Journal of Water Process Engineering 81 (2026) 109239

Contents lists available at ScienceDirect

Journal of Water Process Engineering

journal homepage: www.elsevier.com/locate/jwpe

Valorization of nitrogen and sulfur from digestate and biogas: A closed-loop approach to sulfur-rich microbial protein synthesis

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 21, Naples, 80125, Italy

ARTICLE INFO

Editor: Ze-Xian Low

Keywords:
Direct nitrogen stripping
Hollow fiber membrane bioreactor
Methane oxidizing bacteria
Single-cell protein
Sulfur oxidizing bacteria

ABSTRACT

An integrated route for the conversion of anaerobic digestion outputs: biogas (CH_4/H_2S) and digestate (NH_4^+), into microbial protein (MP), is proposed. The MP production from H_2S-rich biogas and digestate nitrogen through a mixed culture of methane oxidizing- (MOB) and sulfur oxidizing-bacteria (SOB) was performed in two hollow fiber membrane bioreactors. The use of digestate and biogas for the supply of N and S, key nutrients for the microbial fermentation process, was compared to the supply of mineral N and S, and its effects on biomass concentration and yield, gas and nutrient consumption, as well as on the amino acids profile and the microbial community composition of the harvested biomass were investigated. Ammonium nitrogen was provided through direct stripping from the liquid digestate using the recirculating biogas stream from the fermenter. The ammonia stripped from the digestate, with nitrogen stripping rates of up to 109.6 mg NH_4^+-N/L·d obtained with at 45 °C and 4 vvm (gas:digestate), met the nitrogen requirements of the biogas-to-MP conversion process. Sulfur was supplied through the H_2S present in the biogas, that was oxidized into sulfate by SOB. Biomass growth occurred even at high sulfide concentrations in the biogas (260.5 mg/L of volatile suspended solids (VSS) at 6000 ppm_v of equivalent H_2S), with a high protein content (up to 65.4 %) of the biomass, while relying on the sulfate produced by H_2S oxidation as sulfur source. Increasing H_2S concentrations in the biogas resulted in an amino acids profile rich in sulfur-amino acids (140.4 mg/g VSS at 6000 ppm_v).

1. Introduction

Anaerobic digestion (AD) enables the conversion of sewage sludge and multiple organic waste not only into low-value bioenergy (i.e., biogas) and fertilizer (i.e., digestate), but also into high-value products, such as microbial protein (MP) [1]. MP represent a potential alternative to conventional animal- or plant-based protein sources as microbial fermentation processes are a prime source of protein-rich biomass endowed with various and abundant amino acids [2]. The biogas produced during AD can sustain the growth of methane oxidizing bacteria (MOB), also known as methanotrophs, which use CH_4 as their sole carbon and energy source and contain around 50–65 % protein on a dry weight basis [3]. Trace contaminants such as H_2S in raw biogas, typically arising from the digestion of sewage sludge, however, hinder the direct utilization by methanotrophs as they can inhibit the methane oxidation activity, even at low concentrations, e.g., 200 ppm [4], thereby imposing the need for pretreatments such as desulfurization [5]. Combining MOB with sulfur oxidizing bacteria (SOB) enables a direct H_2S-rich biogas-to-MP conversion without requiring desulfurization [6–8]. SOB not only lower the toxic effects of sulfide in biogas, but further allow a more efficient nutrients supply as they oxidize H_2S into sulfate, a macronutrient for microbial growth [6].

The other major by-product of AD, i.e., digestate, often requires the removal of high concentrations of ammonium nitrogen (NH_4^+–N) prior to its use as soil amendment or fertilizer to avoid groundwater contamination and eutrophication [9]. On the other hand, digestate stripping, allowing the transfer of NH_4^+–N from the digestate to a gaseous stream, can provide a nitrogen source for MP production [10]. Alternatively to conventional air stripping techniques using high temperature (70–90 °C) and pH (11–12) to enable high rate nitrogen stripping, the direct utilization of the airflow involved in the aerobic fermentation step can induce the release of CO_2 from the digestate,

* Corresponding author.
E-mail address: silvio.matassa@unina.it (S. Matassa).

https://doi.org/10.1016/j.jwpe.2025.109239
Received 2 October 2025; Received in revised form 24 November 2025; Accepted 29 November 2025
Available online 12 December 2025

resulting in a chemical-free pH increase and in a low-rate stripping of free gaseous ammonia without the use of heat and alkali chemicals [10,11]. In addition to air, also biogas can be used as stripping agent for the on-site ammonia stripping in anaerobic digesters, with rates and efficiencies highly dependent on temperature and pH of the digestate [12].

In the context of waste-to-protein approaches, the use of gaseous substrates, i.e., biogas and ammonia, avoids contact between the MP product and the potentially contaminated waste matrix (e.g., micro-pollutants, heavy metals and pathogens) used as AD feedstock, provided that appropriate gas polishing (e.g. through membrane filtering) and condensate removal is guaranteed [13]. To this end, this study proposes an integrated platform where H_2S-rich biogas from AD of organic waste and ammonia stripped from digestate provide energy, carbon and key nutrients (sulfur and nitrogen) to produce MP through a MOB-SOB mixed culture in hollow fiber membrane bioreactors. The recirculation flow of the gas headspace of the fermentation reactor was used as stripping agent in the digestate stripping unit, thereby avoiding external gas flows. The effectiveness of recovering and supplying nutrients through digestate stripping (nitrogen) and sulfide-laden biogas (sulfur) was evaluated and compared with reference to mineral nutrients (ammonium nitrogen and sulfate) fed through the culture medium. The effects of increasing sulfide levels in the biogas were tested in the reactors as well as through dedicated batch tests. The latter tests were carried out also to assess the effects of the absence of magnesium or sulfate, as these nutrients are often supplied together [14]. Magnesium is indeed crucial for microbial growth, as it influences key enzyme activation and cell membrane stabilization [15]. The performance of the process was monitored and evaluated in terms of biomass growth, gas and nutrient consumption, protein content and amino acids profile as well as rate and efficiency of the nitrogen stripping process. Moreover, the microbial community composition of both the suspended and the attached biomass was characterized, and the techno-economic feasibility of the proposed integrated platform was also preliminarily assessed.

2. Materials and methods

2.1. Anaerobic digester for biogas production

The raw biogas used in the experimental activity was produced through a 10 L lab-scale anaerobic digester kept at 35 °C by means of a thermostatic water bath. Digestate from a full-scale anaerobic digester treating buffalo manure (Eboli, Italy) and fruit and vegetable waste (FVW) were utilized as inoculum and organic substrates, respectively. The AD reactor was operated with a 9 L working volume with an inoculum/substrate ratio of 2 on a volatile solids (VS) basis [16] and was run in fed-batch mode for the whole duration of the experiments. In particular, a portion of the AD reactor was replaced by the same volume of fresh FVW on a weekly basis, thereby setting an organic loading rate of 5 g VS/L·d.

2.2. Inoculum and cultivation medium for microbial protein production

A MOB-SOB mixed culture characterized by the dominance of *Methylocystis* spp. and *Chryseobacterium* spp., enriched as described by Areniello et al. [7], was utilized as inoculum. Ammonium mineral salts (AMS) medium (Table S1) supplemented with a micronutrient solution (0.01 % v/v) prepared according to Sun et al. [17] was used as cultivation medium. A 0.2 M phosphate buffer solution, with 1.148 g/L KH_2PO_4 and 1.64 g/L Na_2HPO_4 (Sigma Aldrich, St. Louis, USA), was used to maintain the pH at 7.0 (±0.2) during the experiments. A 1 M NaOH (Sigma Aldrich, St. Louis, USA) solution was used to further adjust and manually control the pH around neutrality. Since H_2S concentrations in the raw biogas produced in the AD reactor (Section 2.1) were below the detection limit, the presence of H_2S in the biogas was

Table 1
Overview of the operating conditions adopted and process variables tested during the experiments.

Reactor	Phase – objective	Duration (days)	HRT (d)	$Na_2S·9H_2O$ (mg/L)[a]	H_2S equivalent in biogas (ppm_v)	Carbon source (L CH_4/L biogas)[b]	Nitrogen source (mg NH_4^+-N/L)	Sulfur source (mg SO_4^{2-}/L)	Stripping temperature (°C)
1	1 – Acclimation	0–7	FB	31.1	1500	0.54	MM: 278.2	MM: 450.0	N.A.
	2 – Acclimation	7–20	2.5			0.57	MM: 278.2	MM: 450.0	
	3 – Reduced N supply	20–48				0.63	MM: 100.0	MM: 450.0	
	4 – N stripping addition	48–74				0.58	MM: 100.0 + stripped NH_3	MM: 450.0	30
	5 – N stripping as only N source	74–105				0.59	MM: 0.0 + stripped NH_3	MM: 450.0	30
	6 – Effect of higher stripping temperature	105–138				0.64	MM: 0.0 + stripped NH_3	MM: 450.0	45
2	1 – Acclimation	0–7	FB	31.1	1500	0.54	MM: 278.2	MM: 450.0 + S^{2-} in biogas	N.A.
	2 – Acclimation	7–20	2.5			0.57	MM: 278.2	MM: 450.0 + S^{2-} in biogas	
	3 – Reduced sulfate supply	20–40				0.62	MM: 278.2	MM: 100.0 + S^{2-} in biogas	
	4 – Absence of sulfate supply	40–74				0.59	MM: 278.2	MM: 0.0 + S^{2-} in biogas	
	5 – Effect of higher H_2S concentration	74–99		82.8	4000	0.60	MM: 278.2	MM: 0.0 + S^{2-} in biogas	
	6 – Effect of higher H_2S concentration	99–116		124.2	6000	0.62	MM: 278.2	MM: 0.0 + S^{2-} in biogas	
	7 – Effect of absence of H_2S	116–138		0	0	0.64	MM: 278.2	MM: 450.0	

FB: fed batch mode.
MM: supply through the mineral medium.
S^{2-} in biogas: dissolved sulfide (as $Na_2S·9H_2O$) referred to the H_2S equivalent in the biogas.
N.A. Nitrogen stripping not applied.
[a] The dissolved sulfide concentrations equivalent to H_2S content in biogas and the corresponding equivalent concentrations of $Na_2S·9H_2O$ were determined according to Xu et al. [4].
[b] The carbon source provided to the MP production process is the methane present in the influent biogas, which concentration expressed as L CH_4/L biogas is equivalent to the methane volumetric concentration in the biogas (v/v).

simulated through $Na_2S{\cdot}9H_2O$ addition in the liquid medium – equivalent to different H_2S concentrations (0–6000 ppm_v) – based on the H_2S gas dissolution and dissolved H_2S dissociation equilibrium, according to Xu et al. [4] (Section S2 and Table 1).

2.3. Hollow fiber membrane reactors set-up and operation

Two 1.05 L closed glass vessels were used as reactors (R1 and R2) and kept at 30 °C (±1) by means of an external water jacket (Fig. S3). Gas substrates were supplied in each reactor through a dead-end hollow fiber membrane module (membrane P60, Zena s.r.o., Brno, Czech Republic) having 500 polyethylene fiber membranes with a porosity of 0.1 μm, total surface of 1000 cm^2 and a ID/OD ratio of 310/240 μm. The k_La of the membranes (0.029 min^{-1}) was determined by Areniello et al. [8]. Both reactors were filled with 1 L cultivation medium supplied with 10 % (v/v) of the MOB-SOB inoculum.

Table 1 summarizes the experimental conditions tested in both reactors. During the start-up period (days 0–20), the reactors were run as biological replicates under the same operating conditions, while from day 20 onwards the operating conditions of the two reactors were differentiated (Table 1). R1 was used to evaluate the supply of NH_4^+–N either as mineral nitrogen supplied through the cultivation medium (phases 1–3) or by replacing the latter with the gaseous ammonia recovered through digestate stripping by means of the internal gas recirculation flow (phases 4–6) (Section 2.4). The experiments with R2 focused, instead, on the supply of sulfur by providing it either as mineral sulfate (phases 2–4) or through different equivalent H_2S concentrations in the biogas (phases 5–7) (Table 1).

Experiments were run in fed-batch mode during days 0–7, with manual sampling and medium addition through a sampling port. From days 7 to 138, liquid medium was continuously provided through a peristaltic pump (Master-Flex L/S, Radnor, USA), collecting the liquid effluent in an external tank, and thereby setting a hydraulic retention time (HRT) of 2.5 days.

2.4. Reactor gas feeding

The raw biogas produced through the lab-scale anaerobic digester (Section 2.1) was collected into Tedlar® gas sampling bags (Thermogreen® LB-2 septa, Sigma-Aldrich, St. Louis, USA) and used to feed R1 and R2.

Analytical grade oxygen (purity ≥ 99.999 %) sampled from gas cylinders (Nippon gases, Milan, Italy) and collected into Tedlar® gas bags was also utilized. Biogas and oxygen from the gas bags were fed to each reactor through a peristaltic pump (Master-Flex L/S, Radnor, USA) and injected in the hollow membrane modules with a total gas flow rate of 4 L/d (2 L/d of biogas and 2 L/d of oxygen), chosen according to the biogas productivity of the anaerobic digester, resulting in a gas residence time (GRT) of 6 h. The gas present in the headspace of the reactors was recirculated through an additional peristaltic pump (Master-Flex L/S, Radnor, USA) (Fig. S3) with a flow rate of 1 L/min and injected at the bottom of each reactor through a stainless-steel aeration stone to avoid biomass settling and ensure appropriate mixing of the liquid phase. The peristaltic pumps were calibrated once a week to ensure a stable gas supply. The unused gas was collected from the headspace of the reactors into an external gas bag for volume and gas composition measurements. Starting from day 74, the gas recirculation line of R1 was adapted to perform digestate nitrogen stripping (Section 2.5).

2.5. Direct nitrogen stripping from anaerobic digestate

Digestate from the anaerobic digester (Section 2.1) was used for the nitrogen stripping process applied to R1 in phases 4–6. The stripping process was performed under batch conditions in a 500 mL glass bottle filled with 250 mL of liquid digestate (Fig. S3). Digestate was previously centrifuged at 4032 ×*g* to separate the liquid from the solid fraction according to Matassa et al. [10]. A volume of 10 mL of antifoam (Antifoam O-30, Sigma Aldrich, St. Louis, USA) was added to avoid excessive foaming during gas stripping. The bottle with the liquid digestate was placed in a thermostatic bath to keep the temperature at 30 °C during phases 4 and 5 and at 45 °C during phase 6.

The gas collected from the headspace of R1 through the gas recirculation pump (Section 2.4) was used as stripping agent with a flow rate of 1 L/min and sparged in the digestate through an aeration stone placed at the bottom of the bottle. This resulted in a gas-to-digestate ratio, defined as the volume of gas supplied every minute per volume of digestate (vvm) of 4:1 [10]. Condensate from the outflowing gas was collected in a 500 mL glass bottle kept at ambient temperature (Fig. S3).

2.6. Batch tests

Batch cultivation tests were performed to evaluate the influence of the absence of magnesium and sulfate as well as of different equivalent H_2S concentrations on the biogas-to-MP conversion by the reactor biomass, according to Areniello et al. [7]. The tests were carried out by using the same AMS medium amended with the micronutrient solution and phosphate buffer used for the reactors (Section 2.1), adapted based on the different operating conditions to be tested (Table S4). In the first test $MgSO_4{\cdot}7H_2O$, present in the mineral medium and used in the control bottles, was substituted by Na_2SO_4 and $MgCl_2{\cdot}6H_2O$ (Sigma Aldrich, St. Louis, USA) (Table S4) to test the effects of the absence of magnesium and sulfate in the medium, respectively. The second batch test was aimed at assessing the effects of four equivalent H_2S concentrations in the biogas, i.e., 0, 1500, 6000 and 12,000 ppm_v on the fermentation process to identify the sulfide inhibition threshold, with $Na_2S{\cdot}9H_2O$ dosed accordingly in the mineral medium (Table S4).

Each batch test lasted 96 h and was performed in triplicate within 300 mL serum bottles sealed with butyl rubber stoppers and aluminum crimps, incubated at 30 °C and shaken at 120 rpm. Each bottle was operated at 10 % (v/v) of working volume, resulting in a liquid culture of 30 mL and a headspace of 270 mL. Biomass sampled from reactor R1 (6 mL) on days 116 and 130 of reactor operation was used to inoculate each bottle at the start of the first and the second test, respectively.

The headspace of each bottle, containing air, was supplemented with biogas (Section 2.3) to achieve a CH_4:O_2 ratio of 2:3 (v/v) at the start of the experiments, according to Areniello et al. [7]. The initial pH of each bottle was adjusted to 7.0 (±0.2) by dosing 1 M NaOH (Sigma Aldrich, St. Louis, USA).

Liquid samples from each serum bottle were taken at the beginning (0 h), at day 2 (48 h) and at the end (96 h) of each batch test to measure biomass concentration, ammonium nitrogen concentration and protein content of the biomass. The gas headspace was sampled in all triplicate bottles at the start and at the end (96 h) of each test for the evaluation of the gas composition. The pressure of each bottle was monitored daily by using a digital manometer (Leo 1, Keller, Switzerland).

2.7. Analytical procedures

Liquid samples were taken daily from both reactors. The pH was measured 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., NH_4^+–N and sulfate, were measured after centrifugation at 4032 ×*g* and filtration through 0.45 μm Millipore Millex® PTFE syringe filters (EMD Millipore, Burlington, USA). Total (TSS) and volatile (VSS) suspended solids concentrations as well as NH_4^+–N concentration were analyzed according to Standard Methods [18]. Sulfate was 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), with a detection limit of 0.20 mg/L, as described by Areniello et al. [7].

Liquid samples were also stored at −20 °C and then thawed at 50 °C for the subsequent protein determination according to the Lowry

colorimetric method [19]. Gas samples taken daily from the gas bag containing biogas produced through AD (Section 2.1) and from the outlet gas bags of the reactors for feed and excess gas composition analyses were measured through a Star3400 gas chromatograph (Varian, Palo Alto, USA), equipped with a thermal conductivity detector and a ShinCarbon ST80/100 column (Restek, Bellefonte, USA) [20].

Samples of effluent from R2 were taken at the end of phases 4, 6 and 7 for both optical microscopy (Wild M8 6-500× microscope, Leica, Heerbrugg, Switzerland) and sludge volume index (SVI) determination to assess the influence of increasing equivalent H_2S concentrations on biomass settleability and biomass morphology. The SVI was determined according to Standard Methods [18].

To determine the amino acids (AA) profile of the produced MP, the effluent was continuously collected throughout each phase from each reactor to achieve the required amount of biomass representative for the analysis. Biomass was harvested through centrifugation at 4032 ×*g*, dried at 70 °C and grinded into a fine powder. The AA analysis was performed on the dried powder by Teagasc (Moorepark, Ireland) as described by Areniello et al. [6]. Briefly, samples were treated with phenol-containing performic acid prior to microwave hydrolysis in 6 M HCl at 160 °C for 15 min. Sodium bisulfite was used to neutralize the reaction and pH was adjusted to 2.2 with 7.5 N NaOH. Samples were then deproteinized using 24 % (w/v) tri-chloroacetic acid and centrifuged for 10 min. Supernatants were removed and samples were then diluted 1 in 2 with the internal standard, norleucine. Amino acids were quantified using a Jeol JLC-500/V amino acid analyser (Jeol Ltd., Garden city, Herts, UK).

2.8. Microbial community analysis

Biomass samples were collected for analysis of the microbial community at the start of the experiment (day 0) and at the end of each experimental phase in both reactors. Moreover, the biofilm sampled from the membrane modules at the end of the experiment (day 138) was used to evaluate the composition of the attached microbial biomass in both R1 and R2. Samples were stored at −20 °C and the DNA extraction, sequencing and bioinformatic analysis were performed by Fisabio (Valencia, Spain) as described by Areniello et al. [7]. Briefly, samples were homogenized by adding 1 mL of phosphate-buffered saline (PBS) to 2 mL of sample and were centrifuged at 4 °C for 2 min at 2000 rpm and for 30 min at 13200 rpm. DNA extraction was carried out on the pellet using a MAgNa Pure LC robot and a III 3,264,785,001 isolation kit (Roche, Basel, Switzerland). Qubit dsDNA HighSensitivity kit (Qiagen, Hilden, Germany) was used for DNA quantification. DNA was normalized at 5 ng/ul to start the library preparation protocol. The Illumina protocol [21] targeting V3 and V4 regions of the 16S genes was followed for polymerase chain reaction (PCR) amplification, sequencing, and PCR cleanup. Silva138 was used as database for the taxonomic assignation.

2.9. Calculations and data analysis

The volumetric productivity and biomass yield were calculated as described by Areniello et al. [6].

The stripping rate of ammonium nitrogen from the digestate was calculated as follows:

$$\begin{aligned} &\mathrm{NH_4^+ - N\ stripping\ rate}\left(\frac{\mathrm{mg\ NH_4^+ - N}}{\mathrm{L{\cdot}d}}\right) \\ &= \frac{\mathrm{NH_4^+ - N_0 - NH_4^+ - N_t}\left(\frac{\mathrm{mg\ NH_4^+ - N}}{\mathrm{L}}\right)}{\mathrm{t\ (d)}} \\ &= \frac{\mathrm{NH_4^+ - N\ decrease}\left(\frac{\mathrm{mg\ NH_4^+ - N}}{\mathrm{L}}\right)}{\mathrm{t\ (d)}} \end{aligned} \quad (1)$$

where "$NH_4^+ - N$ decrease" is the difference between the initial and the final $NH_4^+ - N$ concentration (mg/L) measured in the digestate in the reference period having a duration of t (days).

The specific sulfide loading rate (SSLR) was calculated as follows:

$$\mathrm{SSLR}\left(\frac{\mathrm{mg\ S^{2-}}}{\mathrm{g\ VSS{\cdot}d}}\right) = \frac{\mathrm{SLR\ (mg\ S^{2-}/d)}}{\mathrm{Biomass\ (g\ VSS)}} \quad (2)$$

where the sulfide loading rate (SLR) is the daily sulfide supply (mg S^{2-}/d) calculated based on the $Na_2S{\cdot}9H_2O$ provided through the influent mineral medium in each phase (considering that each gram of $Na_2S{\cdot}9H_2O$ provides 0.134 g of S^{2-}) and the biomass is calculated considering the average biomass concentration of each experimental phase and the working volume of the reactors of 1 L.

The methane uptake rate was calculated as follows:

$$\mathrm{CH_4\ uptake\ efficiency(\%)} = \frac{\mathrm{CH_4 - in\ (L/d) - CH_4 - out\ (L/d)}}{\mathrm{CH_4 - in\ (L/d)}}{\cdot}100 \quad (3)$$

where the influent and effluent methane flows were evaluated based on the daily measurements of gas volumes and compositions of both the influent and effluent gas.

The mass balances of carbon and sulfur were developed as suggested by Areniello et al. [6] and are shown Tables S6 and S10.

Briefly, the carbon conversion efficiency was calculated as follows:

$$\mathrm{Carbon\ conversion\ efficiency(\%)} = \frac{\mathrm{CH_4 - C\ consumed\ (mg/d) - Biomass\ C\ (mg/d)}}{\mathrm{CH_4 - C\ consumed\ (mg/d)}}{\cdot}100$$

where "$CH_4 - C$ consumed" is the amount of methane daily consumed (mg/d) and "Biomass-C" is the amount of carbon present in the biomass produced daily, calculated considering an average carbon content of the biomass of 0.53 mg C/mg VSS.

The average amounts of nitrogen supplied and assimilated as MP during the experiments are shown in Table S5.

All analyses and measurements were made with analytical triplicates and results are expressed as the mean ± standard deviation. The reference values relating to each experimental phase, lasting at least 4 HRTs to ensure stable process conditions, were calculated by averaging all the data collected during each phase after the first HRT (2.5 d) from the start of the phase in order to exclude any influence from the previous phase. All results from the batch tests were expressed as the mean ± standard deviation of each experimental condition performed in triplicate. The statistical analysis of the experimental data of the batch tests was performed through the analysis of variance (ANOVA) carried out with the MINITAB® software (version 21.3.1, Minitab Inc., State College, USA). The experimental results were considered significantly different when the *p*-value was below or equal to 0.05 ($p\text{-value} \leq 0.05$).

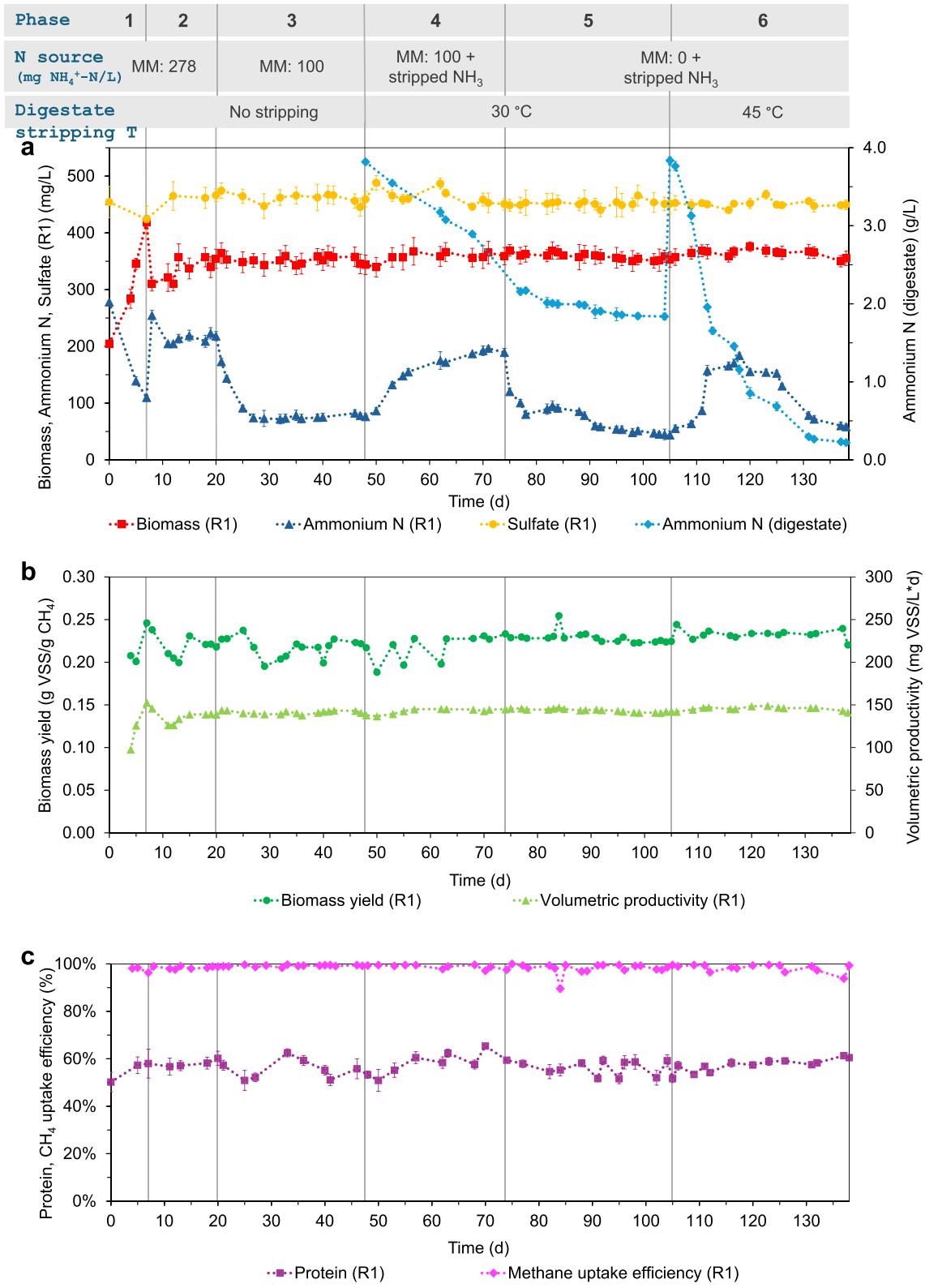

Fig. 1. Performance of the biogas-to-MP conversion process in reactor R1. The performance of the process is expressed in terms of a) biomass, ammonium nitrogen and sulfate concentration in the reactor and ammonium nitrogen concentration in the anaerobic digestate during the stripping process, b) volumetric productivity and biomass yield, and c) methane uptake rate and protein content of the biomass.
MM: mineral ammonium nitrogen supplied through the influent mineral medium.

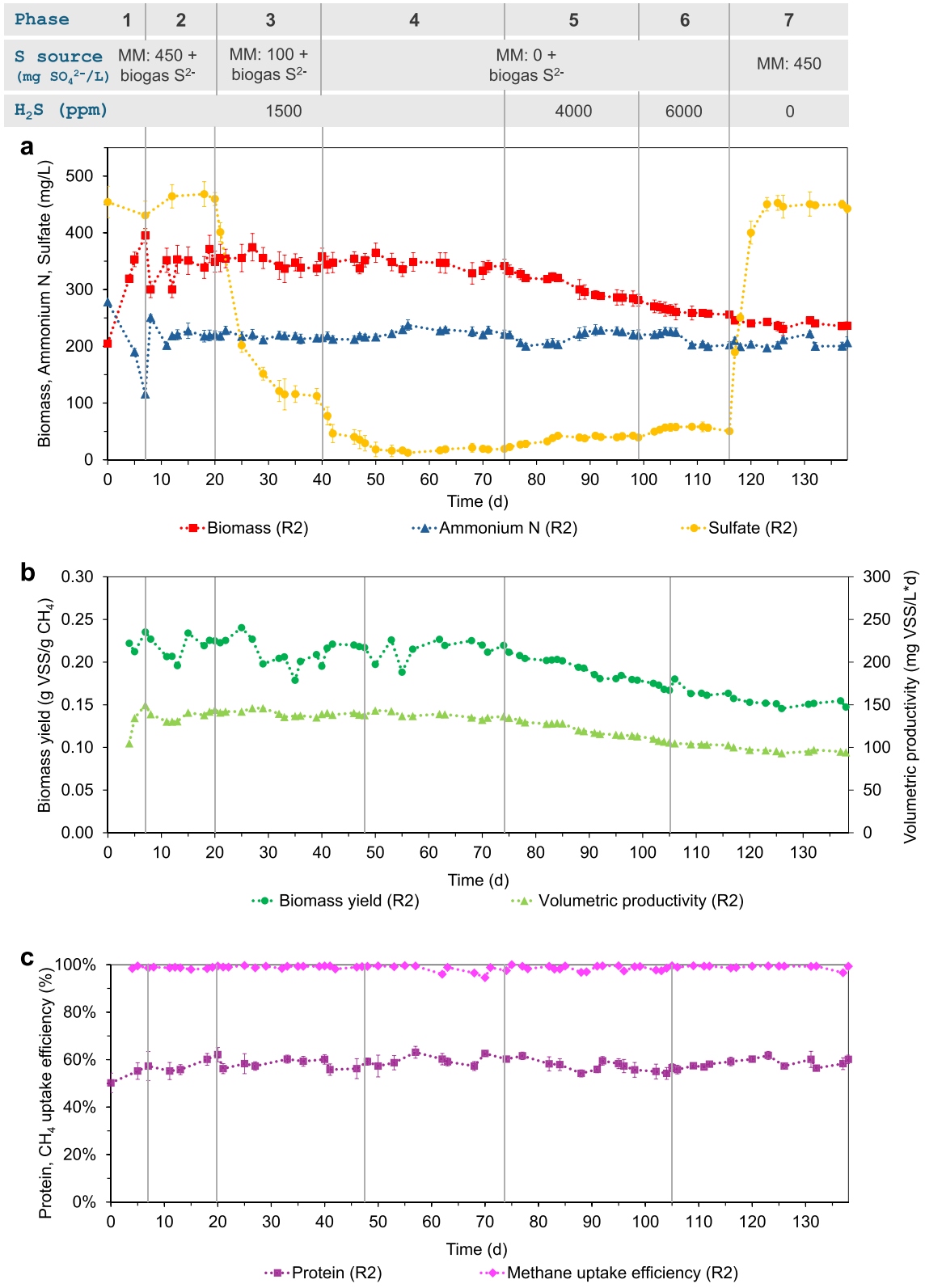

Fig. 2. Performance of the biogas-to-MP conversion process in reactor R2. The performance of the process is expressed in terms of a) biomass, ammonium nitrogen and sulfate concentration in the reactor, b) volumetric productivity and biomass yield, and c) methane uptake rate and protein content of the biomass. MM: mineral sulfate supplied through the influent mineral medium.

3. Results

3.1. Start-up period

During the first 20 days of the start-up (phases 1–2) (Figs. 1a–2a), the reactors, operated as biological replicates, showed very similar performance and reached an average biomass concentration of 342.3 (±19.0) mg VSS/L. Along with steady biomass growth, stable levels of residual NH_4^+–N (averagely 215.5 ± 7.4 mg/L) and sulfate (averagely 464.2 ± 2.9 mg/L) were observed in the reactors at the end of phase 2.

3.2. Optimization of the ammonium nitrogen supply

As high levels of residual NH_4^+–N were observed at the end of phase 2, the influent concentration of mineral NH_4^+–N was decreased to 100 mg NH_4^+–N /L in R1 in phase 3 based on the average consumption of ~60 mg NH_4^+-N /L observed when steady biomass growth was achieved in phase 2 (Fig. 1a). The lower NH_4^+–N supply did not affect the microbial growth as biomass growth remained stable, with an average biomass concentration of 350.3 (±5.6) mg VSS/L and a residual nitrogen concentration of 74.2 (±1.9) mg NH_4^+–N/L at the end of phase 3 (Fig. 1a).

Nitrogen stripping from the anaerobic digestate through the internal gas recirculation line was combined with the mineral nitrogen supply through the influent medium in phase 4 in R1. The NH_4^+–N concentration in the digestate unit progressively decreased (Fig. 1a), with an average stripping rate of 57.3 mg NH_4^+–N/L·d in phase 4, leading to a 43.5 % ammonium recovery and confirming that ammonia was being removed from the digestate, while the NH_4^+–N concentration increased in R1 up to 191.3 (±3.5) mg NH_4^+–N/L as stripped ammonia was solubilized when sparged in the reactor. The pH of the digestate progressively increased while the nitrogen stripping was performed (Fig. 3). The pH in R1, which tended to decrease (up to 6.64) prior to stripping (phases 1–3), became more stable around neutral values from phase 4 onwards (Fig. 3), thereby requiring less frequent pH corrections.

A constant biomass concentration of 356.5 (±7.7) mg VSS/L (Fig. 1a) was observed over the whole phase 4 in R1, along with a biomass yield of 0.218 (±0.015) g VSS/g CH_4 (Fig. 1b).

The levels of residual NH_4^+-N observed in R1 in phase 4 (Fig. 1a) (Table S5) further suggested that the stripping process could supply a sufficient amount of nitrogen. Therefore, mineral NH_4^+-N provision through the influent cultivation medium was discontinued in phase 5 in R1. The nitrogen stripping process slowed down, with the stripping rate decreasing by 75 % compared to phase 4, reaching 14.2 mg NH_4^+–N/L·d (Fig. 1a) likely because of the decreased gradient of NH_4^+-N concentration between the liquid and the gas phase achieved after phase 4 when lower concentrations of ammonia in the liquid digestate and high residual levels of NH_4^+-N in R1 were observed (Fig. 1a). In the same phase, biomass concentration (357.7 ± 5.1 mg VSS/L) and biomass yield (0.228 ± 0.007 g VSS/g CH_4) (Fig. 1a–b) remained constant, resulting in a carbon conversion efficiency of 16.2 %, in line with those observed in the previous phases (Table S6).

Based on the reported beneficial effects of increasing temperature on the stripping process [22], a higher temperature, yet still in the mesophilic range, was applied to the stripping unit in phase 6 by shifting from 30 °C to 45 °C. This resulted in a marked improvement of the ammonium stripping rate, equaling 109.6 mg NH_4^+–N/L·d during phase 6, with a 94 % recovery of the NH_4^+–N available in the digestate achieved in 33 days, compared to the 52 % recovered nitrogen over phases 4–5 in 56 days (Fig. 1a). Also, the pH of the digestate achieved a value of 9.0 during phase 6, higher than the values around 8.1 reached in phase 5 (Fig. 3). The NH_4^+–N concentration in R1 peaked to 184.0 (±4.6) NH_4^+–N/L during phase 6, while reaching a residual concentration of 58.4 (±4.5) NH_4^+–N/L at the end of the phase (Fig. 1a). Biomass productivity and yield were stable during phase 6, with average values of 145.6 (±2.2) mg VSS/L·d and 0.233 (±0.005) g VSS/g CH_4, respectively (Fig. 1a–b).

Constant values of the methane uptake rate (90–100 %) were observed during the whole experiment in R1 (Fig. 1c).

3.3. Optimization of the sulfur supply

The $MgSO_4$ concentration in the mineral medium was decreased to achieve 100 mg SO_4^{2-}/L in phase 3 in R2. Sulfate levels reached values of 114.5 (±1.5) mg SO_4^{2-}/L at the end of phase 3, while the biomass

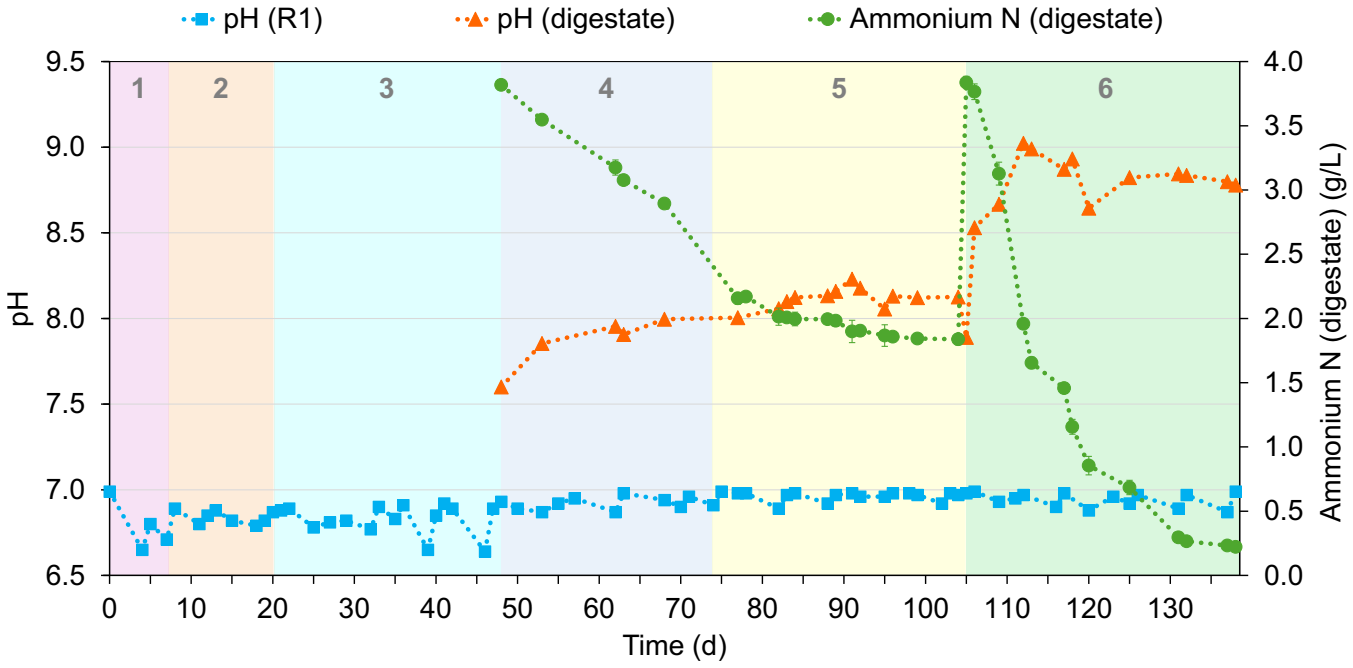

Fig. 3. pH values of R1 effluent and pH values and ammonium nitrogen concentrations of the digestate during the stripping process. The pH in R1 was monitored daily and adjusted by means of 1 M NaOH every time it dropped below 7.0 (±0.2) to maintain neutrality. The pH values detected before NaOH addition are displayed.

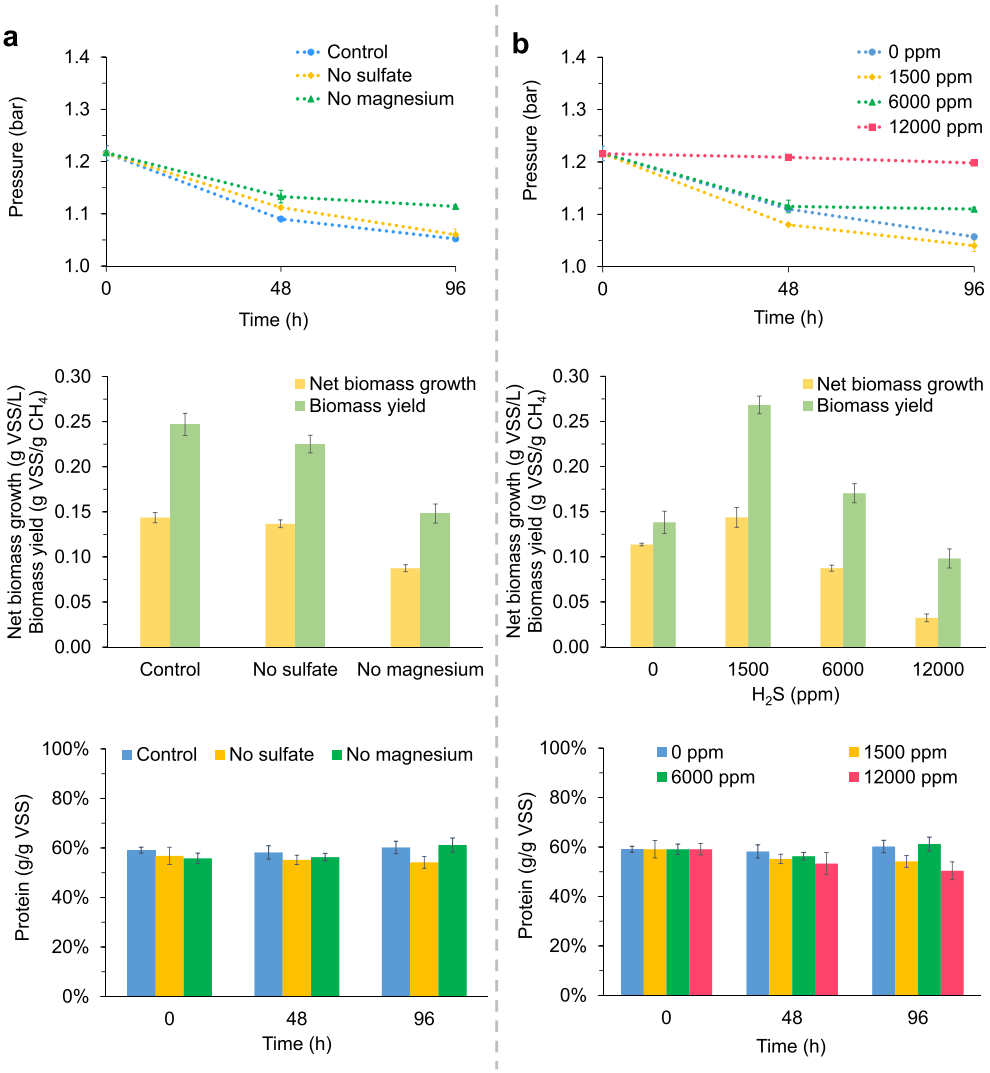

Fig. 4. Performance of the fermentation process of biogas conversion into MP in batch tests. The effects of a) absence of sulfate and magnesium, and b) increasing concentrations of equivalent H_2S in the biogas on the process of biogas conversion into MP in the batch tests expressed in terms of pressure trends, net biomass growth, biomass yield and protein content of the biomass.
*: Significantly different values (*p-value* $\leq$ 0.05).

concentration was constant over the whole phase around 349.7 (±11.9) mg VSS/L (Fig. 2a).

In phase 4, $MgSO_4$ was replaced by $MgCl_2$ to test the absence of influent sulfate while avoiding the lack of magnesium and relying on sulfide ($Na_2S{\cdot}9H_2O$) as the sole source of sulfur (Table S1). Fig. 2a shows constant trends of both biomass concentration and residual NH_4^+–N (344.6 ± 9.3 mg VSS/L and 223.5 (±6.7) mg NH_4^+-N/L, respectively) in R2 in phase 4, with residual sulfate concentrations of 17.6 (±2.3) mg SO_4^{2-}/L still detected. Biomass concentration and yield decreased when $MgSO_4$ was replaced by Na_2SO_4 as sulfate source in the batch tests (Fig. 4a). Indeed, a net biomass growth and biomass yield of 87.4 (±3.9) mg VSS/L and 0.148 (±0.01) g VSS/g CH_4, respectively, were observed in the absence of magnesium, i.e., 39 % and 40 % lower compared to the control condition. Biomass growth was instead not affected in the absence of sulfate, i.e., when $MgSO_4$ was replaced with $MgCl_2$, with a net biomass growth of 136.7 (±4.3) mg VSS/L in the presence of $MgCl_2$ compared to 143.7 (±5.8) mg VSS/L in the presence of $MgSO_4$ (Fig. 4a).

3.4. *Effect of the H_2S concentrations in biogas*

The effect of different concentrations of equivalent H_2S in the biogas as well as the lack of H_2S on the process of biogas-to-MP conversion were tested in phases 4–7 in R2 and in batch screening tests. Biomass concentrations of 297.5 (±14.9) mg VSS/L and 260.0 (±3.2) mg VSS/L were observed in phases 5 and 6 in R2 in the presence of 4000 and 6000 ppm_v of equivalent H_2S, respectively, being 14 % and 25 % lower compared to the presence of 1500 ppm_v of equivalent H_2S (phase 4) (Fig. 2a). The SSLR thus increased, moving from 4.82 mg S^{2-}/g VSS·d in phase 4 to 14.87 mg S^{2-}/g VSS·d in phase 5 and 25.82 mg S^{2-}/g VSS·d in phase 6, thereby being 3.1 and 5.3 times higher in the presence of 4000 and 6000 ppm_v of equivalent H_2S, respectively, compared to 1500 ppm_v (Table S7).

Biomass yield also decreased in response to the increasing sulfide concentrations, moving from 0.216 (±0.010) g VSS/g CH_4 in phase 4 to 0.190 (±0.010) g VSS/g CH_4 in phase 5 and 0.167 (±0.006) g VSS/g CH_4 in phase 6 (Fig. 2b). The latter also resulted in 11 % and 22 % lower carbon conversion efficiencies in the presence of 4000 and 6000 ppm_v of equivalent H_2S, compared to 1500 ppm_v of equivalent H_2S (Table S6). Similarly, the batch tests showed only a partial inhibition of biomass growth at 6000 ppm_v of equivalent H_2S, achieving a net biomass growth of 87.4 mg VSS/L, that is 39 % lower compared to 1500 ppm_v of equivalent H_2S (Fig. 4b). The net biomass growth was reduced by about

76 % (32.3 mg VSS/L) and the biomass yield was 63 % lower (0.098 g VSS/g CH_4) at 12000 ppm_v of equivalent H_2S compared to 1500 ppm_v.

Constant values of the methane uptake rate (95–100 %) were observed in R2 throughout the experiment (Fig. 2c). The residual NH_4^+–N concentrations were quite constant under different H_2S concentrations in R2 (Fig. 2a), with average values of 223.5 (±6.7), 219.0 (±9.3) and 214.2 (±11.1) mg NH_4^+–N/L in phases 4, 5 and 6, respectively (Table S5).

Fig. S8 shows the effects of the increasing sulfide levels on the morphological characteristics of the biomass. The biomass grown at 1500 ppm_v showed a higher tendency to flocculate, while after the increase of the sulfide levels to 6000 ppm_v of equivalent H_2S it tended to lose its flocculated structure forming smaller filaments. The biomass also had lower settleability in response to the increasing sulfide concentrations as the SVI value at 6000 ppm_v of equivalent H_2S amounted to 28.5 mL/g VSS, while it was 16.3 mL/g VSS and 22.7 mL/g VSS at 1500 (end phase 4) and 0 (end phase 7) ppm_v of equivalent H_2S, respectively.

3.5. Protein and amino acid content of the biomass

The protein content of the biomass in both reactors was stable within ranges of 50.5–65.4 % in R1 and 50.2–63.1 % in R2 (Figs. 1c–2c). The harvested biomass showed high total amino acids contents of 391.4–534.8 mg AA/g VSS (Table S9).

The amino acids profile in R1 was overall stable during the experiment, with some AA, i.e., cysteine, threonine and glutamic acid, becoming more abundant when stripped nitrogen was provided (Table S9).

The total AA content in R2 increased from 395.0 mg AA/g VSS in phase 4 to 489.4 mg AA/g VSS in phase 5 and 534.8 mg AA/g VSS in phase 6, thereby increasing by 23.9 % and 35.4 % when shifting from 1500 ppm_v of equivalent H_2S to 4000 and 6000 ppm_v, respectively (Table S9). Particularly, the total content of sulfur-amino acids (S-AAs) (i.e., methionine, cysteine and taurine), which was 58.3 mg AA/g VSS in the absence of sulfide, increased progressively along with the increasing equivalent H_2S concentrations and achieving 87.0, 120.8 and 140.4 mg S-AA/g VSS in the presence of 1500, 4000 and 6000 ppm_v of equivalent H_2S, respectively (Table S10). Indeed, the methionine content was 1.7, 2.4 and 2.7 times higher in the presence of 1500, 4000 and 6000 ppm_v of equivalent H_2S, respectively, compared to the absence of sulfide, i.e., 21.4 mg/g VSS (Fig. 5). A similar trend was observed for the cysteine content, which increased by 1.4, 1.8 and 2.0 times in the presence of the same sulfide concentrations with respect to the value observed in the absence of sulfide (26.7 mg/g VSS). The content of taurine, instead, was 1.3, 2.3 and 2.9 times higher than in the absence of H_2S (10.2 mg/g VSS). The high content of sulfur amino acids resulted in a sulfur content of the harvested biomass of 1.9–4.3 % (w/w of VSS) (Table S10).

Among the other AA, alanine (35.4–48.1 mg/g VSS), aspartic acid (28.0–45.4 mg/g VSS) and glutamic acid (32.7–50.7 mg/g VSS) were particularly abundant in the harvested biomass (Fig. 5).

3.6. Microbial community composition

The inoculum was almost completely characterized by bacteria belonging to the phyla of *Proteobacteria* and *Bacteroidota*, having a relative abundance (RA) of 57.8 % and 33.8 %, respectively (Fig. 6a–b). At the genus level (Fig. 6c–d), the inoculum was dominated by the presence of *Methylocystis* spp. (RA: 30.3 %) and *Chryseobacterium* spp. (RA: 29.4 %). The inoculum was further characterized by the presence of *Methylocaldum* spp., another methylotrophic genus with a marginal RA of 3.6 %, and other side genera, e.g., *Pseudomonas* spp. (RA: 9.1 %), *Xanthomonas* spp. (RA: 3.5 %) and *Hyphomicrobium* spp. (RA: 2.6 %).

The biomass in R1 was characterized by the dominance of *Methylocystis* spp. (RA: 31.9–35.7 %) and *Chryseobacterium* spp. (RA: 29.0–30.8 %) during the experiment, with low variability of the abundance of the other side genera, e.g., *Methylocaldum* spp. (RA: 1.0–2.4 %), *Pseudomonas* spp. (RA: 6.2–8.1 %) and *Hyphomicrobium* spp. (RA: 3.2–6.8 %) (Fig. 6c). The biofilm harvested from the membrane module in R1 at the end of the experiment was characterized by the dominance of *Methylocystis* spp. (RA: 29.8 %), *Chryseobacterium* spp. (RA: 28.1 %) and *Hyphomicrobium* spp. (RA: 12.9 %) (Fig. 6c).

The dominance of *Methylocystis* spp. (RA: 29.7–37.8 %) and *Chryseobacterium* spp. (RA: 25.1–33.0 %) was also observed during R2 operation (Fig. 6d). The presence of *Pseudomonas* spp. and *Hyphomicrobium* spp. increased while moving from 1500 ppm_v of equivalent H_2S to 4000 and 6000 ppm_v in R2. Indeed, *Pseudomonas* spp., having a RA of 7.1 % in

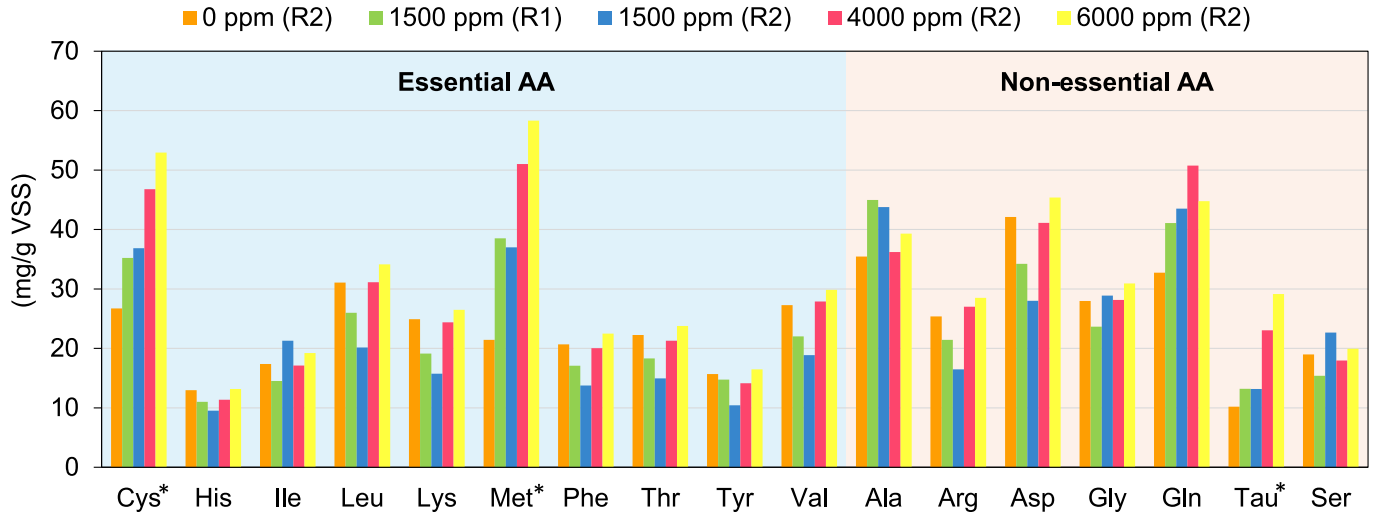

Fig. 5. Amino acid composition (mg AA/g VSS) of the biomass under different H_2S concentrations. The average AA composition of the biomass harvested from R1 during phases 3–6 in the presence of 1500 ppm_v is shown, as well as the AA composition of the biomass harvested from R2 during phases 4–7, in the presence of 0, 1500, 4000 and 6000 ppm_v of equivalent H_2S, respectively.
Amino acids classification between essential and non-essential is in accordance with Hou and Wu [23].
*: sulfur-containing amino acids.
Cys: cysteine, His: histidine, Ile: isoleucine, Leu: leucine, Lys: lysine, Met: methionine, Phe: phenylalanine, Thr: threonine, Tyr: tyrosine, Val: valine, Ala: alanine, Arg: arginine, Asp: aspartic acid, Gly: glycine, Gln: glutamine, Tau: taurine, Ser: serine.

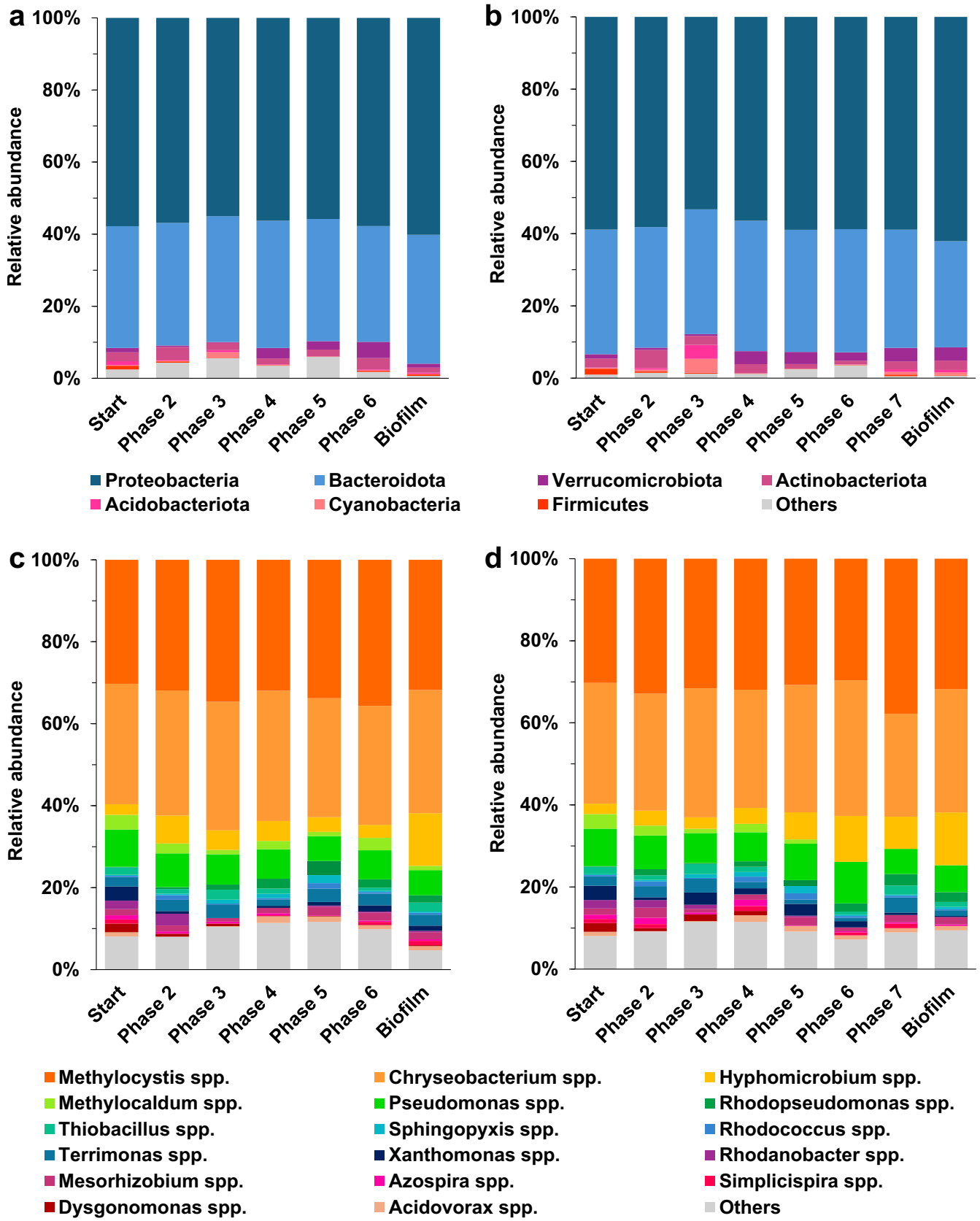

Fig. 6. Microbial community composition of the MOB-SOB biomass. The microbial community composition of the MOB-SOB biomass at the phylum (panels a and b) and genus (panels c and d) level is represented. The composition of the inoculum (Start) is reported as well as the microbial composition at the end of each experimental phase for R1 (panels a and c) and R2 (panels b and d). The composition of the biofilm harvested on the membrane module at the end of the experiment is also shown. Only the phyla and genera having a relative abundance higher than 1 % are reported.

phase 4, reached RAs of 8.9 % and 10.1 % in phases 5 and 6, respectively. Likewise, the abundance of *Hyphomicrobium* spp. increased from 3.9 % in phase 4 to 6.6 % in phase 5 and 11.2 % in phase 6.

Furthermore, a 7.9 % decrease of the RA of *Chryseobacterium* spp. was observed between phase 6 to phase 7, with relative abundances of 33.0 % and 25.1 %, respectively. The same trend was observed in *Pseudomonas* spp. and *Hyphomicrobium* spp., the relative abundances of which reached 6.1 % and 7.1 %, respectively, in phase 7, in the absence of sulfide. The presence of *Methylocaldum* spp. decreased from phase 1 to phase 5 (from a RA of 3.6 % to 1.0 %), after which it became less abundant (RA: <1 %). The attached biomass on the membrane module in R2 was characterized by the dominance of *Methylocystis* spp. (RA: 31.8 %), *Chryseobacterium* spp. (RA: 30.1 %) and *Hyphomicrobium* spp. (RA: 12.9 %) (Fig. 6d).

4. Discussion

4.1. Mineral nitrogen vs stripped ammonia as nitrogen source

The combination of nitrogen stripping and microbial fermentation envisaged in the proposed biogas-to-MP conversion platform represents a key strategy for biogas and digestate valorization towards high-value biobased products. The process of nitrogen stripping met the nitrogen requirements of the microbial fermentation process, while the low residual NH_4^+−N concentrations achieved in the treated digestate (Fig. 1a) highlighted its potential use for agronomic applications with lower environmental impacts, i.e., with reduced risks of run-off or volatilization.

Although better assimilation efficiencies could be obtained by optimizing the nitrogen supply according to the biomass growth, the mass balance on nitrogen (Table S5) demonstrated that the N feeding strategies were comparable within experimental variability, confirming the effectiveness of digestate nitrogen stripping as a valid strategy to provide nitrogen for the MP production. Moreover, the different conditions of nitrogen supply tested in R1 did not affect the microbial community composition (Fig. 6a and c).

Digestate nitrogen stripping also allowed to mitigate the acidification of the cultivation medium that occurred in the phases where nitrogen was provided in the mineral form (phases 1–3) (Fig. 3) and in previous studies on sulfide-rich biogas conversion into MP [6,7]. The propensity of the pH to acidify due to the CO_2 presence in the supplied biogas was in fact alleviated by the presence of NH_3 in the recirculating gas in R1, thereby lowering the need for pH buffering.

However, despite the utilization of the internal recirculating gas for NH_3 stripping has the advantage of not requiring an additional external gas flow, the composition of the recirculating gas might limit the efficiencies of the stripping process with respect to applications where external air is used as stripping agent. In the latter case, an almost complete ammonium stripping from the digestate can be achieved after 18–48 h under similar temperatures and gas-to-digestate ratios [10]. The pH is the main parameter affecting the nitrogen stripping process, with high ammonia recovery levels occurring only at high pH values [24]. The main process driving the pH increase in the digestate is the CO_2 stripping, thus the presence of CO_2 in the recirculation gas collected from the headspace of R1 (Fig. 3) likely limited the CO_2 stripping from the digestate, thereby resulting in a slower and less marked pH increase that might have limited the ammonia stripping [22]. CO_2 was indeed present in the recirculating gas stream used as stripping flow not only because it is present in the biogas supplied to the reactor, but also because it is produced by the MOB through methane oxidation [25], and was likely only partially consumed by autotrophic SOB [26].

Nevertheless, the direct utilization of the biogas/oxygen mixture as stripping agent for ammonia recovery from digestate allowed promising performance (up to 94 % ammonia recovered) when compared to previous studies where biogas was used for the same purpose. As an example, Serna-Maza et al. [12] observed lower efficiencies of NH_4^+-N recovery from digestate when the stripping process was performed at temperatures of 30, 55 and 70 °C (34 %, 42 % and 56 % of nitrogen recovery, respectively). The latter study achieved recovery rates close to those of this study only by adjusting the pH of the digestate up to 10, reaching a 92 % recovery of NH_4^+−N at 70 °C, thus requiring significantly higher expenses for digestate buffering and heating. Although the nitrogen removal efficiencies achieved in the present study were higher than those obtained by Kim et al. [27] (49–75 %), who used biogas as stripping gas for nitrogen recovery from digestate at a gas-to-digestate ratio of 5:1 and at a significantly higher temperature (70 °C), the stripping rates were much lower compared to those obtained in only 8 h by the same authors. This highlights the higher efficiency of the proposed process obtained without the need of pH adjustments of the digestate, meeting the nitrogen requirements of the microbial fermentation process as confirmed by the levels of residual nitrogen observed in the reactor (phases 4–6) (Fig. 1a).

The presence of oxygen in the recirculating gas stream (Fig. S11), which lowered the CO_2 concentration of the supplied biogas, might have limited the negative effects of utilizing a CO_2-rich stream as stripping agent, thereby allowing the promising results achieved in the proposed process. Nevertheless, the performance of the direct nitrogen stripping could be further improved by employing a separated air/oxygen stream as stripping agent, that would then provide the required O_2 to the process of microbial fermentation.

4.2. Mineral vs microbially produced sulfate as sulfur source

This study demonstrated the capability of the MOB-SOB culture to rely solely on the sulfate originating from the oxidation of biogas sulfide as sulfur source in the biogas-to-MP process. Sulfur is commonly supplied as nutrient to the microorganisms in the form of sulfate (SO_4^{2-}) [28]. The latter is produced as the end product of microbial sulfide oxidation [29].

The high levels of the residual sulfate observed in the reactors at the end of phase 2 (Fig. 2a) suggested the need to optimize the supply of mineral sulfate in the influent medium. The influent sulfate concentration was thereby decreased during phase 3 in R2. No influence on the biomass growth was observed by the decreased sulfate supply and the residual sulfate levels in phase 3 were higher than the influent sulfate concentration (100 mg/L) (Fig. 2a). This suggested that the microbial sulfate uptake was lower than the sulfate production through sulfide oxidation, as already experienced in previous experiments on the continuous sulfide-rich biogas-to-MP conversion [6]. Indeed, by comparing the sulfate concentrations measured in the reactors with the influent sulfide concentration (1500 ppm_v of equivalent H_2S), the sulfate increase was comparable to the sulfate potentially produced from the complete sulfide oxidation [29].

During phase 4, $MgSO_4$ was not provided anymore with the influent cultivation medium and was replaced by $MgCl_2$. This did not affect biomass growth and productivity in contrast with a previous study on H_2S-rich biogas-to-MP conversion, where a 20 % depletion of the biomass concentration was observed when $MgSO_4$ supply was discontinued [6]. In that case, however, both magnesium and sulfate were removed simultaneously from the influent medium, thereby suggesting that the main cause of biomass depletion was the lack of Mg, which is an important nutrient for biomass growth [30]. The latter observations were confirmed by the batch tests investigating the role of the absence of magnesium and sulfate separately (Fig. 4a) which showed detrimental effects of the lack of magnesium on biomass growth and yield, while no effects of the absence of sulfate. Despite the absence of mineral sulfate supply, sulfur was nevertheless provided in the form of $Na_2S{\cdot}9H_2O$, thus possibly explaining the SOB-driven sulfate production through sulfide oxidation, also experienced in R2, where measurable sulfate concentrations were detected despite the discontinuation of sulfate feeding in phase 4 (Fig. 2a), confirming the ability of the MOB-SOB biomass to rely on biogas sulfide as the sole sulfur source.

4.3. Influence of H_2S concentration on biogas-to-MP conversion

The H_2S-rich biogas-to-MP conversion process was effectively performed in hollow fiber membrane bioreactors, enabling efficient gas mass transfer and allowing the development of both attached and suspended MOB-SOB biomass tolerant to sulfide toxicity. The membrane-based system allowed to tolerate increasing sulfide levels thanks to the different microbial composition of the biofilm attached to the membranes, which might have allowed the retention of specific SOB mitigating the toxic effects of sulfide and, therefore, enabling a better utilization of the gaseous substrates, confirming the findings of a previous study on MP production from H_2S-rich biogas using hollow fiber membrane bioreactors [8].

The H_2S concentration in biogas is highly variable (0–10,000 ppm_v) and depends on the nature of the organic substrate subjected to AD, with protein-rich substrates inducing particularly high sulfide concentrations [31]. H_2S reportedly inhibits methanotrophic growth by limiting the activity of methane monooxygenase (MMO), the enzyme catalyzing the methane oxidation reaction, which copper sites are inactivated by the precipitation of copper sulfide [32]. The MOB-SOB culture was capable of growing on biogas at high equivalent H_2S concentrations, showing only a marginal inhibitory effect of sulfide on MOB growth (Fig. 2a and b), with clear inhibition occurring only beyond 6000 ppm_v of equivalent H_2S as shown in the batch tests (Fig. 4b). The latter is confirmed by the higher carbon conversion efficiencies observed in this study in the presence of high equivalent H_2S concentrations (Table S6) compared to those obtained with a bubble column reactor type (7.6 % and 6.0 % in the presence of 3000 and 4000 ppm_v of equivalent H_2S) [6]. Shifting from 1500 to 4000 ppm_v of equivalent H_2S caused a 40 % decrease of the biomass concentration when the process of biomass-to-MP conversion with the same MOB-SOB culture was performed in bubble column reactors [6], while in the present study the decrease of biomass concentration was limited to 15 %.

The sulfide inhibition on the MOB-SOB mixed culture was clearly lower than that observed in other studies on methanotrophic-driven biogas conversion to MP thanks to the microbial community composition of the MOB-SOB culture. The most abundant genera were *Methylocystis* spp. and *Chryseobacterium* spp. during the whole experiment in both reactors (Fig. 6). *Methylocystis* spp. is a methanotroph type II, which demonstrates inhibition in the presence of low concentrations of H_2S in the biogas when cultivated alone, even at 500 ppm_v [33] or 200 ppm_v [34].

Chryseobacterium spp., a heterotroph commonly found in sulfide-rich environments and in sulfide oxidation systems [35,36], was the other dominant strain of the mixed culture used in the experiment. The combination of MOB with several bacterial groups capable of sulfide oxidation, e.g., *Chryseobacterium*, *Pseudomonas*, *Thiobacillus* and *Hyphomicrobium* [34,37], significantly improved the resistance to high equivalent H_2S concentrations in biogas (1500–6000 ppm_v).

The enhanced performance of H_2S-rich biogas-to-MP conversion in hollow fiber membrane reactors and the improved resistance to sulfide toxicity was further favored by the presence of the biomass under both attached and suspended forms. The biofilm biomass harvested on the membrane modules in R1 and R2, apart from the dominance of *Methylocystis* spp. and *Chryseobacterium* spp., showed also a particularly abundant presence of *Hyphomicrobium* spp. which had higher RAs than in the suspended biomass harvested at the same time (end of phase 6 in R1 and of phase 7 in R2). Thus, as already mentioned above, the significant dominance in the biofilm biomass of SOB strains, e.g., *Chryseobacterium* spp., *Hyphomicrobium* spp. and *Pseudomonas* spp., might have enhanced the resistance to high sulfide concentrations in biogas.

H_2S inhibition of methanotrophic growth has been reported already at concentrations as low as 200 ppm_v [3]. The extent of sulfide inhibition varies among different methanotrophic cultures and is higher in pure cultures compared to mixed microbial cultures. As an example, the MOB genus dominant in the present study, i.e., *Methylocystis* spp., showed a 85 % lower growth in the presence of 500 ppm_v of H_2S in the biogas than in the absence of sulfide when cultivated as pure culture [33]. Xu et al. [4] instead experienced sulfide inhibition for the growth of a pure culture of *Methylocapsa acidiphila* above 1000 ppm_v of H_2S in biogas. A 44–60 % inhibition of the growth of *Methylocaldum* spp. was caused by a biogas containing 1000 ppm_v of H_2S, with a more severe inhibition occurring at 3000 ppm_v [31]. In another study, the growth of *Methylophilus* spp. was inhibited by 44 % in the presence of 1000 ppm_v H_2S in the biogas and, after a further increase of the concentration to 5000 ppm_v, the biomass concentration decreased by 90 % [38]. Lee et al. [34] observed inhibition of the activity of *Methylocystis* spp. already at 200 ppm_v, with a 35 % lower methane oxidation rate than in the absence of sulfide.

The variable sulfur supply tested in R2 affected the microbial community composition (Fig. 5b and d). Indeed, the RA of *Methylocaldum* spp. was negatively affected by the increasing concentrations of equivalent H_2S in biogas (phases 4–6) (Fig. 5d). Rising levels of equivalent H_2S in the biogas stimulated instead the presence of some SOB, e.g., *Pseudomonas* spp. and *Hyphomicrobium* spp., i.e., heterotrophic bacteria which might have grown on the organic metabolites produced by MOB during methane oxidation. They might thus have contributed to the growth of the mixed culture as methanotrophic activity is potentially hindered by the accumulation of certain organic metabolites [39]. The mixed culture also comprised other heterotrophs frequently detected in aerobic systems, including *Terrimonas* spp. and *Xanthomonas* spp. [40,41].

The biomass yields observed in this study (up to 0.218 g VSS/g CH_4), despite being close to those achieved in other studies for biogas-to-MP production in membrane reactors [42], are rather lower compared to other studies for continuous biogas-to-MP production [43,44]. This could be explained by the lower biomass yield that typically characterize type II methanotrophs [45], in contrast to the higher growth performance shown by type I methanotrophs [8]. The latter aspect emphasizes how the design of dedicated microbial consortia could help improving biomass yield, although this might affect the robustness and the stability of the biological process.

4.4. Biomass quality: protein content and amino acid profile

Despite the various operational changes, a stable and high biomass protein content was observed in both reactors (Figs. 1c and 2c), confirming the potential of the employed MOB-SOB culture of acquiring high protein concentrations in line with those typically found in MOB biomass grown on biogas [4,46], and H_2S-rich biogas [6,7]. The protein content and AA profile of the biomass was comparable to that of conventional protein sources, e.g., fishmeal or soybean [47], thereby confirming the suitability of the produced MP for feed or food applications.

The AA profile of the harvested biomass was overall stable with respect to the nitrogen supply strategy in R1, with only slight variations of the concentration of some AA (Table S9), thereby confirming that using NH_4^+–N from digestate stripping did not affect the protein and AA content of the biomass. In contrast, the AA profile was markedly influenced by the increasing sulfide concentrations tested in R2, which enhanced the total AA content of the biomass (Table S9) as already reported by Areniello et al. [6]. This contrasts studies using pure methanotrophic cultures showing detrimental effects of sulfide in biogas on protein and AA synthesis [4].

The most relevant effect of the increasing biogas equivalent H_2S concentration was observed on the S-AA, i.e., cysteine, methionine and taurine (Fig. 5), confirming the beneficial influence of sulfide on S-AA synthesis [48]. This result is particularly encouraging as MP are commonly deficient in S-AA, thus supplementation of methionine and cysteine is often required for MP used as feed ingredient [49]. Moreover, methionine and cysteine are essential AA lacking in most of the conventional plant-based sources, e.g., legumes [50]. The methionine and cysteine (Met + Cys) concentrations obtained in the harvested biomass

(96.2 ± 210.2 mg/g protein) are particularly promising compared to the nutritional daily requirements for Met + Cys for infants and adults that are 42 and 25 mg/g protein, respectively. The latter makes the produced MP biomass competitive against common alternative protein sources, i.e., soybean meal and fishmeal, having average Met + Cys contents of 28 and 30 mg/g protein, respectively [51,52]. The attained S-AA content resulted in a sulfur content of the harvested biomass (Table S10) higher than what is reported in the literature for microbial biomass (around 1 % g S/g VSS) [53].

Glutamic acid (32.7–50.7 mg/g VSS) confirmed to be among the most abundant AA in methanotrophic biomass [40,54,55]. Alanine, although not essential, is important for the intracellular muscle buffering [38] and was present at high concentrations (35.4–48.1 mg/g VSS). The increased equivalent H_2S concentrations also benefitted the synthesis of lysine, an essential AA largely utilized as diet supplement in fishmeal and animal feed as it favors animal growth and muscle development [40].

4.5. Potential advantages and bottlenecks of the integrated conversion of digestate nitrogen and H_2S-rich biogas to MP

The economic performance of the integrated platform developed in this study, coupling digestate nitrogen recovery with the conversion of H_2S-rich biogas to MP, can be preliminarily evaluated drawing on previous works that assessed the techno-economic feasibility of on-site upcycling of biogas carbon and mineral nitrogen recovered from anaerobic digestion for MP production. Although a comprehensive techno-economic assessment lies beyond the scope of this study, preliminary evaluations can provide valuable insights into the advantages of the proposed integrated strategy. A detailed breakdown of these considerations is provided in Section S12 and Tables S12 and S13.

Verbeeck et al. [50], in their assessment of biogas-to-MP conversion via a MOB consortium using ammonia recovered from anaerobic digestate (through a stripping process), estimated an MP production cost of 1.54 €/kg.

Based on their estimations, by eliminating the need for biogas desulfurization, MP production costs could be reduced by about 4 %, reaching 1.48 €/kg (Section S12 and Table S13). Furthermore, the direct use of H_2S-rich biogas would obviate the need for sulfur supplementation with chemicals such as $MgSO_4$, offering an additional cost advantage.

Conventional stripping processes for ammonium recovery from digestate generally rely on external airflow as the stripping agent. In contrast, the platform proposed here employs the recirculation of unused headspace gas from the fermentation unit, using the existing recirculation pump, thus avoiding the need for additional air supply (Fig. S3). This also eliminates the requirement for chemicals (e.g., NaOH) to increase digestate pH. Avoiding both air-blowing equipment and chemical dosing reduces the total MP production cost to 1.22 €/kg (Table S13).

Assuming an average protein content of 60 % in MOB biomass, the corresponding price per kilogram of protein is 2.03 €. This is particularly promising when benchmarked against conventional protein sources such as fishmeal (1.6 €/kg product, ~60 % protein) and soybean meal (0.42 €/kg product, ~40 % protein) [53,54], which correspond to protein prices of 2.67 €/kg and 1.15 €/kg, respectively [55]. It is also worth noting that this preliminary evaluation does not account for potential savings in externalized costs associated with MP production, such as reduced water consumption, land use, nitrogen pollution, and greenhouse gas emissions, which would further improve the relative competitiveness of MP compared to conventional protein sources.

On the other hand, dealing with gaseous substrates constitutes a major technological bottleneck for cost-effective production and poses the need for an efficient bioreactor design capable of enhancing mass transfer between gas and liquid phases [8]. Therefore, the scalability of biogas-to-MP conversion is tightly linked to the optimization of bioreactor configuration, which must ensure maximized substrate conversion rates and yields. In parallel, tailoring microbial consortia according to the metabolic traits of selected strains is equally crucial. Together, these advancements would contribute to improving essential performance indicators, including protein and biomass yields and productivities, thereby strengthening the overall techno-economic viability of the process.

5. Conclusions

The feasibility of the combined valorization of H_2S-rich biogas and digestate nitrogen into sulfur-rich microbial protein was demonstrated in this study. The MP production process could rely solely on the nitrogen stripped from anaerobic digestate and on the sulfate produced by the biogas H_2S oxidation performed by the SOB as nitrogen and sulfur sources, respectively. The nitrogen stripping from anaerobic digestate through the internal biogas recirculation flow achieved nitrogen stripping rates of 109.6 mg NH_4^+−N/L·d at 45 °C, with residual NH_4^+−N levels in the digestate of 58.4 (±4.5) NH_4^+−N/L, making it suitable for agronomic use.

Magnesium was found to be a crucial nutrient to support microbial growth, therefore highlighting the need to not discontinue its supply along with that of sulfate. The extent of H_2S inhibition was clearly lower than in previous studies testing the methanotrophic growth on H_2S-containing biogas, mainly thanks to the microbial community of the biofilm biomass, which was comprised of *Methylocystis* spp. and several SOB. The increasing H_2S levels were beneficial for the synthesis of amino acids, and in particular for the accumulation of sulfur-containing amino acids, having a pivotal importance in view of the utilization of the produced MP biomass for nutritional applications. The potential of the proposed platform to replace conventional protein production, e.g., fishmeal and soybean meal, was demonstrated, although a proper design of microbial consortia and bioreactor configurations would improve biomass/protein productivities.

CRediT authorship contribution statement

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

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by Science Foundation Ireland (SFI) through the MaREI Centre for Energy, Climate and Marine research and innovation under Grants No. 12/RC/2302_P2 and 16/SP/3829 as well as the SFI Research Professorship Programme entitled Innovative Energy Technologies for Biofuels, Bioenergy and a Sustainable Irish Bioeconomy (IETSBIO^3; grant number 15/RP/2763) and the Research Infrastructure research grant Platform for Biofuel Analysis (Grant Number 16/RI/3401). This research was carried out within the framework of the MedInCircle project. MedInCircle is part of the PRIMA program supported by the European Union. This project received funding from ANR (France), MUR (Italy), STDF (Egypt) and TUBITAK (Turkey) as part of the PRIMA program. The content of the present article reflects only the author's view, and the PRIMA Foundation is not

responsible for any use that may be made of the information it contains.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jwpe.2025.109239.

Data availability

Data will be made available on request.

References

[1] B. Khoshnevisan, P. Tsapekos, Y. Zhang, B. Valverde-Pérez, I. Angelidaki, Urban biowaste valorization by coupling anaerobic digestion and single cell protein production, Bioresour. Technol. 290 (2019) 121743, https://doi.org/10.1016/j.biortech.2019.121743.

[2] S. Matassa, N. Boon, I. Pikaar, W. Verstraete, Microbial protein: future sustainable food supply route with low environmental footprint, Microb. Biotechnol. 9 (2016) 568–575, https://doi.org/10.1111/1751-7915.12369.

[3] R. Li, X.L. Fan, Y.F. Jiang, R.N. Wang, R.B. Guo, Y. Zhang, S.F. Fu, From anaerobic digestion to single cell protein synthesis: a promising route beyond biogas utilization, Water Res. 243 (2023) 120417, https://doi.org/10.1016/j.watres.2023.120417.

[4] M. Xu, H. Zhou, X. Yang, I. Angelidaki, Y. Zhang, Sulfide restrains the growth of *Methylocapsa acidiphila* converting renewable biogas to single cell protein, Water Res. 184 (2020) 116138, https://doi.org/10.1016/j.watres.2020.116138.

[5] Z. Yang, P. Tsapekos, Y. Zhang, Y. Zhang, I. Angelidaki, W. Wang, Bio-electrochemically extracted nitrogen from residual resources for microbial protein production, Bioresour. Technol. 337 (2021) 125353, https://doi.org/10.1016/j.biortech.2021.125353.

[6] M. Areniello, S. Matassa, G. Esposito, P.N.L. Lens, H2S-laden biogas triggers sulfur amino acids production in microbial protein synthesized during mixed culture fermentation by methane and sulfur oxidizing bacteria, Chem. Eng. J. 490 (2024) 151648, https://doi.org/10.1016/j.cej.2024.151648.

[7] M. Areniello, S. Matassa, G. Esposito, P.N.L. Lens, Microbial protein production from sulfide-rich biogas through an enrichment of methane- and sulfur-oxidizing bacteria, Bioresour. Technol. 383 (2023) 129237, https://doi.org/10.1016/j.biortech.2023.129237.

[8] M. Areniello, S. Matassa, G. Esposito, P.N.L. Lens, Sulfur-containing microbial protein production from sulfide-rich biogas in hollow fiber membrane bioreactors, J. Environ. Chem. Eng. 13 (2025) 115264, https://doi.org/10.1016/j.jece.2024.115264.

[9] K. Verbeeck, J. De Vrieze, I. Pikaar, W. Verstraete, K. Rabaey, Assessing the potential for up-cycling recovered resources from anaerobic digestion through microbial protein production, Microb. Biotechnol. 14 (2021) 897–910, https://doi.org/10.1111/1751-7915.13600.

[10] S. Matassa, V. Pelagalli, S. Papirio, C. Zamalloa, W. Verstraete, G. Esposito, F. Pirozzi, Direct nitrogen stripping and upcycling from anaerobic digestate during conversion of cheese whey into single cell protein, Bioresour. Technol. 358 (2022) 127308, https://doi.org/10.1016/j.biortech.2022.127308.

[11] Q.B. Zhao, J. Ma, I. Zeb, L. Yu, S. Chen, Y.M. Zheng, C. Frear, Ammonia recovery from anaerobic digester effluent through direct aeration, Chem. Eng. J. 279 (2015) 31–37, https://doi.org/10.1016/j.cej.2015.04.113.

[12] A. Serna-Maza, S. Heaven, C.J. Banks, Biogas stripping of ammonia from fresh digestate from a food waste digester, Bioresour. Technol. 190 (2015) 66–75, https://doi.org/10.1016/j.biortech.2015.04.041.

[13] M. Areniello, S. Matassa, G. Esposito, P.N.L. Lens, Biowaste upcycling into second-generation microbial protein through mixed-culture fermentation, Trends Biotechnol. 41 (2023) 197–213, https://doi.org/10.1016/j.tibtech.2022.07.008.

[14] S. Upadhyaya, S. Tiwari, N. Arora, D.P. Singh, Microbial protein: a valuable component for future food security, in: J.S. Singh, D.P. Singh (Eds.), Microbes and Environmental Management, Studium Press, USA, 2016, pp. 260–279, https://doi.org/10.13140/RG.2.1.1775.8801.

[15] G.M. Walker, The roles of magnesium in biotechnology, Crit. Rev. Biotechnol. 14 (1994) 311–354, https://doi.org/10.3109/07388559409063643.

[16] S. Papirio, Á. Trujillo-Reyes, S. di Perta, A. Kalogiannis, G. Kassab, S. Khoufi, S. Sayadi, L. Frunzo, G. Esposito, F.G. Fermoso, K. Stamatelatou, Exploring the biochemical methane potential of wholesale market waste from Jordan and Tunisia for a future scale-up of anaerobic digestion in Amman and Sfax, Waste Biomass Valoriz. 13 (2022) 3887–3897, https://doi.org/10.1007/s12649-022-01790-1.

[17] M.T. Sun, Z.M. Yang, S.F. Fu, X.L. Fan, R.B. Guo, Improved methane removal in exhaust gas from biogas upgrading process using immobilized methane-oxidizing bacteria, Bioresour. Technol. 256 (2018) 201–207, https://doi.org/10.1016/j.biortech.2018.02.020.

[18] A. APHA, Standard Methods for the Examination of Water and Wastewater, American Public Health Association, American Water Works Association, and Water Environment Federation, Washington, DC, 2005.

[19] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275, https://doi.org/10.1016/S0021-9258(19)52451-6.

[20] C. Moscariello, S. Matassa, F. Pirozzi, G. Esposito, S. Papirio, Valorisation of industrial hemp (Cannabis sativa L.) residues and cheese whey into volatile fatty acids for single cell protein production, Environ. Sci. Ecotechnol. 21 (2024) 100439, https://doi.org/10.1016/J.ESE.2024.100439.

[21] Inc. Illumina, 16S Metagenomic Sequencing Library, Illumina.Com, 2013, pp. 1–28. http://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf.

[22] S. Guštin, R. Marinšek-Logar, Effect of pH, temperature and air flow rate on the continuous ammonia stripping of the anaerobic digestion effluent, Process Saf. Environ. Prot. 89 (2011) 61–66, https://doi.org/10.1016/j.psep.2010.11.001.

[23] Y. Hou, G. Wu, Nutritionally essential amino acids, Adv. Nutr. 9 (2018) 849–851, https://doi.org/10.1093/advances/nmy054.

[24] D. Yang, Q. Chen, R. Liu, L. Song, Y. Zhang, X. Dai, Ammonia recovery from anaerobic digestate: state of the art, challenges and prospects, Bioresour. Technol. 363 (2022) 127957, https://doi.org/10.1016/j.biortech.2022.127957.

[25] M.G. Kalyuzhnaya, O.A. Gomez, J.C. Murrell, The Methane-Oxidizing Bacteria (Methanotrophs), 2019, https://doi.org/10.1007/978-3-319-60053-6_10-1.

[26] T. Ito, K. Sugita, S. Okabe, Isolation, characterization, and in situ detection of a novel chemolithoautotrophic sulfur-oxidizing bacterium in wastewater biofilms growing under microaerophilic conditions, Appl. Environ. Microbiol. 70 (2004) 3122–3129, https://doi.org/10.1128/AEM.70.5.3122-3129.2004.

[27] D.G. Kim, S. Witherrite, L. Yu, Q. Zhao, S. Chen, Novel ammonia recovery from anaerobic digestion by integrating biogas stripping and gypsum absorption, Process Saf. Environ. Prot. 180 (2023) 48–55, https://doi.org/10.1016/j.psep.2023.09.059.

[28] M.G. Fox-Powell, C.S. Cockell, Building a geochemical view of microbial salt tolerance: halophilic adaptation of Marinococcus in a natural magnesium sulfate brine, Front. Microbiol. 9 (2018) 1–10, https://doi.org/10.3389/fmicb.2018.00739.

[29] D. Pokorna, J. Zabranska, Sulfur-oxidizing bacteria in environmental technology, Biotechnol. Adv. 33 (2015) 1246–1259, https://doi.org/10.1016/j.biotechadv.2015.02.007.

[30] D.W. Tempest, J.T.M. Wouters, Properties and performance of microorganisms in chemostat culture, Enzym. Microb. Technol. 3 (1981) 283–290, https://doi.org/10.1016/0141-0229(81)90001-6.

[31] W. Zhang, X. Ge, Y.-F. Li, Z. Yu, Y. Li, Isolation of a methanotroph from a hydrogen sulfide-rich anaerobic digester for methanol production from biogas, Process Biochem. 51 (2016) 838–844, https://doi.org/10.1016/j.procbio.2016.04.003.

[32] J.D. Semrau, A.A. Dispirito, S. Yoon, Methanotrophs and copper, FEMS Microbiol. Rev. 34 (2010) 496–531, https://doi.org/10.1111/j.1574-6976.2010.00212.x.

[33] M. Càceres, J.C. Gentina, G. Aroca, Oxidation of methane by *Methylomicrobium album* and Methylocystis sp. in the presence of H2S and NH3, Biotechnol. Lett. 36 (2014) 69–74, https://doi.org/10.1007/s10529-013-1339-7.

[34] E.H. Lee, K.E. Moon, T.G. Kim, S.D. Lee, K.S. Cho, Inhibitory effects of sulfur compounds on methane oxidation by a methane-oxidizing consortium, J. Biosci. Bioeng. 120 (2015) 670–676, https://doi.org/10.1016/j.jbiosc.2015.04.006.

[35] L.S. Azevedo, I.M.P. Castro, C.D. Leal, J.C. Araújo, C.A.L. Chernicharo, Performance and bacterial diversity of bioreactors used for simultaneous removal of sulfide, solids and organic matter from UASB reactor effluents, Water Sci. Technol. 78 (2018) 1312–1323, https://doi.org/10.2166/wst.2018.403.

[36] R. Khanongnuch, F. Di Capua, A.M. Lakaniemi, E.R. Rene, P.N.L. Lens, Effect of N/S ratio on anoxic thiosulfate oxidation in a fluidized bed reactor: experimental and artificial neural network model analysis, Process Biochem. 68 (2018) 171–181, https://doi.org/10.1016/j.procbio.2018.02.018.

[37] J. Das, H. Ravishankar, P.N.L. Lens, Biological removal of gas-phase H2S in hollow fibre membrane bioreactors, J. Chem. Technol. Biotechnol. 97 (2022) 1149–1161, https://doi.org/10.1002/jctb.6999.

[38] P. Tsapekos, B. Khoshnevisan, X. Zhu, X. Zha, I. Angelidaki, Methane oxidising bacteria to upcycle effluent streams from anaerobic digestion of municipal biowaste, J. Environ. Manag. 251 (2019) 109590, https://doi.org/10.1016/j.jenvman.2019.109590.

[39] H. Bothe, K. Møller Jensen, A. Mergel, J. Larsen, C. Jørgensen, H. Bothe, L. Jørgensen, Heterotrophic bacteria growing in association with Methylococcus capsulatus (Bath) in a single cell protein production process, Appl. Microbiol. Biotechnol. 59 (2002) 33–39, https://doi.org/10.1007/S00253-002-0964-1.

[40] M. Xu, D. Zhao, X. Zhu, Y. Su, I. Angelidaki, Y. Zhang, Biogas upgrading and valorization to single-cell protein in a bioinorganic electrosynthesis system, Chem. Eng. J. 426 (2021) 131837, https://doi.org/10.1016/j.cej.2021.131837.

[41] K. Vikrant, S.K. Kailasa, D.C.W. Tsang, S.S. Lee, P. Kumar, B.S. Giri, R.S. Singh, K. H. Kim, Biofiltration of hydrogen sulfide: trends and challenges, J. Clean. Prod. 187 (2018) 131–147, https://doi.org/10.1016/j.jclepro.2018.03.188.

[42] B. Valverde-Pérez, W. Xing, A.A. Zachariae, M.M. Skadborg, A.F. Kjeldgaard, A. Palomo, B.F. Smets, Cultivation of methanotrophic bacteria in a novel bubble-free membrane bioreactor for microbial protein production, Bioresour. Technol. 310 (2020) 123388, https://doi.org/10.1016/j.biortech.2020.123388.

[43] Y. Ma, T. Liu, Z. Yuan, J. Guo, Single cell protein production from methane in a gas-delivery membrane bioreactor, Water Res. 259 (2024) 121820, https://doi.org/10.1016/j.watres.2024.121820.

[44] B. Khoshnevisan, L. He, M. Xu, B. Valverde-Pérez, J. Sillman, G.C. Mitraka, P. G. Kougias, Y. Zhang, S. Yan, L. Ji, M. Carbajales-Dale, S.N. Elyasi, H. Marami, P. Tsapekos, H. Liu, I. Angelidaki, From renewable energy to sustainable protein sources: advancement, challenges, and future roadmaps, Renew. Sust. Energ. Rev. 157 (2022), https://doi.org/10.1016/j.rser.2021.112041.

[45] P.J. Strong, S. Xie, W.P. Clarke, Methane as a resource: can the methanotrophs add value? Environ. Sci. Technol. 49 (2015) 4001–4018, https://doi.org/10.1021/es504242n.
[46] D. Van Der Ha, L. Nachtergaele, F.M. Kerckhof, D. Rameiyanti, P. Bossier, W. Verstraete, N. Boon, Conversion of biogas to bioproducts by algae and methane oxidizing bacteria, Environ. Sci. Technol. 46 (2012) 13425–13431, https://doi.org/10.1021/es303929s.
[47] F.M. Kerckhof, M. Sakarika, M. Van Giel, M. Muys, P. Vermeir, J. De Vrieze, S. E. Vlaeminck, K. Rabaey, N. Boon, From biogas and hydrogen to microbial protein through co-cultivation of methane and hydrogen oxidizing bacteria, Front. Bioeng. Biotechnol. 9 (2021) 1–17, https://doi.org/10.3389/fbioe.2021.733753.
[48] K. Soda, Microbial sulfur amino acids: an overview, Methods Enzymol. 143 (1987) 453–459, https://doi.org/10.1016/0076-6879(87)43080-2.
[49] M. Sharif, M.H. Zafar, A.I. Aqib, M. Saeed, M.R. Farag, M. Alagawany, Single cell protein: sources, mechanism of production, nutritional value and its uses in aquaculture nutrition, Aquaculture 531 (2021) 735885, https://doi.org/10.1016/j.aquaculture.2020.735885.
[50] P. Tsapekos, X. Zhu, E. Pallis, I. Angelidaki, Proteinaceous methanotrophs for feed additive using biowaste as carbon and nutrients source, Bioresour. Technol. 313 (2020) 123646, https://doi.org/10.1016/j.biortech.2020.123646.
[51] M. Øverland, A.H. Tauson, K. Shearer, A. Skrede, Evaluation of methane-utilising bacteria products as feed ingredients for monogastric animals, Arch. Anim. Nutr. 64 (2010) 171–189, https://doi.org/10.1080/17450391003691534.
[52] L. Gasco, F. Gai, G. Maricchiolo, L. Genovese, S. Ragonese, T. Bottari, G. Caruso, Fishmeal alternative protein sources for aquaculture feeds, in: Feeds for the Aquaculture Sector, Springer International, New York, NY, USA, 2018, pp. 1–28. https://link.springer.com/content/pdf/10.1007%2F978-3-319-77941-6.pdf.
[53] E. Metcalf, E. Eddy, Wastewater Engineering: Treatment and Reuse, McGraw Hill Companies, Inc., New York, 2003.
[54] Z. Rasouli, B. Valverde-Pérez, M. D'Este, D. De Francisci, I. Angelidaki, Nutrient recovery from industrial wastewater as single cell protein by a co-culture of green microalgae and methanotrophs, Biochem. Eng. J. 134 (2018) 129–135, https://doi.org/10.1016/j.bej.2018.03.010.
[55] B. Khoshnevisan, M. Dodds, P. Tsapekos, E. Torresi, B.F. Smets, I. Angelidaki, Y. Zhang, B. Valverde-Pérez, Coupling electrochemical ammonia extraction and cultivation of methane oxidizing bacteria for production of microbial protein, J. Environ. Manag. 265 (2020), https://doi.org/10.1016/j.jenvman.2020.110560.