB), also known as methanotrophs, utilize methane as carbon and energy substrate, and are prime candidates for MP production thanks to their high biomass yield and productivity (Strong et al., 2015). MP production from natural gas, i.e. the fossil-fuel counterpart of biomethane, is indeed a well-established and commercially available technology (Banks et al., 2022). In order to move away from the utilization of fossil fuels and enable renewable MOB-based MP production from biogas, several challenges still need to be addressed, e.g., its higher production costs with respect to that of the fossil counterparts.

Recent research has focused on the possibility of turning raw biogas into MP to avoid the expensive step of biogas upgrading, mainly by investigating the effect of CO_2 or that of recovered nitrogen sources, such as anaerobic digestates which are rich in ammonium nitrogen (Acosta et al., 2020; Tsapekos et al., 2019). Nevertheless, more efforts are required to evaluate the suitability of raw biogas streams that are rich in trace contaminants such as hydrogen sulfide (H_2S). H_2S can be present in biogas with concentrations between 500–5000 ppm (Cherosky and Li, 2013), and even after the desulfurization unit its concentrations can still be as high as 200 ppm (Xu et al., 2020). H_2S constitutes a problem for biogas valorization since it may inhibit bacterial activity by limiting cell respiration, with relevant inhibitory effects occurring already at concentrations close to 1000 ppm, especially when performed through conventional mono-culture fermentation processes (Xu et al., 2020).

In this scenario, shifting from a pure towards a mixed-culture based fermentation approach could allow exploiting the cooperative interactions established between the different microbial actors, thereby enabling a more efficient utilization of substrates and the removal of potentially inhibitory compounds (Ghosh et al., 2016). As a matter of fact, besides being a potential inhibitory compound, H_2S could represent also a valuable energy and nutrient source for MP production.

A cooperative interaction could thus arise between MOB and SOB as the H_2S oxidation performed by SOB could support the MOB in withstanding higher H_2S concentrations and, on the other hand, SOB could utilize as carbon source either the CO_2 (autotrophic SOB) or the organic metabolites (heterotrophic SOB) produced by MOB during methane oxidation (Pokorna and Zabranska, 2015). Indeed, the combination of methanotrophs and heterotrophs has already been shown beneficial (Nunes et al., 2016), as the heterotrophs help mitigating the potential inhibitory The developed MOB-SOB s of metabolites on the growth of methanotrophs (Bothe et al., 2002; Ho et al., 2014). At the same time, the association of methanotrophs and autotrophic SOB could help increasing the carbon capture potential, therefore maximizing the valorization of the carbon present in the biogas.

With the aim of enabling the conversion of sulfide-rich biogas into MP, the present study investigated the potential of MOB and SOB to coevolve in a well performing MOB-SOB mixed culture. To this end, a long-term enrichment of the microbial consortium was performed by using a biomethane-air gas mixture in the absence or in the presence of sodium sulfide salts (Na_2S) as source of sulfide, thus promoting the enrichment of MOB alone or in combination with SOB, respectively. Upon obtaining stable enrichments, several batch cultivation tests were carried out to identify the most optimal operating parameters in terms of CH_4:O_2 ratio, starting pH value, sulfide level and nitrogen source. The MOB-SOB enriched culture was benchmarked against the biological performance of MOB alone, by monitoring gas consumption rate, biomass growth, biomass yield and protein content.

2. Materials and methods

2.1. Enrichment of MOB and MOB-SOB consortia

The material sampled from the bed of a biological desulfurization filter was used as inoculum source of MOB and SOB. The filter serves a full-scale anaerobic digestion plant treating agri-food waste and is operated by Acqua & Sole Srl (Vellezzo Bellini, Italy). Two separate microbial enrichments were developed in parallel under the following conditions: *a)* MOB: no addition of sulfide, *b)* MOB-SOB: addition of sulfide. For this purpose, one serum bottle, sealed with a butyl rubber stopper and an aluminum crimp, was prepared for each enrichment. The total volume of each serum bottle was 315 mL, with a working volume of 40 mL. Each serum bottle was initially inoculated with 10 g of the same inoculum. A nitrate mineral salts (NMS) medium supplemented with a micronutrient solution (0.1% v/v), prepared according to Sun et al. (2018), was used as nutrient source for the growth of the microorganisms. To stabilize the pH during the experiments, a phosphate buffer solution of 0.272 g/L of KH_2PO_4 and 0.284 g/L of Na_2HPO_4 (Sigma Aldrich, USA) was added to the NMS medium. Following the procedure adopted by Xu et al. (2020), sodium sulfide salts were used as source of sulfide in the experiments. Thus, 31.05 mg/L of sodium sulfide nine hydrate ($Na_2S\cdot 9H_2O$) (Sigma Aldrich, USA), equivalent to 10.09 mg/L of Na_2S, was diluted in the liquid medium of the MOB-SOB enrichment to simulate a H_2S concentration of 1500 ppm in the headspace. The latter concentration was chosen as representative of an averagely high H_2S concentration in biogas (Cherosky and Li, 2013). The required Na_2S dosage was calculated, as proposed by Xu et al. (2020), through the dissolved H_2S dissociation equilibrium.

Biomethane collected by treating the biogas from a lab-scale anaerobic digester through NaOH absorption was used as methane source. CO_2 was removed from the biogas, although not necessary in the envisioned real scale application of the raw biogas-to-MP process, in order to enable monitoring the amount of CO_2 evolving from the biomethane in the presence or in the absence of sulfide.

The headspace (215 mL) of each bottle containing air was supplemented with biomethane to achieve a CH_4:O_2 ratio of 2:3 (v/v). The serum bottles were incubated in a thermostatic bath at 30 °C and shaken at 120 rpm to favor the solubilization of the gas in the liquid phase. The enrichments were performed by exchanging half of the liquid culture volume with fresh medium, adjusting the pH to 7.0 (±0.1) through 1 M sodium hydroxide (Sigma Aldrich, USA) addition and by refreshing the gas headspace on a weekly basis. Once stable conditions in terms of both gas consumptions and biomass growth were reached, the enrichments were maintained in the same conditions described above and used as inoculum for the batch tests.

2.2. Batch screening tests

Batch screening cultivation tests were performed to evaluate the influence of the CH_4:O_2 ratio, starting pH value, Na_2S concentration and nitrogen source on gas consumption, biomass growth and protein content of the MOB and MOB-SOB enrichments (section 2.1). Each batch test lasted 96 h and was performed in triplicate within 150 mL serum bottles sealed with butyl rubber stoppers and aluminum crimps. Each bottle was operated with a 10% (v/v) working volume, resulting in a liquid culture of 15 mL and a headspace of 135 mL. Each bottle was inoculated with 2.3 mL of active inoculum sampled from the enrichments, incubated at 30 °C and shaken at 120 rpm.

Based on previous research (AlSayed et al., 2018; Xu et al., 2020;

Zhang et al., 2016), a series of parameters, summarized in Table 1, were tested for both enrichments (MOB and MOB-SOB). Briefly, the effect of three CH_4:O_2 ratios (2:3, 2.5:3 and 2:3.5), four initial pH values (7.0, 6.7, 6.4 and 5.8), three nitrogen sources (Nitrate, Ammonium, Urea) and five Na_2S concentrations (0, 10.09, 20.18, 26.91 and 40.37 mg/L, equivalent to 0, 1500, 3000, 4000 and 6000 ppm in the gas phase, respectively) were tested to identify the optimal growth conditions and to compare the performance of the two enrichments. The tests were carried out by using the same NMS medium, micronutrient solution and phosphate buffer already described for the microbial enrichment (section 2.1). The nitrogen source test was performed by replacing nitrate with ammonium chloride as source of ammonium nitrogen, and urea as source of organic nitrogen. The initial pH of each bottle was adjusted to 7 by dosing 1 M NaOH or 1 M HCl (Sigma Aldrich, USA), while different phosphate buffer compositions were used to set lower starting pH values (see Supplementary Material).

2.3. Sampling and analytical methods

The pressure of each bottle was monitored daily by using a digital manometer (Leo 1, Keller, Switzerland). The gas headspace was sampled in all triplicate bottles at the start and at the end (96 h) of each test, while a single bottle per triplicate was alternatively sampled at 24, 48 and 72 h. The gas samples were analyzed through a Star 3400 gas chromatograph (Varian, Palo Alto, USA) equipped with a ShinCarbon ST80/100 (Restek, Bellefonte, USA) column and a thermal conductivity detector. Argon was used as the eluent gas. To restore the gas content, 6 mL of the initial methane-air mixture were injected in each bottle after gas sampling.

Liquid samples from each serum bottle were taken at the beginning and at the end of each batch test. The pH measurements were performed on the unfiltered samples through a WTW Multi 3410 instrument equipped with a SenTix® 940 pH electrode (WTW, Weilheim in Oberbayern, Germany). Soluble compounds, i.e. chemical oxygen demand (COD), nitrates and ammonium nitrogen, were measured by first centrifuging and filtering the samples through 0.2 μm Millipore Millex® PTFE syringe filters (EMD Millipore, Burlington, USA). The COD, which was measured spectrophotometrically according to the Standard Methods (APHA, 2005), was analyzed both as soluble (sCOD) and total COD (tCOD). The latter was measured on the uncentrifuged and unfiltered samples. Nitrates were measured through ion chromatography (IC) using a Metrohm 883 Basic IC Plus system equipped with a Metrosep A Supp 5–150/4.0 column (Metrohm, Herisau, Switzerland), total ammonium nitrogen (TAN) was determined spectrophotometrically through the indophenol blue method (Aminot et al., 1997), while total organic nitrogen was determined through Kjeldahl analysis according to Standard methods (APHA, 2005). Liquid samples were taken at the end of each test, stored at −20 °C and then thawed at 50 °C for the subsequent protein determination according to Lowry et al. (1951). The measured protein concentration (g/L) was then divided by the final biomass concentration (g VSS/L) to determine the biomass protein content (g protein/g VSS).

Table 1
Overview of the operating condition tested in the batch screening tests.

Test	Variable	MOB enrichment	MOB-SOB enrichment
Gas composition	CH_4:O_2	2:3.5; 2:3; 2.5:3	
	Initial pH	7.0	
	N-source	Nitrate	
	Na_2S	0 mg/L	10.09 mg/L
	H_2S equivalent	0 ppm	1500 ppm
pH	CH_4:O_2	2:3	
	Initial pH	5.8; 6.4; 6.7; 7.0	
	N-source	Nitrate	
	Na_2S	0 mg/L	10.09 mg/L
	H_2S equivalent	0 ppm	1500 ppm
Sulfide concentration	CH_4:O_2	2:3	
	Initial pH	7.0	
	N-source	Nitrate	
	Na_2S	0 mg/L; 10.09 mg/L; 20.18 mg/L; 26.91 mg/L; 40.37 mg/L	
	H_2S equivalent	0 ppm; 1500 ppm; 3000 ppm; 4000 ppm; 6000 ppm	
Nitrogen source	CH_4:O_2	2:3	
	Initial pH	7.0	
	N-source	Nitrate; ammonium; urea	
	$Na_2S\cdot 9H_20$	0 mg/L	10.09 mg/L
	H_2S equivalent	0 ppm	1500 ppm

2.4. Microbial community analysis

Samples from the enrichment batch tests taken at the start (day 7) and at the end (day 250) of the enrichment tests, and stored at −20 °C, were used to monitor the composition and the evolution of the cultures by means of microbial community analysis. DNA extraction, sequencing and bioinformatics analysis were performed by Fisabio (Valencia, Spain) as described by Oliva et al. (2022). Briefly, samples were homogeneized and centrifuged, DNA extraction was done on the pellet with MAgNa Pure LC robot and a III 3,264,785,001 isolation kit (Roche, Basel, Switzerland), and a Qubit dsDNA High Sensitivity kit (Qiagen, Hilden, Germany) was used for DNA quantification. The Illumina protocol (Illumina, 2013) was followed to perform polymerase chain reaction (PCR) amplification, sequencing, and PCR cleanup to target the V3 and V4 regions of the 16S genes. Silva138 was used as database for taxonomic assignation.

2.5. Statistical analysis

The results were expressed as the mean ± standard deviation of each experimental condition performed in triplicate. The statistical analysis of the experimental data was performed through the MINITAB® software (version 21.3.1, Minitab Inc., State College, USA). General linear models with a confidence interval of 95% were used to perform the analysis of variance (ANOVA). The experimental results were considered significantly different when the *p-value* was below or equal to 0.05 ($p\text{-value} \leq 0.05$).

2.6. Calculations

The COD measurement was used as proxy of carbon fixation and biomass growth within the tests. The difference between total and soluble COD was used to estimate the biomass concentration as follows:

$$\text{Biomass concentration (mg VSS/L)} = \frac{\text{tCOD (mg/L) - sCOD (mg/L)}}{\text{1.42 (mg COD/mg VSS)}} \tag{1}$$

where 1.42 mg COD/mg VSS represents a factor commonly used to convert the COD fraction that is due to microbial biomass into volatile suspended solids (VSS) (Irvine and Bryers, 1985).

The biomass yield was used to evaluate the biomass growth through methane oxidation and was calculated as follows:

$$\text{Biomass yield (g VSS/g CH}_4\text{-COD)} = \frac{\text{Net biomass growth (g VSS/L)}}{\text{CH}_4\text{-COD}_{\text{consumed}}\ \text{(g CH}_4\text{-COD/L)}} \tag{2}$$

where the net biomass growth was calculated as difference between the final and initial biomass content, while the CH_4-$COD_{consumed}$ was calculated by multiplying the moles of methane consumed within each test by a conversion factor of 64 g COD/mol CH_4.

The maximum methane consumption rate ($\nu_{\text{m-max}}$) was evaluated by considering the maximum daily methane consumption calculated for each experimental condition. Since the triplicate gas measurements were performed only at the start and at the end of the test, the $\nu_{\text{m-max}}$ calculated at 24, 48 and 72 h could not be expressed as mean ± standard deviation.

The nitrogen balance was used to benchmark the nitrogen consumption, measured experimentally, against the theoretical nitrogen assimilated and converted into MP.

The nitrogen consumption was calculated as follows:

$$\text{N-consumption (mg)} = (\text{N})_{\text{i}} - (\text{N})_{\text{f}} \tag{3}$$

were $(\text{N})_{\text{i}}$ and $(\text{N})_{\text{f}}$ represent, respectively, the initial and residual nitrate-nitrogen (N-NO_3^-), ammonium nitrogen (N-NH_4^+) or organic nitrogen.

The theoretical nitrogen assimilated and converted into MP was instead calculated as follows:

$$\text{N biomass (g)} = \text{Net biomass growth (g VSS)} \cdot \text{\% Protein (g protein/g VSS)} \cdot 0.16 \text{ (g N/g protein)} \tag{4}$$

where 0.16 g N/g protein represents the average nitrogen content of protein (Krul, 2019).

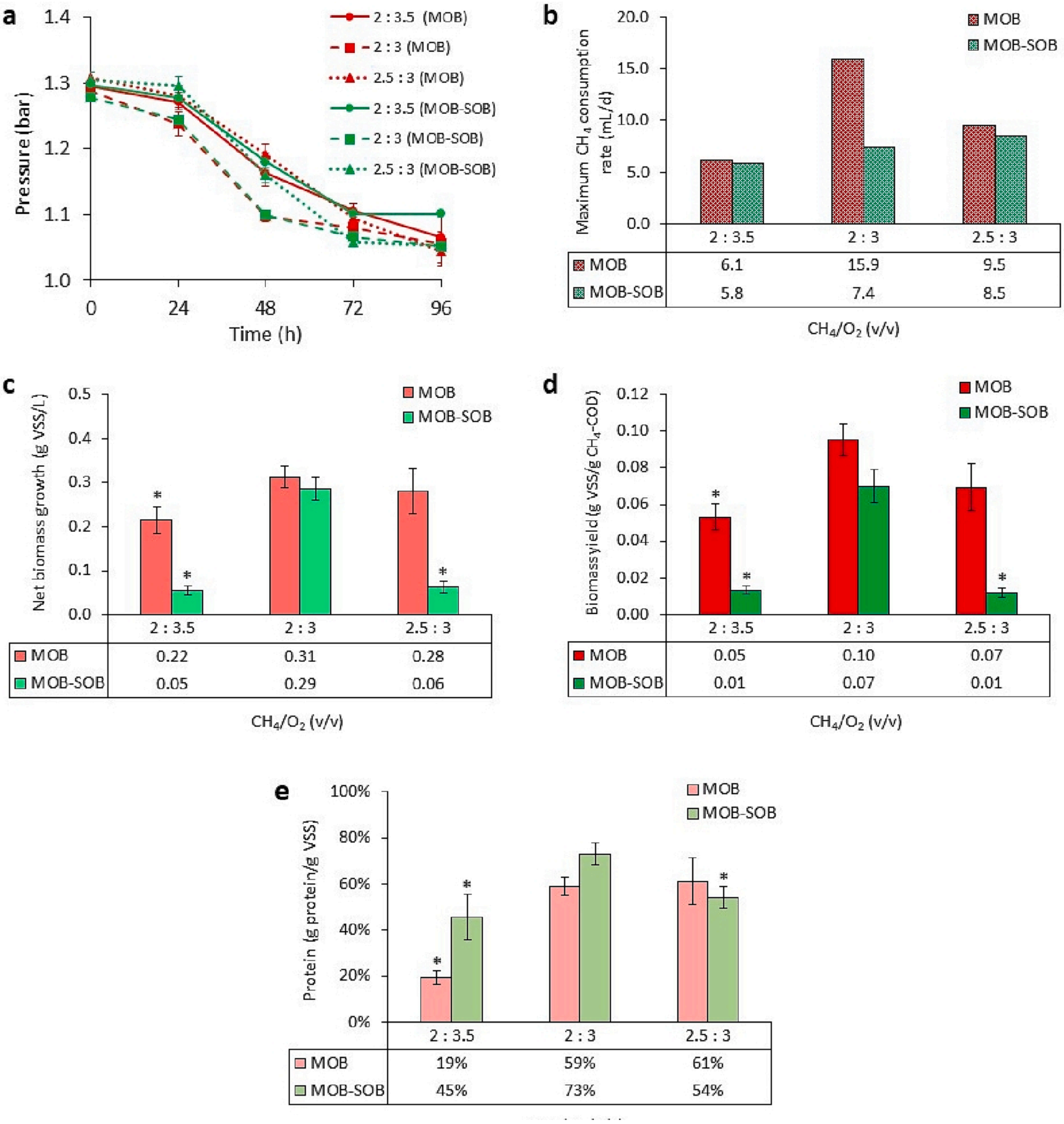


Fig. 1. Effect of the CH_4:O_2 ratio on: a) pressure trend, b) maximum methane consumption rate, c) net biomass growth, d) biomass yield on methane, and e) biomass protein content. *: Significantly different values (*p-value* ≤ 0.05).

3. Results and discussion

3.1. Effect of CH_4:O_2 ratio

The CH_4:O_2 ratio is known to affect the methane oxidation process and its stoichiometry (Tsapekos et al., 2019), and thus represents an important process parameter to be carefully considered and evaluated. Therefore, in view of the direct upcycling of raw biogas into MP, the variability of the methane content, resulting in the variability of the methane/oxygen ratio during the fermentation process, must be taken into account. Also, the explosive nature of the CH_4/O_2 mixture imposes the need to operate the process with a methane content always outside the 5–15% range, which represent, respectively, the lower and upper explosion limits of methane in air (Casson Moreno et al., 2018). In view of the above, the influence of different CH_4:O_2 ratios on the MOB and MOB-SOB enrichments during MP production was studied.

In terms of gas consumption, the pressure trends (Fig. 1a) show that, for all the conditions tested, a first acclimation phase was required by both enrichments, as demonstrated by the negligible pressure drop that occurred during the first 24 h of each test. Most of the gas consumption occurred between 24 and 72 h in all the tested conditions. In the case of a CH_4:O_2 ratio of 2:3, a milder initial lag phase was followed by the highest $\nu_{\text{m-max}}$ of 15.9 and 7.4 mL CH_4/d for the MOB and MOB-SOB cultures, respectively (Fig. 1b).

The analysis of the COD values (see Supplementary Material) shows how the CH_4:O_2 ratio of 2:3 also led to the highest ratio between tCOD:sCOD, thereby confirming the importance of this parameter for the maximization of the anabolic activity, especially in the presence of sulfide. Indeed, despite the gas consumption was comparable to the other conditions, low net biomass growths were detected with both the highest and the lowest CH_4:O_2 ratio invetigated (Fig. 1c). The latter is reflected by the significantly lower (*p-value* < 0.05) tCOD:sCOD ratios (more than 11 times lower) measured with both the highest and lowest CH_4:O_2 ratios, which suggest the predominance of catabolic activity towards the production of soluble metabolites under those conditions.

The CH_4:O_2 ratio of 2:3 was also optimal for the biomass yield (Fig. 1d), and especially for the MOB-SOB inoculum enriched and cultivated with sulfide, which could not effectively grow under the other test conditions. Indeed, significantly lower yields (*p-value* < 0.05) were reached with low (both enrichments) and high (only for MOB-SOB) CH_4:O_2 ratios with respect to the yields of 0.10 (±0.01) and 0.07 (±0.01) g VSS/g CH_4-COD achieved at a CH_4:O_2 ratio of 2:3 for the MOB and MOB-SOB enrichments, respectively. The latter results are in accordance with data from the literature showing that, stoichiometrically, a mixture of oxygen and methane at a ratio of 1.45 v/v is required to aerobically bio-oxidize natural gas (Khoshnevisan et al., 2019). Furthermore, a comparable biomass yield on methane (0.10 g VSS/g CH_4-COD) was achieved at a CH_4:O_2 ratio of 2:3.7 in a previous study investigating a membrane reactor for methanotrophic biomass cultivation (Valverde-Pérez et al., 2020).

Along with anabolic activity and biomass yield, the CH_4:O_2 ratio of 2:3 maximized also the protein production (Fig. 1e), particularly for the MOB-SOB enrichment, which achieved a significantly higher protein content of 73 (±5)% (*p-value* < 0.05) under this gas feeding condition. The latter value is notably higher than, for instance, the 53% obtained in previous studies investigating the semi-continuous fermentation of methanotrophs at the same CH_4:O_2 ratio (Tsapekos et al., 2020). On the other hand, increasing the CH_4:O_2 ratio did not produce any significant effect (*p-value* < 0.05) on the protein content of the MOB enrichment cultivated without sulfide, as values of 59 (±4)% and 61 (±10)% were reached for the 2:3 and 2.5:3 ratio, respectively. Conversely, decreasing the CH_4:O_2 ratio led to a significant decrease (*p-value* < 0.05) in the protein content also for the MOB enrichment (19 ± 3%). Similar negative effects of the excess presence of oxygen over methane on protein accumulation were reported for a mixed culture of *Methylomonas* and *Methylophilus* spp., which reached a protein content of ~ 42% on a VSS basis when cultivated with a 2:4 CH_4:O_2 ratio (Zha et al., 2021).

3.2. Effect of starting pH

The pH is among the most critical process parameters as the optimal range for microbial growth is often quite narrow. This is particularly relevant for methane oxidation as the medium tends to acidify due to the generation of CO_2 (Xu et al., 2020). Indeed, higher biomass yields were detected when methanotrophs were cultivated by manually adjusting the initial pH to limit the consequences of CO_2 acidification (Khoshnevisan et al., 2019). Moreover, the pH plays a critical role in the presence of sulfide as it affects the equilibrium between undissociated (H_2S) and dissociated (HS^- and S^{2-}) sulfide in the liquid phase. Sulfide speciation is highly relevant for microbial activity as undissociated sulfide diffuses through the cell membrane and causes severe inhibition (Pokorna and Zabranska, 2015). In view of this, different starting pH values were tested to evaluate the influence of acidic pH conditions on methanotrophic protein production.

In terms of gas consumption, the initial pH value did not influence the process of methane oxidation. As shown in Fig. 2a, a lag phase of 24 h was present in all test conditions, followed by a rapid gas depletion between 24 h and 48 h. The highest $\nu_{\text{m-max}}$ of 15.9 mL CH_4/d was observed at pH 7.0 for the MOB culture (Fig. 2b), while the MOB-SOB culture achieved its $\nu_{\text{m-max}}$ of 12.8 mL CH_4/d at pH 5.8, indicating that the combination of acidic pH and sulfides did not negatively influence the process of aerobic methane oxidation.

Concerning the net biomass growth and biomass yield (Fig. 2c and 2d), only the MOB culture was significantly affected (*p-value* < 0.05) by the lower initial pH values of 6.4 and 5.8, as net biomass growth values were 45 and 26% lower, respectively, and the yield values were 50 and 39% lower, respectively, than the values achieved at a neutral pH. The latter result points toward the inability of the selected methanotrophic strains to withstand acidic pH values. Indeed, the activity of methane monooxygenase (MMO), the enzyme responsible for methane oxidation in methanotrophs, is higher at neutral and alkaline pH conditions than at acidic ones (Park and Kim, 2019).

Despite this generally accepted assumption, the optimal pH value for methane oxidation is highly dependent on the microbial species involved and, according to the literature, more than 90% methanotrophs prefer to grow in pH ranges from 5.5 to 8 (AlSayed et al., 2018). Indeed, while *Methylococcus capsulatus* showed a significantly suppressed growth when the pH of the medium was below 7.0, other more acidophilic species such as *Methylocapsa acidiphila* displayed maximum growth at pH 4.5–5.8 (Xu et al., 2020). Similarly, Càceres et al. (2014) obtained comparable results in terms of biomass growth when cultivating *Methylomicrobium album* and *Methylocystis* sp. at pH 6.0 and 6.8.

The lower starting pH values did not significantly affect (*p-value* $<$ 0.05) the biomass yields of the MOB-SOB culture, which ranged between 0.06 (±0.01) and 0.07 (±0.03) g VSS/g CH_4-COD, showing a higher versatility in terms of pH. However, while pH did not significantly affect the protein content of the MOB culture (from 40 ± 7 to 59 ± 4%) (Fig. 2e), the acidic pH values led to a consistent decrease in the protein content of the MOB-SOB culture, where the protein content of 40 (±2)% and 45 (±7)% were observed with pH values of 5.8 and 6.4, respectively, which were significantly (*p-value* < 0.05) lower than the maximum value of 73 (±5%) obtained at pH 7.0.

3.3. Effect of sulfide concentration

To evaluate the potential of the direct valorization of raw biogas, several Na_2S concentrations were tested to assess the influence of different H_2S concentrations on the bioprocess performance of both the unacclimated enrichment (MOB) and the one enriched in the presence of 10.09 mg Na_2S/L (MOB-SOB).

Fig. 3 shows how the elevated sulfide concentration affected the overall biological process performance. The increasing H_2S

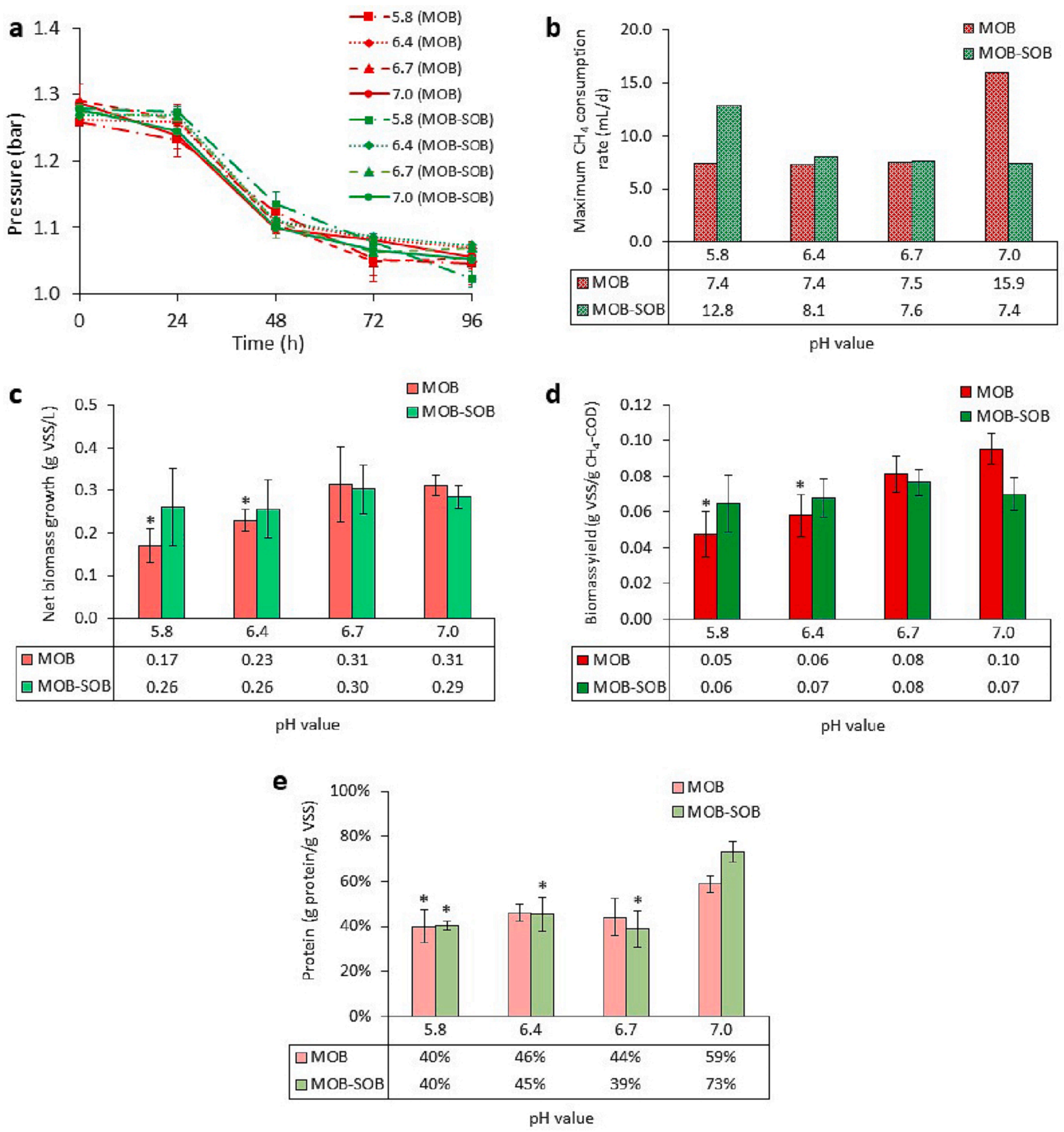


Fig. 2. Effect of the starting pH on: a) pressure trend, b) maximum methane consumption rate, c) net biomass growth, d) biomass yield on methane, and e) final protein content. *: Significantly different values (*p-value* ≤ 0.05).

concentration led to a lower gas consumption in the MOB culture, as a significantly lower pressure drop was detected in the presence of 40.37 mg Na_2S/L (6000 ppm of H_2S) (Fig. 3a). The negative effects of the presence of sulfide on the unacclimated MOB culture were already starting from 10.09 mg Na_2S/L (1500 ppm of H_2S) (Fig. 3b, 3c and 3d). Indeed, the biomass yields decreased significantly (*p-value* < 0.05) by 61, 49, 50 and 66%, in the presence of 10.09, 20.18, 26.91 and 40.37 mg Na_2S/L (1500, 3000, 4000 and 6000 ppm of H_2S), respectively, with respect to the yield of 0.10 (±0.01) g VSS/g CH_4-COD obtained without Na_2S. The COD measurements (see Supplementary Material) confirm this result as the tCOD:sCOD ratio significantly decreased (*p-value* < 0.05) when sulfide was added to the MOB culture: 76%, 68%, 69%, and 83% lower ratios with 10.09, 20.18, 26.91 and 40.37 mg Na_2S/L, respectively, with respect to the ratio obtained without Na_2S.

These results are in line with those reported by Xu et al. (2020) who observed how the presence of sulfide led to a progressively stronger inhibition of the growth of *Methylocapsa acidiphila,* with the inhibition in terms of biomass growth occurring already at 1000 ppm of H_2S equivalent. Additional research works further demonstrated how the methanotrophic activity is clearly inhibited even at lower H_2S concentrations of 200–1000 ppm (Lee et al., 2011; Tsapekos et al., 2019; Zhang et al., 2016). Therefore, the extent of H_2S inhibition varies among different methanotrophic cultures, and strongly depends on the microbial species present. Càceres et al. (2014) observed the inhibition of *Methylomicrobium album* and *Methylocystis* sp. already in the presence of 500 ppm of H_2S, which led to a 37–85% reduction of microbial growth. A higher resistance to sulfide was previously reported when cultivating a H_2S-tolerant strain of *Methylocaldum gracile* which showed a decreased biomass growth of 17% at 500 ppm. The same microorganism was, nevertheless, completely inhibited at 3000 and 5000 ppm of H_2S with no or minimal cell growth (Zhang et al., 2016).

The mixed MOB-SOB culture enriched with sulfide in the present study demonstrated a high tolerance towards H_2S, achieving a biomass yield of 0.06 (±0.00) VSS/g CH_4-COD at concentrations as high as 4000 ppm (26.91 mg Na_2S/L), which was comparable to that measured with lower or no sulfide presence (Fig. 3d). A 53% decrease of the biomass yield occurred instead at 6000 ppm, indicating the onset of severe H_2S inhibition also on the acclimated MOB-SOB enrichment. These results suggest that the enrichment and acclimation procedure used to develop the MOB-SOB culture was effective in overcoming the limitations linked

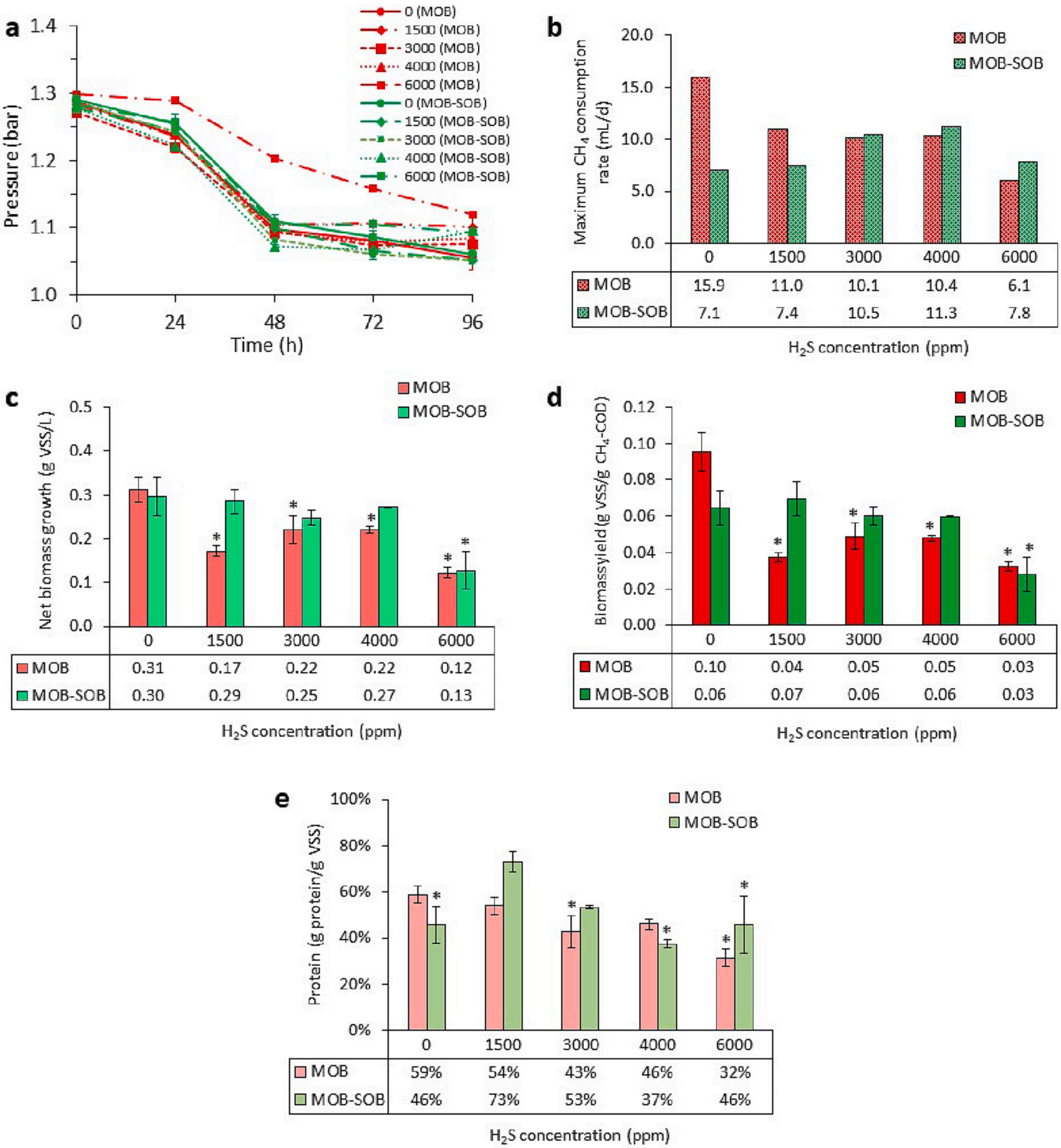
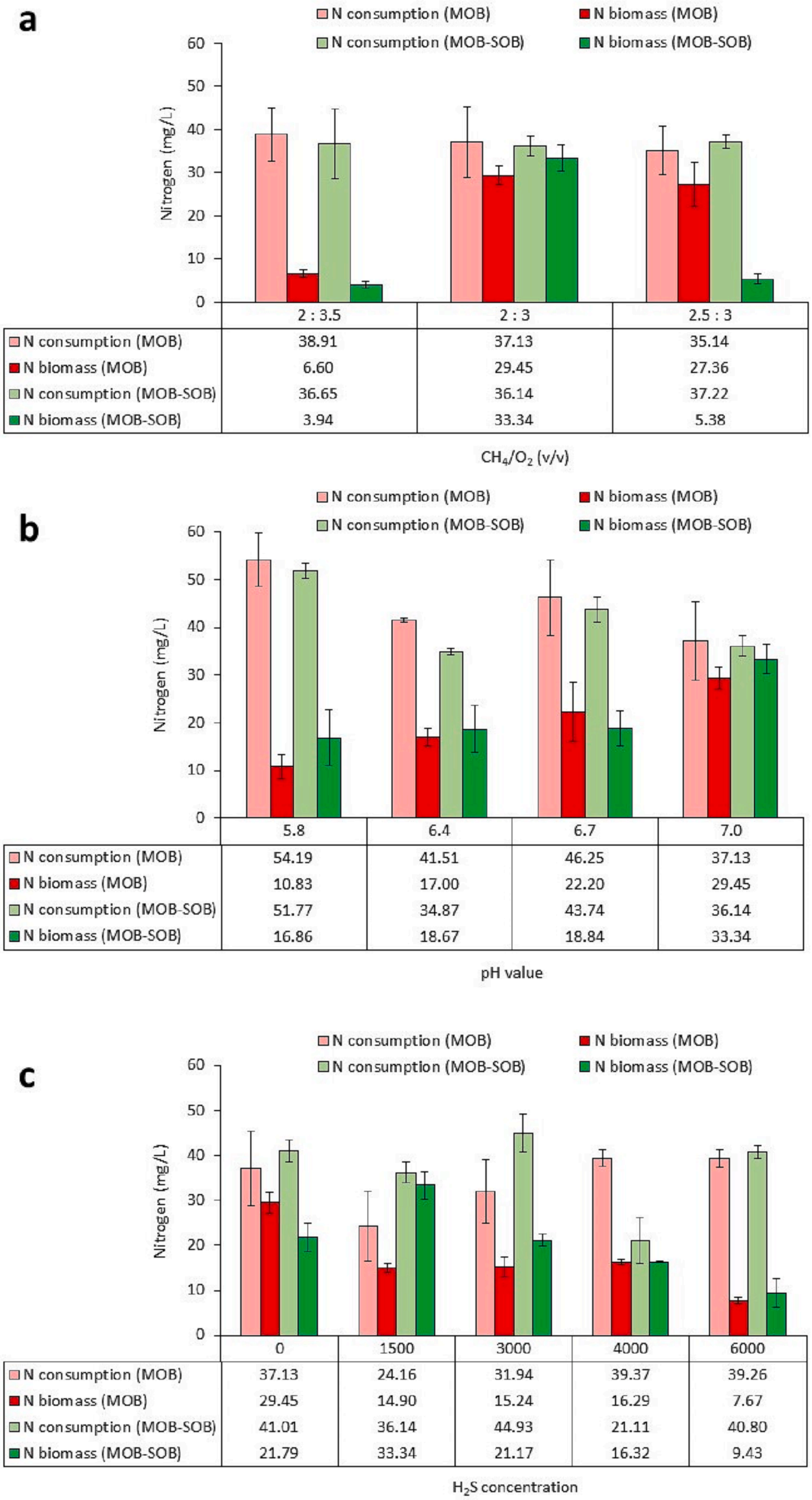


Fig. 3. Effect of sulfide concentration on: a) pressure trend, b) maximum methane consumption rate, c) net biomass growth, d) biomass yield on methane, and e) final protein content. *: Significantly different values (*p-value* $\leq$ 0.05).

to unacclimated methanotrophic cultures, especially in the presence of 10.09 mg Na_2S/L (1500 ppm of H_2S), where the biomass yield of the MOB-SOB culture almost doubled compared to that of the unacclimated MOB. This has relevant practical implications as H_2S levels as high as 1500 ppm, which are often found in raw biogas (Xu et al., 2020), would strongly limit the possibility of direct biogas conversion into MP through conventional MOB cultivation.

Regarding the protein content (Fig. 3e), similarly to what was already observed for the biomass yield, the MOB culture was negatively affected by the presence of H_2S, as shown by the 27% lower (*p-value* $<$ 0.05) protein content obtained with 20.18 mg Na_2S/L (3000 ppm of H_2S), as compared to that of 59 ($\pm$4)% measured in the absence of sulfide. Although not significantly different, a lower protein content (46 $\pm$ 2%) was achieved also with 26.91 mg Na_2S/L (4000 ppm of H_2S). Interestingly, the protein content of the MOB-SOB culture was negatively affected not only by the increase of the Na_2S concentration, as suggested by the significantly lower protein content attained at 4000 and 6000 ppm, but also by the absence of Na_2S. Indeed, without sulfide, a 37% decrease of the protein content was observed as compared to the highest value of 73 ($\pm$5)% reached at 1500 ppm, i.e. the condition under which the MOB-SOB culture was enriched. Xu et al. (2020) observed that sulfide alters the biomass composition by reducing the protein content, as they measured the highest amino acids content when *M. acidiphila* were cultivated in the absence of sulfide, detecting an almost twice higher amino acids content than that obtained with sulfide. However, the same authors (Xu et al., 2020) also found that in the presence of



CH$_4$/O$_2$ (v/v)	2 : 3.5	2 : 3	2.5 : 3
🟥 N consumption (MOB)	38.91	37.13	35.14
🟥 N biomass (MOB)	6.60	29.45	27.36
🟩 N consumption (MOB-SOB)	36.65	36.14	37.22
🟩 N biomass (MOB-SOB)	3.94	33.34	5.38

pH value	5.8	6.4	6.7	7.0
🟥 N consumption (MOB)	54.19	41.51	46.25	37.13
🟥 N biomass (MOB)	10.83	17.00	22.20	29.45
🟩 N consumption (MOB-SOB)	51.77	34.87	43.74	36.14
🟩 N biomass (MOB-SOB)	16.86	18.67	18.84	33.34

H_2S concentration	0	1500	3000	4000	6000
🟥 N consumption (MOB)	37.13	24.16	31.94	39.37	39.26
🟥 N biomass (MOB)	29.45	14.90	15.24	16.29	7.67
🟩 N consumption (MOB-SOB)	41.01	36.14	44.93	21.11	40.80
🟩 N biomass (MOB-SOB)	21.79	33.34	21.17	16.32	9.43

Fig. 4. Nitrogen consumption and theoretical nitrogen assimilation in the tests on: a) CH_4:O_2 ratio, b) starting pH, and c) sulfide concentration.

sulfide the proportion of essential amino acids was slightly higher than that obtained without sulfide, probably due to the contribution of sulfide to the synthesis of S-containing amino acids such as cysteine and methionine. Therefore, the presence of sulfide at a concentration equal to that of the enrichment might have played a beneficial role in promoting a higher protein content in the MOB-SOB enrichment.

3.4. Nitrogen balance

Fig. 4 compares the nitrate nitrogen consumption with the theoretical nitrogen assimilated and converted into MP during the tests on the CH_4:O_2 ratio, starting pH and sulfide concentration. Overall, especially under those conditions where biomass growth was low, nitrogen consumption was much greater than that theoretically assimilated and converted into MP. Indeed, the CH_4:O_2 ratio of 2:3, which was found optimal for biomass growth and protein production, allowed an almost complete nitrogen assimilation, with only the 21 (±2)% and 8 (±2)% of the consumed nitrogen exceeding that required for biomass synthesis by the MOB and MOB-SOB enrichment, respectively. On the other hand, the lower CH_4:O_2 ratio of 2:3.5, led to a nitrogen consumption far greater than the estimated nitrogen assimilation, as only the 17 (±4)% and 11 (±2)% of the consumed nitrogen would have been required to support

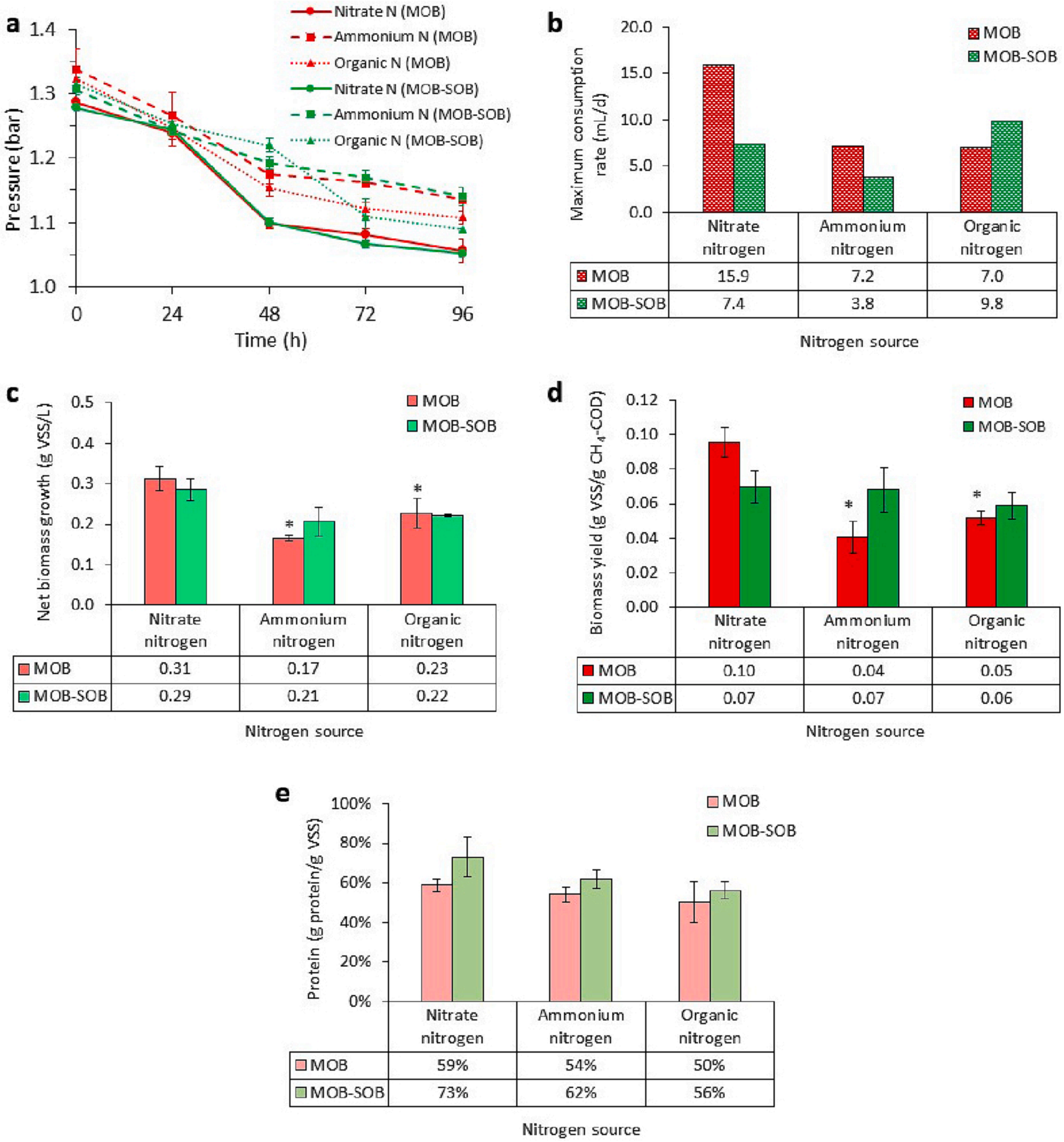


Fig. 5. Effect of the nitrogen source on: a) pressure trend, b) maximum methane consumption rate, c) net biomass growth, d) biomass yield on methane, and e) final protein content. *: Significantly different values (*p-value* ≤ 0.05).

biomass synthesis. In the case of a higher CH_4:O_2 ratio of 2.5:3, a significantly higher (*p-value* < 0.05) nitrogen uptake with respect to that theoretically assimilated was found only in the case of the MOB-SOB culture (Fig. 4a), in line with the lower biomass yield observed with respect to that of the MOB culture under the same conditions (Fig. 1).

Significant discrepancies in the nitrogen balance were found within the test performed at different starting pH values (Fig. 4b). The neutral pH, which was optimal for both biomass yield and protein content, maximized nitrogen assimilation with respect to the more acidic starting pH values. Indeed, while at pH 7 almost all the nitrogen consumed (79% and 92% for the MOB and MOB-SOB cultures, respectively) fits with that theoretically utilized for biomass synthesis, the more acidic pH values led to a higher N imbalance as the theoretic nitrogen utilized for growth was only 20% and 33% of that effectively consumed, respectively.

The increasing sulfide concentrations also produced detrimental effects on the nitrogen uptake (Fig. 4c), as the difference between the theoretically assimilated nitrogen and that consumed became significant when the Na_2S concentration in the liquid medium increased. Indeed, up to 80 and 77% of the total N consumption exceeded the theoretical N requirements for the MOB and MOB-SOB cultures, respectively, in the presence of sulfide concentrations equivalent to 6000 ppm of H_2S. This could mean that high sulfide levels set unfavorable conditions for biomass growth while not affecting catabolic activity, as demonstrated by the higher production of soluble catabolites (estimated through tCOD and sCOD measurements) in most of the conditions with high sulfide concentrations (see supplementary material).

3.5. Effect of nitrogen source

It is worth noting that, in the cases where the MOB-SOB culture showed high biomass yields but low protein contents (e.g., pH 5.8–6.7 or 0, 3000 and 4000 ppm of H_2S equivalent), a significant imbalance in the nitrogen uptake/utilization occurred (Fig. 4). This suggests that when the growth conditions were not optimal, nitrate nitrogen assimilation for biomass synthesis was not the predominant metabolism, but other dynamics might have occurred. For instance, nitrate might have also served as electron acceptor in concomitance with low oxygen levels in the headspace (see supplementary material), thereby shifting from aerobic methane oxidation to anoxic methane oxidation through nitrate. Indeed, methanotrophs possess a flexible response towards different nitrogen sources under different oxygen conditions, thus having the ability to nitrify and denitrify (AlSayed et al., 2018). For this reason, a further screening test was carried out to evaluate the influence of different nitrogen sources, i.e., ammonium nitrogen and organic nitrogen, on the enriched cultures under the optimal growth conditions identified in the previous tests (Fig. 5).

In general, the best biological performances were still achieved when nitrate nitrogen was present in the cultivation medium as the gas substrates, i.e. methane and oxygen, were consumed to a lower extent (Fig. 5a) and at lower rates (Fig. 5b) when ammonium- and organic nitrogen were utilized, so probably the change of nutrient source had negatively affected the gas uptake. Changing the nitrogen source did produce significant (*p-value* < 0.05) effects on the biomass growth of the MOB culture (Fig. 5c and 5d), as biomass yield decreased from the 0.10 ($\pm$0.01) g VSS/g CH_4-COD measured in the presence of nitrates to 0.04 ($\pm$0.01) and 0.05 ($\pm$0.01) g VSS/g CH_4-COD with ammonium and urea, respectively. Indeed, it has been reported that ammonium can partially inhibit methanotrophic growth because of the competition between ammonium on MMO or the accumulation of excessive toxic hydroxylamine or nitrite, whereas nitrates support higher methanotrophic growth rates (AlSayed et al., 2018).

On the other hand, the changes of the nitrogen source did not produce any significant effect (*p-value* < 0.05) on the growth performances of the MOB-SOB culture (Fig. 5c and 5d), which achieved yields of

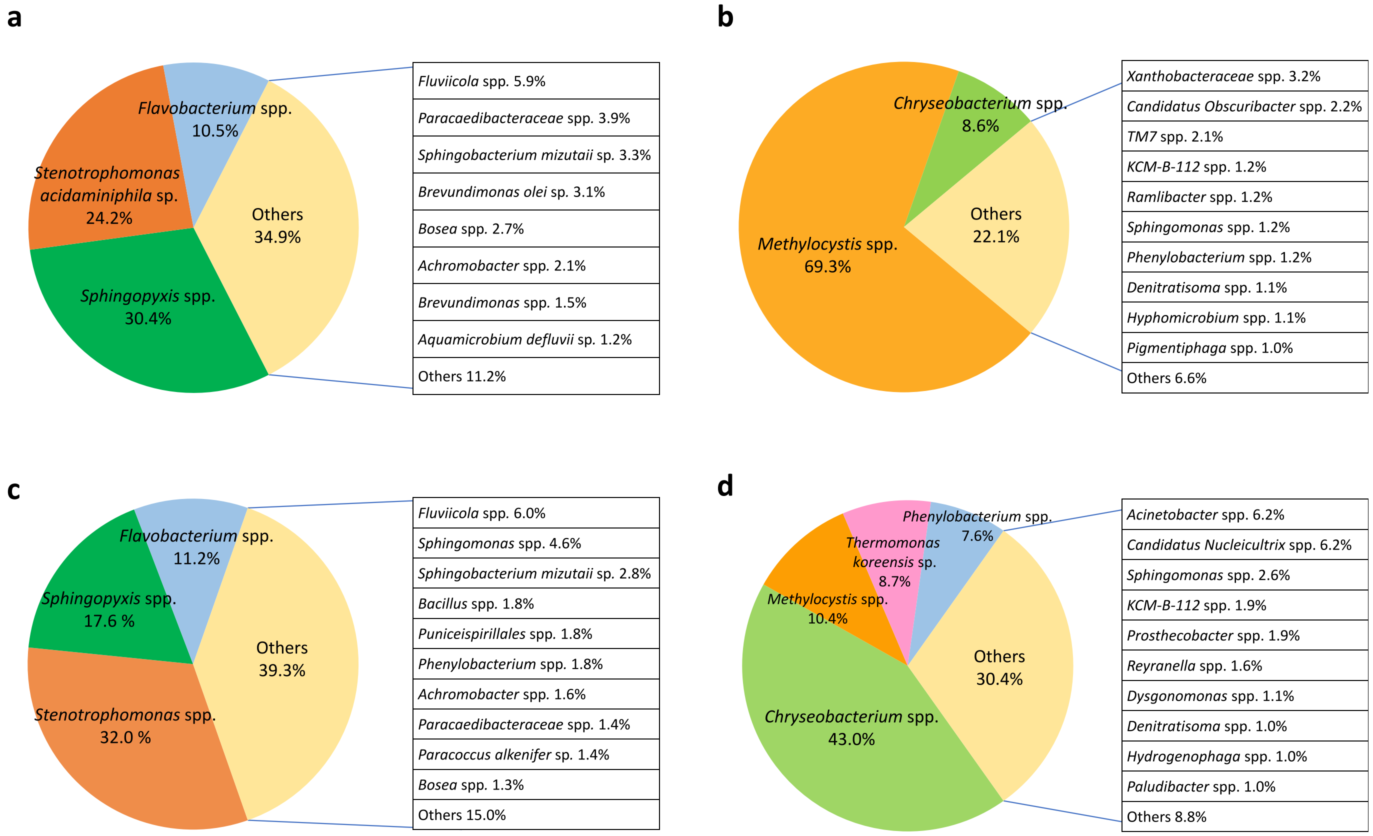


Fig. 6. Microbial community composition of: a) MOB culture at the start of the enrichment, b) MOB culture at the end of the enrichment, c) MOB-SOB culture at the start of the enrichment, and d) MOB-SOB culture at the end of the enrichment. All bacterial strains having a relative abundance higher than 1% are reported.

0.06–0.07 g VSS/g CH_4-COD regardless of the nitrogen source present in the medium. Xu et al. (2020) observed on *M. acidiphila* a similar performance in terms of biomass yields for nitrate and ammonium when grown in the presence of sulfide.

3.6. Microbial community analysis

Fig. 6 shows the evolution of the microbial community from the initial inoculum (Fig. 6a and 6c) to that achieved after the enrichment and used to perform the batch tests (Fig. 6b and 6d). The microbial community of the inoculum was characterized by the dominant presence of *Stenotrophomonas* spp., having a relative abundance (RA) of 24–32%, *Sphingopyxis* spp. (RA of 18–30%) and *Flavobacterium* spp. (RA of 11%).

The enriched MOB and MOB-SOB microbial communities were then characterized by a rather high diversity. *Methylocystis* spp. were present in both enrichments, reaching RAs of 69.3% and 10.4% in the MOB and MOB-SOB cultures, respectively. The genus *Methylocystis*, belonging to methanotrophs type II (Càceres et al., 2014; Jiang et al., 2016), was thus the dominating bacterium in the MOB enrichment (Fig. 6b). *Methylocystis* can be found in a wide range of environments as they possess a greater metabolic flexibility than other methanotrophic genera (Dam et al., 2013). The relevant presence of *Methylocystis* further supports the previously mentioned hypothesis about the potential presence of denitrification dynamics (Section 3.4), as they have been reported as capable of reducing nitrates to nitrous oxide and further to nitrogen gas under oxygen limiting conditions (Dam et al., 2013).

Due to the selective pressure imposed by the presence of Na_2S, *Chryseobacterium*, a bacterial genus commonly found in sulfide-rich environments (Azevedo et al., 2018), became dominant in the MOB-SOB culture, reaching a RA of 43.0%. A lower abundance (8.6%) of *Chryseobacterium* was found in the MOB culture. Fig. 6d, showing the composition of the enriched MOB-SOB culture, displays how the presence of multiple S-utilizing and potentially sulfide tolerant bacteria enabled the culture to withstand higher sulfide concentrations than the MOB alone. Indeed, the MOB-SOB enrichment was further characterized by bacterial genera commonly associated with sulfur-oxidation such as *Thermomonas* (RA: 8.6%) and *Sphingobacteria* (RA: 2.6%) (Vannini et al., 2008), or *Phenylobacterium* spp. (RA: 7.6%) (Hui et al., 2022). Interestingly, members of the genus *Thermomonas*, such as *Thermomonas koreensis*, were classified as sulfide-dependent denitrifying bacteria (Tong Wu et al., 2021), suggesting that nitrate could have partially served as electron acceptor for sulfide-driven autotrophic denitrification in those cases where the nitrogen balance suggested that other nitrogen dynamics prevailed.

Both enriched microbial communities were generally comprised of common heterotrophs frequently detected in aerobic systems, including genera of the *Xanthomonadaceae* (*Strenotrophomonas, Thermomonas*) (RA: 1–10%) and the *Chitinophagaceae* (RA: 1–6%) families (Hülsen et al., 2018), which growth could have been supported by the production of organic metabolites, i.e. acetate or methanol, by the methanotrophs.

4. Conclusions

The potential of a MOB-SOB enrichment to produce MP though methane oxidation in the presence of high sulfide levels was demonstrated and benchmarked against that of an unacclimated MOB culture. The optimal operating conditions in the presence of sulfide were CH_4:O_2 of 2:3, starting pH of 7 and an equivalent gas phase H_2S concentration of 1500 ppm. The developed MOB-SOB enrichment could withstand gas phase H_2S concentrations as high as 4000 ppm, thereby suggesting its suitability for direct conversion of sulfide-rich biogas into MP. Further research is needed to assess the techno-economic feasibility of the process on a real scale.

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

CRediT authorship contribution statement

Marica Areniello: Investigation, Conceptualization, Methodology, Validation, Formal analysis, Writing – original draft, Writing – review & editing. **Silvio Matassa:** Conceptualization, Methodology, Validation, Supervision, Writing – review & editing. **Giovanni Esposito:** Conceptualization, Resources, Validation, Supervision, Writing – review & editing. **Piet N.L. Lens:** Conceptualization, Resources, Validation, Supervision, Writing – review & editing, Project administration.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Marica Areniello reports financial support was provided by Science Foundation Ireland (SFI) through the MaREI Centre for Energy, Climate and Marine research and innovation..

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2023.129237.

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