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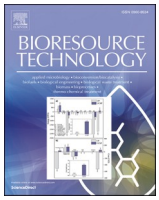

Novel photo-chemoautotrophic system combining microalgae and hydrogen oxidizing bacteria for microbial protein production from carbon dioxide☆

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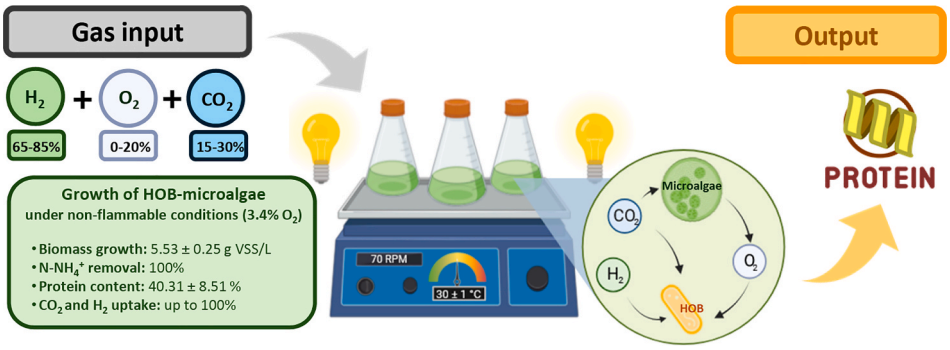
HIGHLIGHTS

- Photo-chemoautotrophic microbial protein production from CO_2 was investigated.
- Co-cultivating microalgae and hydrogen-oxidizing bacteria led to synergic effects.
- A gas mixture with a $H_2/O_2/CO_2$ ratio of 68/3.4/16 led to the highest biomass growth.
- The microbial consortium reached a biomass concentration up to 5.5 ± 0.3 g VSS/L.
- The produced biomass was characterized by a protein content of 40.3 ± 8.5 %.

GRAPHICAL ABSTRACT



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ABSTRACT

This study investigated the production of microbial protein (MP) using a novel microbial consortium composed of microalgae and hydrogen-oxidizing bacteria (HOB). This photo-chemoautotrophic consortium was aimed at capturing and valorising carbon dioxide (CO_2) while overcoming typical limitations of each microbial group. Batch tests were run under varying gas mixtures of oxygen (O_2), CO_2 and hydrogen (H_2) to assess process performance in terms of biomass growth, nitrogen assimilation, gas consumption, final biomass and microbial community composition. The consortium was compared to the single microalgae and HOB cultures grown under photo- and chemoautotrophic conditions, respectively. The consortium achieved significant growth (5.5 g VSS/L) under a non-explosive gas mixture, reaching a protein content of 40.3 ± 8.5 %. The consortium achieved up to 1.8- and 10-times higher biomass growth compared to the single microalgae and HOB cultures, respectively, showing a higher CO_2 consumption and stimulating photosynthetic O_2 production.

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1. Introduction

Global population growth, expected to reach up to more than 9 billion people by 2050 (Areniello et al., 2023), together with the increasingly widespread economic wellbeing, are boosting the demand for protein-rich foods. In this context, meeting the future global protein demand will require an increase by up to 78 % of the current protein production potential (Areniello et al., 2023; Henchion et al., 2017). Meeting this growing demand through conventional protein sources (i.e., animal and vegetal) would further exacerbate the impact of anthropogenic greenhouse gases (GHG) emissions arising from livestock, which already account for 14.5 % of the global GHG emissions (Bateki et al., 2023).

For these reasons, the development of systems capable of combining alternative protein production with carbon capture and utilization (CCU) techniques is increasingly attracting interest. A potential solution in this sense is represented by microbial protein (MP), which constitute the dried microbial cells of microalgae, yeasts, fungi, and bacteria, obtained from autotrophic, CO_2-fixing microorganisms (Ritala et al., 2017). Microalgae are a well-established form of autotrophic MP as they are in average characterised by a protein content of about 40 %, which can reach up to 70 %, and low nucleic acid levels (Spiller et al., 2020). Being photoautotrophs, microalgae fix inorganic CO_2 through light and convert it into protein-rich biomass while producing oxygen (O_2) as a result of the photosynthetic activity (Cardol et al., 2011). Despite the interesting features of microalgae, their cultivation can be limited by many factors, such as a low light penetration in the presence of dense cultures (self-shading) or the inhibition of light receptors by strong light radiation (photoinhibition) (McGinn et al., 2011). Another factor that could lead to a limitation of microalgal growth is the accumulation of high O_2 concentrations, which could lead to the photo-oxidation of chlorophyll when paired with an excess of light (Hong et al., 2020), and/or trigger the onset of photorespiration. Photorespiration activates in presence of high O_2:CO_2 ratios when the oxygenase enzyme reacts with O_2 instead of CO_2, ultimately leading to a higher energy expense and limited biomass growth (Kliphuis et al., 2011). Moreover, the activation of other metabolic pathways such as that of photoreduction, which can enhance the growth of microalgae in the presence of hydrogen (H_2) gas by consuming it through the activation of the hydrogenase enzyme, is also negatively affected by the presence of oxygen (Sleutels et al., 2020). In addition to microalgae, bacteria are also particularly suited for the production of autotrophic MP thanks to their high protein content (up to 80 % protein over dry weight) and rapid growth (Areniello et al., 2023). Chemoautotrophic bacteria, of which the aerobic hydrogen oxidizing bacteria (HOB), or Knallgas bacteria, are an example, are already being used by several companies for the production of both feed and food (Pander et al., 2020). Being chemoautotrophs, the HOB are able to fix carbon dioxide and synthesize new biomass by using the chemical energy released through hydrogen oxidation (Ishizaki and Tanaka, 1990). Although light is not required, the main issue with HOB cultivation stands in their need for a gas mixture composed of both H_2 and O_2, which poses flammability hazards when the volumetric percentage of O_2 in the atmosphere of H_2 is higher than 5 % (Zlochower and Green, 2009). To overcome the limitations linked to the cultivation of the single microbial groups alone, a microbial consortium of HOB and microalgae could offer several advantages and potential, yet unexplored, synergies. As a matter of fact, culturing microalgae together with HOB could help limiting the accumulation of oxygen, thereby mitigating the negative effects of photorespiration as well as enabling effective photoreduction in the presence of H_2 gas. H_2, which is necessary for the growth of HOB, could be oxidized by these bacteria thanks to the O_2 continuously produced by microalgae through their photosynthetic metabolism, thereby avoiding the need of supplying high volumes of potentially explosive gas mixtures. In light of the above, the present study explored the possibility of combining MP production to CCU through a photo-chemoautotrophic system implementing a HOB-microalgae microbial consortium exposed to light and fed with variable CO_2, H_2, and O_2 levels. The main objective of the experimental activity was to optimize the growth of the HOB-microalgae consortium in batch photobioreactors and identify the benefits of cultivating the HOB-microalgae consortium in terms of yielded biomass, protein production, as well as CO_2 and H_2 consumption. The combined photo-chemoautotrophic microalgae-HOB consortium was cultivated under different gas compositions and compared to controls where the microalgae and the HOB were allowed to grow alone under their typical photo- and chemoautotrophic growth conditions, respectively. Headspace pressure, gas composition, biomass concentration, and ammonium nitrogen concentration were monitored, while the macromolecular biomass composition was evaluated in terms of protein and carbohydrates content. Finally, the microbial community composition of the developed microalgae-HOB consortium was also evaluated.

2. Materials and methods

2.1. Microbial inocula and mineral medium

The microalgae-HOB consortium was obtained by combining two inocula composed of mixed cultures previously enriched with HOB and green microalgae. The HOB inoculum, dominated by the *Paracoccus* genus, was obtained from a mixed HOB culture used for the aerobic conversion of syngas into MP (Pelagalli et al., 2024). The HOB culture was enriched from active compost sampled from a composting facility located in the Campania region, Italy. In brief, the supernatant obtained by suspending the compost in a mineral medium prepared according to Yu et al. (2013), was inoculated at 10 % (v/v) in 120 mL of the same fresh medium. The enrichment was obtained by supplying a gas mixture of $H_2/O_2/CO_2$ with a composition of 65/20/15 (v/v) to the inoculated medium until constant and repeatable gas consumption and biomass growth were observed. The green microalgae inoculum was obtained from a sample collected in a lake located in the Campania region, Italy. The enrichment procedure used bold basal medium (BBM) (Andersen, 2005), atmospheric carbon dioxide through aeration and artificial light as growth substrates. The predominance of the *Scenedesmus* genus was assessed through microscopic analysis. The inocula were kept active and enriched throughout the experiment in 250 mL serum bottles, using a mineral medium that combined the macro- and micro- nutrients used in the BBM and in the medium for the growth of the HOB (Yu et al., 2013). This mixed medium was composed by (per litre of solution): 2.3 g KH_2PO_4, 2.3 g Na_2HPO_4, 2.0 g NH_4Cl, 0.5 g $NaHCO_3$, 0.01 g $CaCl_2.H_2O$, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.05 g ferric citrate, 2.5 g NaCl, 1 mL trace metals solution, 1 mL EDTA solution, 1 mL acidified iron solution, 1 mL boric acid solution. The trace metals solution contained (per litre of solution): 8.82 g $ZnSO_4{\cdot}7H_2O$, 1.44 g $MnCl_2{\cdot}4H_2O$, 0.71 g MoO_3, 1.57 g $CuSO_4{\cdot}6H_2O$, 0.40 g $CoCl_2{\cdot}6H_2O$, 0.04 g $NiCl_2{\cdot}6H_2O$ The other solutions were prepared by mixing (per litre of solution): 50 g of EDTA and 31 g of KOH (EDTA solution), 4.89 g $FeSO_4{\cdot}7H_2O$ and 1 mL H_2SO_4 (acidified iron solution) and 11.42 g H_3BO_3 (boric acid solution). The mineral medium was characterised by a neutral pH (7.1 ± 0.1). The liquid phase consisted in 25 mL, while the remaining 225 mL of headspace was filled with a gas mixture composed at a ratio of 65/20/15 of either H_2, O_2, and CO_2 for the HOB, or Argon (Ar), O_2, and CO_2 for the microalgae. The serum bottles (Ochs, Germany) were kept at 30 ± 1 °C by means of a thermostatic bath equipped with thermal probes (Hydor, Italy) and agitated at 75 rpm through an oscillating shaker (Edmund Bühler, Netherlands). LED lights were installed around the transparent walls of the thermostatic bath as a light source for the microalgae, while the HOB inoculum was kept in the dark by wrapping the serum bottles in aluminium foil. Light was provided through cycles of 12 h followed by 12 h of dark, simulating a day/night cycle. The light intensity measured in the thermostatic bath was of 43.8 μmol/m^2/s.

2.2. Experimental setup

A series of batch tests was implemented to study the performances of the microalgae-HOB consortium as compared to those of the HOB and microalgae cultures alone. The tests were performed in 250 mL serum bottles with 25 mL of working volume of the same mineral medium described in Section 2.1 inoculated at 10 % (v/v) with either the mixed culture, the HOB culture alone or the microalgae culture alone. The microalgae-HOB inoculum was obtained by mixing equal volumes (1.25 mL) of the HOB and the microalgal inocula. The optical density of the culture at the beginning of each test was 0.8 ± 0.1, measured using a WTW photoLab 7600 UV–Vis spectrophotometer (WTW, Germany) set at a wavelength of 680 nm. The 225 mL headspace of each bottle containing either the microalgae-HOB culture or the HOB alone was purged with H_2 gas. After that, O_2 and CO_2 were injected to reach a certain volumetric composition, depending on the condition tested. In the bottles containing the microalgae inoculum, argon gas was used instead of H_2 to keep the volumetric composition of O_2 and CO_2 unaltered. The CO_2 (Rivoira, Nippon Gases, Turin, Italy) and Ar (Nippon Gases, Milan, Italy) gas were characterised by a purity of 99.999 %. The O_2 and H_2 gas were produced through electrolysis using an electrolytic cell (R06 Separator Kit, Hydrobullet Hydrogen Technology, Athens, Greece). Every two days a liquid volume of 5 mL was sampled from the serum bottles and exchanged with fresh medium, while 10 mL of gas from the headspace was also withdrawn. After sampling, therefore every two days, the gas headspace was restored to its initial composition, depending on the tested condition. This was done to provide always fresh gas substrates to the cultures and to investigate how their composition could influence the growth and behaviour of the cultures along a consistent time span of 20–23 days.

The pH was controlled manually at 7.0 ± 0.2 by using a 3 M NaOH solution. The serum bottles were kept at 30 ± 1 °C by means of a thermostatic bath and agitated at 75 rpm through an oscillating shaker. LED lights were installed around the transparent walls of the thermostatic bath as a light source for the microalgae. Light was provided through cycles of 12 h followed by 12 h of dark, simulating a day/night cycle. The light intensity measured in the thermostatic bath was of 43.8 μmol/m^2/s. To guarantee reproducibility, biological triplicates were performed for each microbial culture and experimental condition tested.

Table 1
Operating conditions tested to evaluate the growth of the microalgae-HOB consortium.

Test	Condition	Gas composition [%v/v]				Light time exposure	Favoured culture
		Ar	**H_2**	**O_2**	**CO_2**		
1	1.1	0	65	20	15	12 h of light/ 12 h of dark	HOB-microalgae (test)
	1.2	65	0	20	15	12 h of light/ 12 h of dark	Microalgae (control)
	1.3	0	65	20	15	24 h of dark	HOB (control)
2	2.1	0	50	20	30	12 h of light/ 12 h of dark	HOB-microalgae (test)
	2.2	50	0	20	30	12 h of light/ 12 h of dark	Microalgae (control)
	2.3	0	50	20	30	24 h of dark	HOB (control)
3	3.1	0	85	0	15	12 h of light/ 12 h of dark	HOB-Microalgae (test)
	3.2	85	0	0	15	12 h of light/ 12 h of dark	Microalgae (control)
4	4.1	0	68	3.4*	16	12 h of light/ 12 h of dark	HOB-microalgae (test)
	4.2	68	0	3.4*	16	12 h of light/ 12 h of dark	Microalgae (control)

*O_2 injected as air, present at 16%v/v.

The effect of the headspace gas composition on the growth of microalgae-HOB consortium was evaluated by means of different tests (Table 1). Each test lasted between 20 and 23 days. Test 1 was performed by providing the optimal gas composition for the growth of HOB, with 65 % H_2, 20 % O_2 and 15 % CO_2, as well as for *Scenedesmus* sp., that dominate the mixed microalgae inoculum, as a concentration of 15 % of CO_2 is reported as being optimal for the growth of these microalgae (Matassa et al., 2015; Patil and Kaliwal, 2017). Test 2 was characterised by a higher percentage of CO_2 in the headspace (30 % v/v), with the aim of challenging the growth of the microalgal culture, since microalgae are usually limited by higher percentages of CO_2 (Solovchenko and Khozin-Goldberg, 2013). Test 3 was characterised by a complete absence of O_2 in the feed gas mixture, aiming at evaluating the growth of the HOB as dependent only on the O_2 produced by the microalgae. In test 4, finally, the growth of the microalgae-HOB consortium was evaluated in the presence of oxygen concentrations, provided as air, below the flammability threshold of 5 % (Zlochower and Green, 2009). Control conditions were set by evaluating the growth of the HOB and the microalgae alone in three different bottles each. In order to enable the growth of HOB alone, the three inoculated bottles were covered with aluminium foil to avoid light penetration. The growth of the microalgae alone, instead, was guaranteed by replacing the same volume of H_2 with Ar as an inert gas in the headspace, while having equal volumes of O_2 and CO_2. In test 3, the control with the growth of the sole HOB culture was not performed as they are not able to oxidize hydrogen in the absence of molecular oxygen. Also in test 4, the control with HOB alone was not performed because preliminary tests showed that the low O_2 concentration that was injected in the headspace did not allow a measurable growth of the HOB under the set conditions.

2.3. Sampling and analytical methods

Sampling was performed every two days on both the liquid and gaseous volumes of each bottle. The total and soluble oxygen demand (tCOD and sCOD) were measured according to the APHA Standard Methods (APHA, 2007). The sCOD was measured on the sample centrifuged at 14,000 RPM for 10 min and filtered through 0.45 μm Merck Millex™ MCE syringe filters (Milan, Italy). The ammonium nitrogen concentration was analysed according to the indophenol-blue method (Aminot et al., 1997). Analyses of the produced biomass were also performed to evaluate its composition in terms of protein, through the Lowry method (Lowry et al., 1951), and carbohydrates, through the DuBois method (DuBois et al., 1956). The analysis of the gas, which was sampled every two days, was carried out 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. The pH of the liquid fraction was measured using a WTW Multi 3410 instrument equipped with a SenTix® 940 pH electrode (WTW, Germany), while the pressure of each bottle's headspace was monitored by means of a Leo 1 pressure meter (Keller, Switzerland). Light intensity was measured through a Lutron Electronic Enterprise LX-107 lightmeter (Taipei, Taiwan).

2.4. Microbial community analysis

Samples for the identification of the microbial community were collected from the serum bottles at the end of each test and stored at −20 °C. DNA extraction, sequencing and bioinformatic analyses were performed by Biomarker Technologies (BMK) (Münster, Germany). Total genomic DNA was extracted using the TGuide S96 Magnetic Soil/Stool DNA Kit (Tiangen Biotech Co., Ltd., Beijing) according to manufacturer's instructions. The hypervariable region V3-V4 of the bacterial 16S rRNA gene was amplified with primer pairs F: 5′-ACTCCTACGGGAGGCAGCA-3′ and R: 5′-GGACTACHVGGGTWTCTAAT-3′, while the hypervariable region V4 of the 18S rRNA gene was amplified with primer pairs F: 5′-CCAGCASCYGCGGTAATTCC-3′ and R: 5′-

ACTTTCGTTCTTGATYRA-3′. The polymerase chain reaction (PCR) products were checked on agarose gel and purified through the Omega DNA purification kit (Omega Inc., Norcross, GA, USA). The purified PCR products were collected, and the paired ends (2 × 250 bp) were performed on the Illumina Novaseq 6000 platform. Sequences that fitted in the 97 % similarity thresholds were assigned to one operational taxonomic unit (OUT) using USEARCH (version 10.0). Taxonomy annotation of the OTUs and the amplicon sequence variants (ASV) were performed according to the Naïve Bayes classifier in QIIME2 and using the SILVA database (release 138.1). The confidence threshold was set at 70 %. Alpha diversity and Beta diversity were respectively calculated to identify the complexity of species diversity of each sample and to understand the differences between samples for species complexity by principal coordinate analysis (PCoA). Abundance and diversity were compared using the one-way analysis of variance. Linear discriminant analysis (LDA) coupled with effect size (LEfSe) was applied to evaluate the differentially abundant taxa. The online platform BMKCloud was used to analyse the sequencing data.

2.5. Statistical analysis

All results were expressed as the mean ± standard deviation. To determine the statistical significance of the differences in terms of biomass growth among the various tests and among each test and the relative controls, a one-way ANOVA statistical analysis was performed by using the Microsoft Excel (version 2405) (Office 365, Microsoft Corporation, Redmond, USA) statistical package. The results were considered significantly different when the p-value was below or equal to 0.05 (p-value $\leq$ 0.05).

2.6. Calculations

The COD measurement was used as a proxy of biomass concentration in terms of volatile suspended solids (VSS) according to the following equation:

$$Biomassconcentration(mgVSS/L) = \frac{\text{tCOD (mg/L)- sCOD (mg/L)}}{\text{1.42 (mg COD/mg VSS)}} \quad (1)$$

where 1.42 mg COD/mg VSS represents the COD content of a generic microbial biomass sample (Irvine and Bryers, 1985).

Biomass yield on hydrogen (Y_{H2}) was calculated as grams of biomass per grams of H_2 gas COD equivalent (H_2-COD) as represented in the following equation:

$$YH2(mgVSS/mgH2-COD) = \frac{\text{Biomass concentration increase (mg VSS/L)} \bullet Liquidvolume(L)}{H_2 \text{ gas uptake (mmol) - 16 (mg } H_2\text{-COD/mmol)}} \quad (2)$$

considering that the *biomass concentration increase* was calculated as the difference between the final and initial biomass concentration and the *H_2 gas uptake* indicates the total moles of H_2 gas that were consumed between each sampling and restoration of the initial gas composition in the serum bottles during the test. According to Pelagalli et al. (2024), a factor of 16 was considered for the conversion from mole of H_2 gas consumed to grams of H_2-COD.

The consumption of H_2 was expressed as a percentage of the initial H_2 supply, according to the following equation:

$$H2consumption(\%mol/mol) = \frac{H_2gasuptake(mol)}{H_2initialsupply(mol)} \bullet 100 =$$

$$= \frac{H_2initialsupply(mol) - H_2residual(mol)}{H_2initialsupply(mol)} \bullet 100 \quad (3)$$

Similarly, the consumption of CO_2 and O_2 were calculated as:

$$CO2consumption(\%mol/mol) = \frac{CO_2gasuptake(mol)}{CO_2initialsupply(mol)} \bullet 100 =$$

$$= \frac{CO_2initialsupply(mol) - CO_2final(mol)}{CO_2initialsupply(mol)} \bullet 100 \quad (4)$$

$$O2consumption(\%mol/mol) = \frac{O_2gasuptake(mol)}{O_2initialsupply(mol)} \bullet 100 =$$

$$= \frac{O_2initialsupply(mol) - O_2final(mol)}{O_2initialsupply(mol)} \bullet 100 \quad (5)$$

The protein content of the biomass was calculated and expressed as the percentage of protein over biomass as VSS:

$$Protein\,content\,(\%protein/biomass) = \frac{Protein\,concentration\,(\text{mg protein/L})}{Biomass\,concentration\,(\text{mg VSS/L})} \bullet 100 \quad (6)$$

Similarly, the carbohydrate content was calculated as:

$$Carbohydrate\ content\ (\%\ carbohydrates/biomass) =$$

$$= \frac{Carbohydrate\,concentration\,(\text{mg carbohydrates/L})}{Biomass\,concentration\,(\text{mg VSS/L})} \bullet 100 \quad (7)$$

The light intensity values were measured in lux and converted to photosynthetic photon flux density (PPFD) which is measured in μmol/m^2/s through the conversion factor of 0.0146 related to 3500 K white LED lights according to the following equation (Ashdown, 2015):

$$PPFD(\mu mol/m2/s) = Lightintensity(Lux) \bullet 0.0146(\mu mol/m2/s/Lux) \quad (8)$$

3. Results and discussion

3.1. Growth under optimal gas composition

The experimental conditions characterizing test 1 represent the optimal scenario for the growth of the HOB, since a gas mixture with a $H_2/O_2/CO_2$ ratio of 65/20/15 was set in both conditions 1.1 (HOB + microalgae) and 1.3 (HOB alone). Furthermore, a 15 % v/v CO_2 concentration in the headspace should be optimal for the growth of the *Scenedesmus* sp. microalgae, which were dominant in the microalgal inoculum (Huang et al., 2020; Patil and Kaliwal, 2017; Singh and Singh, 2014). Condition 1.2 (microalgae alone) presented the same gas mixture, yet H_2 was replaced with Ar. Fig. 1a shows a high final biomass concentration in condition 1.1, where the growth of the microalgae-HOB consortium was tested, reaching up to 4.7 g VSS/L after 16 days. This biomass concentration value is higher than what obtained in conditions 1.2 and 1.3, where around 2.6 and 1.4 g VSS/L (*p-value* < 0.05) were achieved by microalgae and HOB alone, respectively. This indicates how the microalgae-HOB culture reached an approximately 80 % higher biomass concentration than that of microalgae alone and more than three times that of the HOB culture alone. The highest biomass concentration value of 4.7 g VSS/L reached by the microalgae-HOB

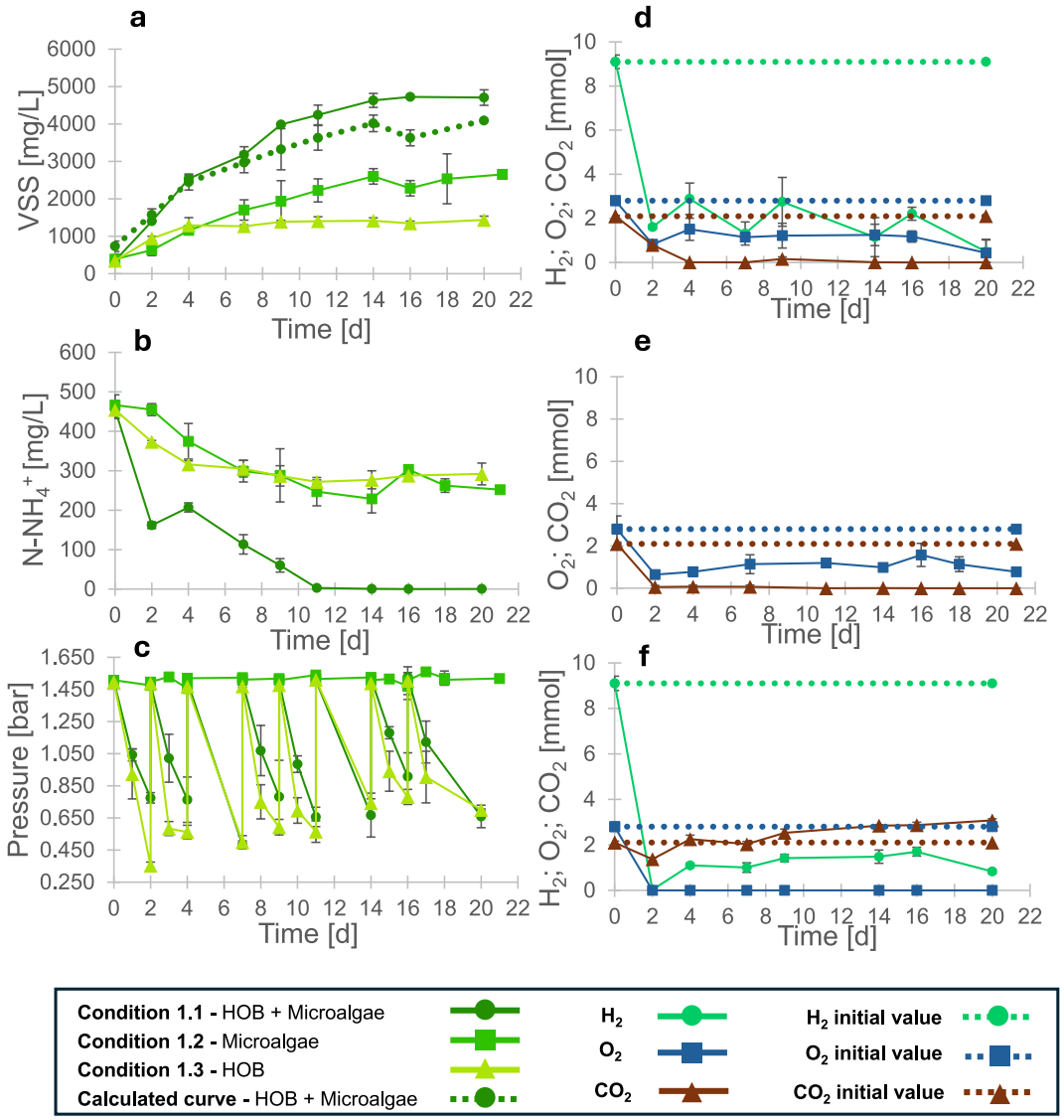

Fig. 1. Effect of the gas composition for the growth of HOB and microalgae on a) biomass growth, b) ammonium-nitrogen consumption, c) headspace pressure. Trends of gas concentrations measured in d) condition 1.1 (HOB + microalgae), e) condition 1.2 (microalgae alone), f) condition 1.3 (HOB alone).

consortium was also around 6.4 times and 1.7 – 2.2 times higher than the highest biomass concentrations of microalgae and HOB, respectively, observed by Noreen et al. (2021) and Nyyssölä et al. (2021) in batch tests. Fig. 1b shows the trends of ammonium nitrogen (NH_4^+-N) concentration under all tested conditions. In condition 1.1, the microalgae-HOB consortium consumed all available NH_4^+-N after eleven days, when also the biomass concentration stabilized. This likely points to a possible nitrogen limitation on the growth of the consortium, which could have therefore achieved even higher biomass concentrations if provided with additional nitrogen. As shown in Fig. 1c, the pressure trend measured in condition 1.2 was constant, while the pressure decreases repeatedly both in conditions 1.1 and 1.3, meaning that hydrogen was consumed both by the microalgae-HOB consortium and by the HOB culture alone. It is worth pointing out how the pressure trend was similar between conditions 1.1 and 1.3, meaning that the extent of hydrogen consumption by the microalgae-HOB consortium was comparable to that of the HOB culture alone. This was confirmed through the calculation of the average uptake of H_2 that occurred every two days after refreshing the gas headspace. The uptake of H_2 in condition 1.1 and condition 1.3 was equal to 81 % and 88 % of the provided H_2, respectively. In condition 1.1 the consumption of O_2 and CO_2 every two days stabilized respectively at around 62 % and 93 % of the initial headspace gas composition throughout the test (Fig. 1d). In condition 1.2, the headspace pressure did not show relevant fluctuations (Fig. 1c) since the predominance of Ar as an inert gas likely mitigated pressure variations linked to the consumption and production of CO_2 and O_2. Nonetheless, the gas analyses of condition 1.2 (Fig. 1e) did not only show a consumption of CO_2, but also of O_2 (respectively 99 and 64 % of the initial value restored after each sampling). These results are similar to what shown by condition 1.1 (Fig. 1d), with the main difference that the uptake of CO_2 and O_2 was also due to the presence of the HOB, while the consumption of O_2 in condition 1.2 might be a consequence of the photorespiration metabolic path. Photorespiration was likely triggered by the onset of low CO_2/O_2 ratios once most of the CO_2 present in the headspace was consumed. Therefore, even though condition 1.1 and 1.2 showed a similar O_2 and CO_2 consumption, the microalgal growth in condition 1.2 was likely slowed down by photorespiration, while the growth of the microalgae-HOB consortium reached a high biomass concentration of 4.7 g VSS/L (Fig. 1a). A constant and full O_2 uptake from the HOB was detected in condition 1.3 (Fig. 1f), pointing to potential O_2 limitations that probably hampered substantial growth and CO_2 fixation by HOB. Based on the H_2 consumption and biomass concentration observed in condition 1.3, a biomass yield of the HOB on H_2 (Y_{H2}) equal to 0.07 ± 0.03 mg VSS/mg H_2-COD (Table S1) can be

calculated according to Eq. (2). This value is on the lower end of the range of 0.07–0.28 mg VSS/mg H_2-COD reported by Pelagalli et al. (2024), thereby indicating the potential predominance of a maintenance metabolism (Yu and Lu, 2019) under the set growth conditions, as also confirmed by the low CO_2 uptake detected (Fig. 1f). On the contrary, under the same headspace gas composition (i.e. 65 % H_2, 20 % O_2, and 15 % CO_2), the HOB-microalgae consortium in condition 1.1 reached a 15 % higher biomass concentration than that calculated by summing the HOB and microalgae cultures alone (Fig. 1a), pointing towards a possible synergistic effect derived from the combination of the two cultures. It is possible that, by having the microalgae and the HOB growing together, the concentration of dissolved oxygen is kept low through its consumption by HOB, therefore preventing the onset of photorespiration in microalgae.

3.2. Growth under high carbon dioxide concentrations

Test 2 was characterised by a higher CO_2 concentration (i.e. 30 %), aimed at evaluating the performance of the microalgae-HOB consortium under conditions which are typically not optimal for microalgae. As a matter of fact, the growth of microalgae can be limited by high CO_2 concentrations due to the acidifying effect that CO_2 gas has on the culture medium. Therefore, the gas mixture employed in the headspace in conditions 2.1 (HOB + microalgae) and 2.3 (HOB alone) was composed of 50 % H_2, 20 % O_2, and 30 % CO_2, which was the same of that used in condition 2.2 (microalgae alone), yet with H_2 replaced by Ar. In terms of biomass growth (Fig. 2a), condition 2.1 reached a VSS concentration of 4.2 g/L, i.e. 10 % lower (*p-value* > 0.05) than the value obtained in condition 1.1. The growth trend of condition 2.1 is coherent with the consumption of ammonium nitrogen, as reported in Fig. 2b, which was almost completely depleted and likely limited the growth of the microalgae-HOB consortium from day 14. In condition 2.2, microalgae alone showed similar trends to condition 2.1, in terms of both ammonium nitrogen consumption (Fig. 2b) and biomass concentration (Fig. 2a), with the latter reaching up to 3.8 g VSS/L (*p-value* > 0.05). Even though the higher concentration of CO_2 in the headspace was supposed to limit the growth of microalgae alone, the biomass in condition 2.2 did not show signs of inhibition and resulted in a 41 % higher biomass growth than in condition 1.2 (*p-value* < 0.05). Indeed, although high CO_2 concentrations are generally inhibitory for many microalgae, the actual effect of CO_2 strongly depends on several parameters (e.g. temperature, headspace volume, medium volume, rate of replacement,

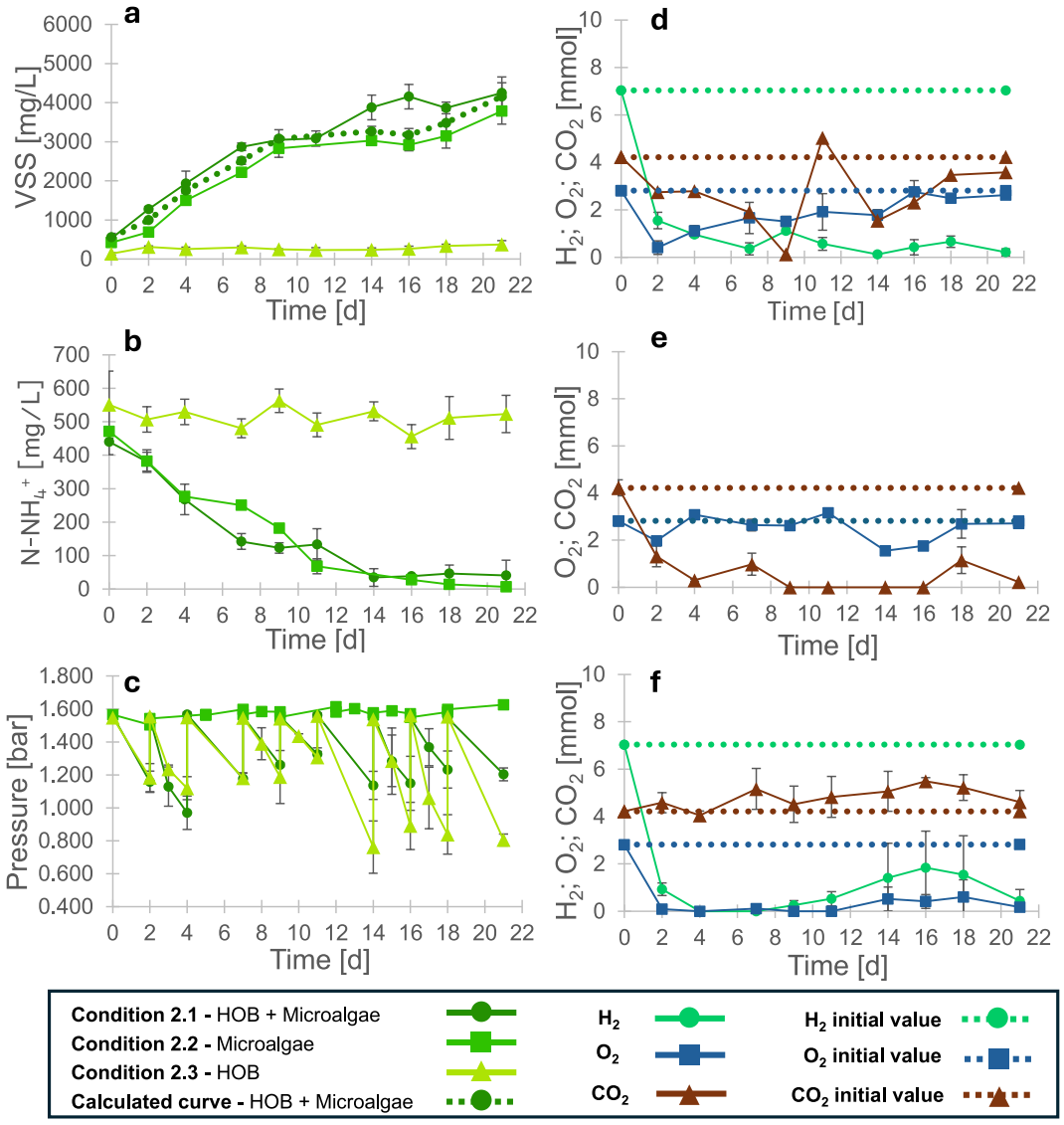

Fig. 2. Effect of high CO_2 concentrations on: a) biomass growth, b) ammonium nitrogen consumption, c) headspace pressure. Trends of gas concentrations measured in d) condition 2.1 (HOB + microalgae), e) condition 2.2 (microalgae alone), f) condition 2.3 (HOB alone).

etc.) that influence its solubility (Huang et al., 2020; Solovchenko and Khozin-Goldberg, 2013). In condition 2.2, thus, not only the excess of CO_2 seemed to not affect negatively the process, but its combination with the O_2 accumulated in the headspace likely limited photorespiration and, thus, improved biomass growth. The HOB culture alone (condition 2.3) showed, on the other hand, a rather low biomass growth compared to condition 2.1 (*p-value* < 0.05), reaching a stable biomass concentration below 0.5 g VSS/L, i.e. about three times less than that reached in condition 1.3. Complementary to the low HOB growth, ammonium nitrogen consumption was also negligible. Even though the recommended optimal CO_2 concentration usually ranges between its atmospheric concentration (0.038 % v/v) and 10 % v/v for most microalgal species (Zhao and Su, 2014), the growth of *Scenedesmus* sp. is usually studied at CO_2 concentrations higher than 10 % v/v. In this study, a CO_2 concentration of 30 % (condition 2.2) instead of 15 % v/v (condition 1.2) led to a better growth of the microalgae rather than the HOB, despite being outside of the range 10–20 % v/v for optimal biomass growth of the *Scenedesmus* sp. (Huang et al., 2020; Patil and Kaliwal, 2017; Singh and Singh, 2014). Also, the HOB were clearly limited by the lower presence of H_2 in the headspace in condition 2.3. The beneficial effect of the higher CO_2 concentration on the growth of the microalgae could have led to a high O_2 production through photosynthesis, which could possibly explain how the headspace pressure in condition 2.1 (Fig. 2c) could not reach the same lowest value as in condition 2.3 (0.97 and 0.76 bar, respectively). Regarding the gas composition, Fig. 2d shows a slow yet steady increase of O_2 concentration in condition 2.1, which was likely due to the high activity of the microalgae in the consortium, eventually exceeding the O_2 uptake by HOB. According to Fig. 2e, the microalgae in condition 2.2 completely consumed the CO_2 provided every two days, differently than what happened in condition 2.1, where CO_2 was always present in excess (Fig. 2d), which indicates a higher carbon conversion efficiency by the microalgae-HOB consortium with respect to the microalgae alone. The lower concentration of H_2 in the headspace of condition 2.3 (Fig. 2f), which resulted in an unbalanced H_2/O2/CO2 ratio and in having less than 23 % H_2 available compared to condition 1.3, led to an averagely 50 % lower biomass concentration. Nonetheless, the H_2 uptake between conditions 1.3 (Fig. 1f) and 2.3 (Fig. 2f) was comparable. As a result, the

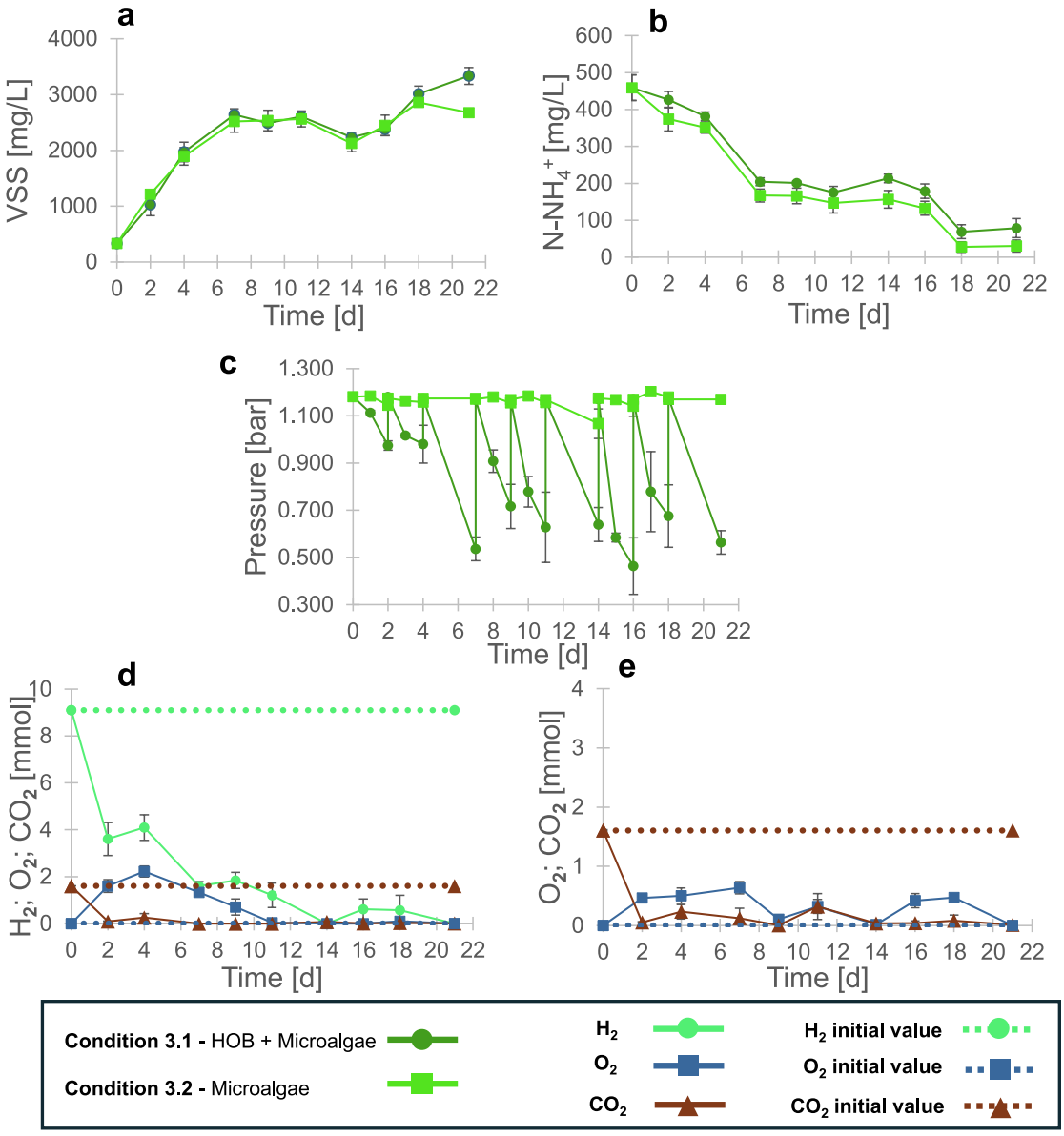

Fig. 3. Effect of the absence of external O_2 addition on: a) biomass growth, b) ammonium nitrogen consumption, c) headspace pressure. Trends of gas concentrations measured in: d) condition 3.1 (HOB + microalgae), e) condition 3.2 (microalgae alone).

biomass yield on H_2 (Y_{H2}) calculated in condition 2.3 was as low as 0.02 ± 0.01 mg VSS/mg H_2-COD (see Supplementary Material), that is more than three times lower than what obtained in condition 1.3 (0.07 ± 0.03 mg VSS/mg H_2-COD), confirming the limited anabolic activity observed with the HOB culture alone. Similarly to condition 1.3, also in condition 2.3 both H_2 and O_2 were always completely consumed after two days from being restored in the headspace (Fig. 2f), and the biomass concentration rapidly stabilized. Differently from Test 1, the synergistic effect showed by the microalgae-HOB consortium in condition 1.1 was not detected in condition 2.1, as the biomass growth reached under condition 2.1 was in line with that calculated by combining the growth of the HOB and microalgae alone (Fig. 2a). As previously discussed, this could be due to the lower concentrations of H_2 (condition 2.1), which reduced the extent of HOB activity while that of microalgae was promoted by the higher CO_2 concentration (Yu et al., 2013).

3.3. Growth in the absence of external oxygen addition

The results obtained in conditions 1.1 (HOB + microalgae with 65 % H_2, 20 % O_2, 15 % CO_2) and 2.1 (HOB + microalgae with 50 % H_2, 20 % O_2, 30 % CO_2) showed that oxygen was not limiting for the growth of HOB in the microalgae-HOB consortium. Therefore, the growth of the microalgae-HOB culture was tested in condition 3.1 without the external addition of O_2, using a gas mixture of 85 % H_2 and 15 % CO_2. This headspace gas composition forced the HOB to rely only on the oxygen produced by the microalgae. Regarding condition 3.2, in which only the microalgae were grown, the headspace gas mixture was composed of 85 % of Ar and 15 % of CO_2. Fig. 3a reports the VSS trend for condition 3.1 (HOB + microalgae) and 3.2 (microalgae alone). The two cultures show the same growth trend for the first 18 days, after which condition 3.1 reached up to 3.3 g VSS/L, while condition 3.2 settled at 2.7 g VSS/L (*p-value* < 0.05). Compared to previous tests, the microalgae-HOB consortium grew less efficiently than in condition 1.1 (*p-value* < 0.05) and 2.1 (*p-value* > 0.05), in which the highest VSS concentrations were around 40 and 30 % higher than that obtained in condition 3.1, respectively. The concentration of ammonium nitrogen (Fig. 3b) in conditions 3.1 and 3.2 followed a similar trend, with the two cultures having comparable values and, ultimately, reaching an almost complete depletion of ammonium nitrogen on day 18. Regarding the pressure trend (Fig. 3c), condition 3.1 (HOB + microalgae) showed slight pressure reductions during the first days, while the pressure values decreased substantially during the rest of the test. This trend could have

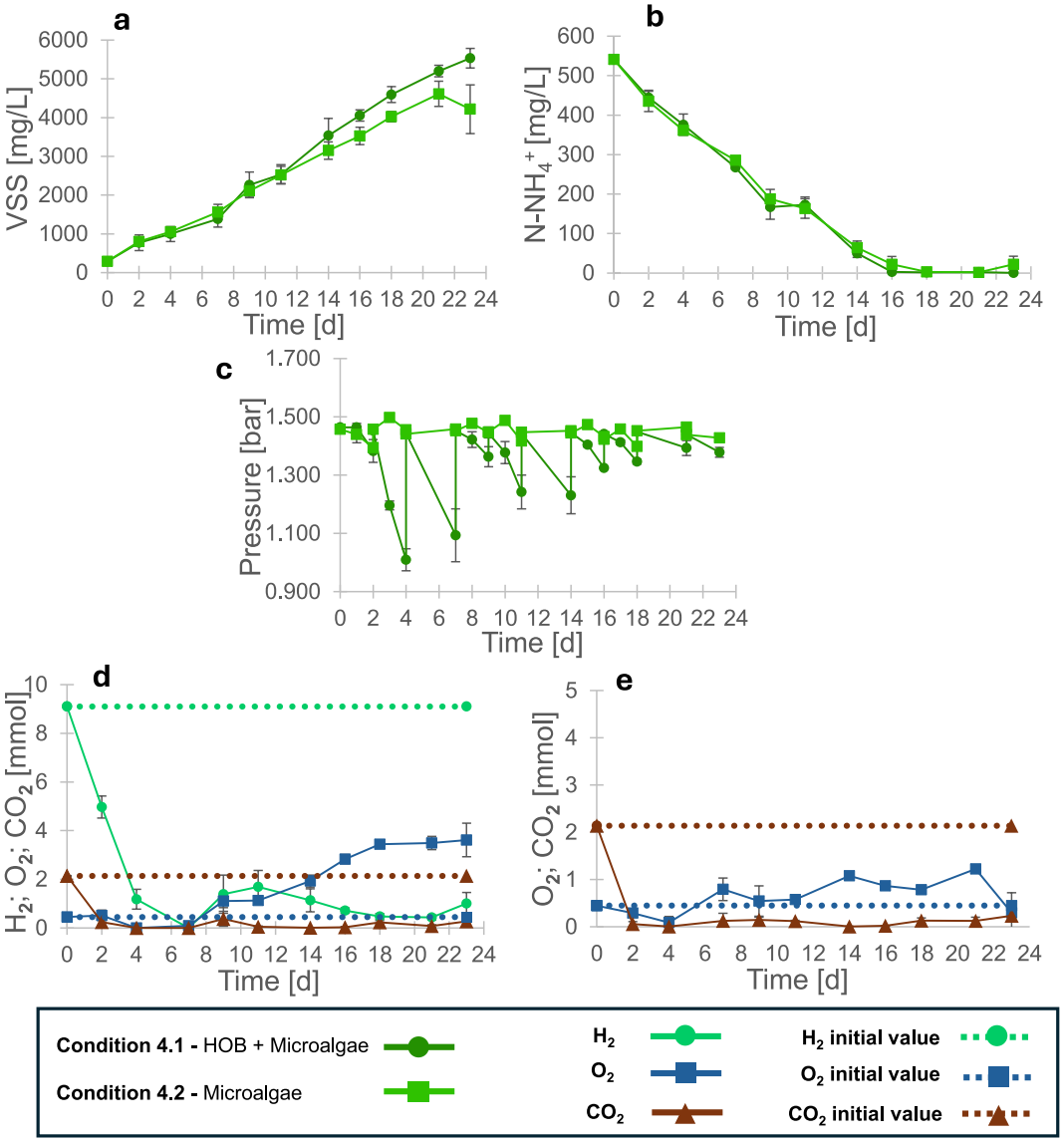

Fig. 4. Effect of sub-stoichiometric and non-explosive O_2 levels on: a) biomass growth, b) ammonium nitrogen consumption, c) headspace pressure. Trends of gas concentrations measured in: d) condition 4.1 (HOB + microalgae), e) condition 4.2 (microalgae alone).

resulted from the O_2 produced by the microalgae and accumulated in the headspace during the first days of the test (Fig. 3d), therefore preventing the pressure to reach lower values. As shown in Fig. 3d, H2 was being almost completely consumed after each refreshing of the headspace only after the first week of the test. It is possible that the initial production of O_2 by microalgae could not match the demand by HOB, leading to a lower consumption of H_2 and likely limiting their growth. After this initial acclimation phase, the HOB increased both H_2 and O_2 consumption, which led to a better growth of the microbial consortium and to a higher final biomass concentration value compared to condition 3.2. CO_2 was always being completely depleted by the mixed culture after each gas sampling and refreshing of the gas headspace, similarly to condition 1.1 (65 % H_2, 20 % O_2, 15 % CO_2). During the first eleven days of the test, condition 3.2 showed a low production of O_2 and a complete CO_2 uptake until the end of the test, plausibly triggering photorespiration as in condition 1.2 (65 % Ar, 20 % O_2, 15 % CO_2), while still consuming all carbon dioxide (Fig. 3f).

3.4. Growth under sub-stochiometric and not-explosive oxygen levels

The gas composition used for test 4 included the injection of air instead of pure O_2 gas in the headspace (68 % H_2, 16 % air, 16 % CO_2 in condition 4.1 and 68 % Ar, 16 % air, 16 % CO_2 in condition 4.2) (Table 1). Air was chosen as a less expensive source of oxygen, and its addition at O_2 concentrations below 5 % allowed to have a non-flammable gas mixture without significantly affecting the concentrations of the other gases. As displayed in Fig. 4, the microalgae-HOB consortium reached a biomass concentration of 5.5 g VSS/L, i.e. 30 % higher than the maximum value of 4.2 g VSS/L reached by the microalgal culture in condition 4.2 (p-value > 0.05). This test resulted in the highest growth for both the microalgae-HOB culture and the microalgal culture, reaching a 17 % higher biomass concentration than in condition 1.1 (p-value < 0.05), 30 % higher than condition 2.1 (p-value < 0.05) and around 65 % higher than condition 3.1 (p-value < 0.05). Accordingly, ammonium nitrogen was completely depleted already from days 15 to 20 under both condition 4.1 and 4.2, as reported in Fig. 4b. Regarding the headspace pressure, as displayed by Fig. 4c, an interesting trend could be observed in condition 4.1. Until day 7, the pressure constantly decreased by about 30 % from the initial pressure value. Starting from day 8, the pressure decrease was only 15 % and, after day 16, only 5 %. This trend could be explained by looking at Fig. 4d which shows that, even though the microalgae-HOB consortium consumed most of the provided H_2, which was supplied every two days through headspace gas refreshment, the high production of O_2 by the microalgae likely compensated the gas consumption by HOB. This aspect also indicates that the oxygen produced by the microalgae, together with that provided through air in the gas mixture every two days, met the oxygen demand from the HOB. In condition 4.1 (HOB + microalgae), the O_2 production after day nine was higher than in condition 4.2 (microalgae alone) (Fig. 4e), suggesting a better growth of the microalgae, and possibly of the HOB. The better growth of the microalgae-HOB consortium in condition 4.1 than in condition 1.1 (65 % H_2, 20 % O_2, 15 % CO_2, HOB + microalgae) could be explained by the same synergistic effect described in paragraph 3.1 regarding the growth of the consortium in condition 1.1 itself. In condition 4.1, the low amount of oxygen provided in the headspace likely limited the onset of photorespiration over photosynthesis in microalgae. Fig. 4d shows that there was no O_2 in the headspace until the end of the first week, meaning that the HOB managed to consume the oxygen produced through photosynthesis, thereby keeping the amount of dissolved oxygen low, differently than what observed in condition 1.1 (Fig. 1d). The low dissolved O_2 levels could have improved the growth of the microalgae in the consortium, which would explain the subsequent increase of O_2 production that occurred after the first week of the test (Fig. 4d). The high amount of O_2 detected in the last thirteen days of the test could have consequently enhanced the growth of the HOB, which could justify the higher growth of the microalgae-HOB consortium (condition 4.1) with respect to the microalgae alone in condition 4.2 (Fig. 4a).

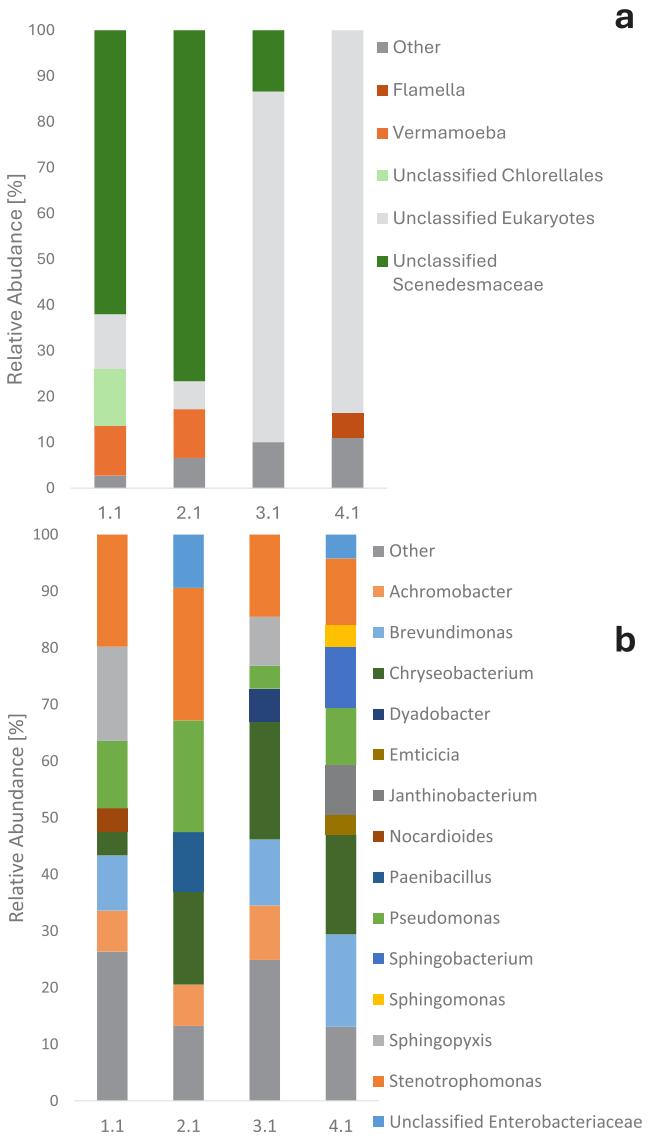

Fig. 5. Microbial community composition of the microalgae-HOB consortium grown in conditions 1.1, 2.1, 3.1, and 4.1 in terms of relative abundances of: a) eukaryotes; b) prokaryotes.

3.5. Microbial community analysis

A characterization of the microbial community was performed to evaluate the composition of the microalgae-HOB consortium, which developed in conditions 1.1, 2.1, 3.1, and 4.1 (Fig. 5). The eukaryotic microbial community in condition 1.1 was dominated by *Scenedesmaceae* at a relative abundance (RA) of 62 %, while *Chlorellales* were present at 12 % of RA (Fig. 5a). Regarding the prokaryotic microbial community, the most abundant genera were *Stenotrophomonas* and *Sphingopyxis* with a RA of 20 and 17 %, respectively, while the genus *Pseudomonas* had a RA of 12 % (Fig. 5b). All the aforementioned genera and/or their family were detected in HOB cultures enriched in previous studies, of which those of Barbosa et al. (2021), and Yang et al. (2021) are an example. Interestingly, *Brevundimonas* was also present (RA of 10 %) despite being reported as able to produce bio-H_2 through the hydrogenase enzyme (Wang et al., 2019). Being a bidirectional enzyme,

the hydrogenase can also be involved in the consumption of H_2. Furthermore, *Pseudomonas*, *Achromobacter*, *Stenotrophomonas*, and *Brevundimonas* were found as able to promote the growth of microalgae in microalgae-bacterial symbiotic systems (Alavianghavanini et al., 2024; Xie et al., 2018), and several strains of bacteria belonging to those genera were identified in association with a culture of *Scenedesmus* (Dao et al., 2018). When lowering the H_2 and increasing the CO_2 content in the headspace in condition 2.1, microalgae belonging to the *Scenedesmaceae* reached a RA of 77 % (Fig. 5a). It is likely that higher concentrations of CO_2 favoured the growth of the *Scenedesmacea* which can withstand CO_2 concentration as high as 50 % better than the *Chlorellales*, despite being both highly tolerant to high CO_2 concentrations (Huang et al., 2020). Regarding the bacterial community (Fig. 5b), *Paenibacillus* and *Chryseobacterium* genera of the family of the *Weeksellaceae,* of which some representatives are identified as HOB according to the study of Barbosa et al. (2021), were identified at a RA of 11 and 16 %. *Enterobacteriaceae,* (RA of 9 %), and *Paenibacillus*, reported as H_2 producers in the literature (Lal et al., 2012), were only found in condition 2.1, suggesting a potential higher tolerance to CO_2. As showed in Fig. 5b, in condition 3.1, which was characterised by a complete absence of O_2 provided in the headspace, the majority of the bacterial population consisted of the *Chryseobacterium* genus with a RA of 21 %, possibly pointing out that O_2 deprivation could provide better conditions for their growth. *Stenotrophomonas, Sphingopyxis, Brevundimonas* and *Achromobacter* were also present, as in condition 1.1 composition (RA of 15, 9, 12, and 10 %, respectively). Regarding the eukaryotic community (Fig. 5a), *Scendesmaceae* were detected at a lower RA of 13 %, while other unclassified eukaryotic species were detected with a RA of 72 %. The RA of the unclassified eukaryotic species lowered to a value of 48 % in condition 4.1, while the *Scenedesmaceae* were only detected at a RA of 3 % (Fig. 5a). The *Chryseobacterium* consisted in the majority of the prokaryotic community in condition 4.1 (Fig. 5b) with a RA of 18 %. Possibly, a low O_2:CO_2 ratio might improve the growth of *Chryseobacterium*, as suggested by their high RA in condition 2.1, 3.1, and 4.1, while only being present in condition 1.1 with a RA below 5 %. These aerobic bacteria have been reported to improve the growth of microalgae thanks to complex synergistic interactions of which the exchange of O_2 and CO_2 gases is just an example (Li et al., 2024). *Pseudomonas* and *Stenotrophomonas* genera were also present, even though with lower RAs of 10 % and 12 %, respectively, together with bacteria of the *Sphingobacterium* genus (11 %), aerobic chemoorganotrophic bacteria that were already identified in enriched hydrogen-oxidizing microbiomes (Ehsani et al., 2019).

3.6. Biomass composition

The biomass composition was analysed in terms of protein and carbohydrates content on a VSS-basis (see Supplementary Material). According to the scientific literature, microalgal cultures are characterised by a protein content between 5 and 70 % of protein (usually around 40 %) and between 8 and 70 % of carbohydrates over dry weight (Debnath et al., 2021). HOB usually present a content of protein and carbohydrates over dry weight in the range of 60–70 % and 5.0–6.7 %, respectively (Volova and Barashkov, 2010). The protein content of the microalgae-HOB culture obtained in this study was closer to the typical protein content of microalgae rather than that of HOB. Contrarily to the aforementioned literature values, the HOB culture alone managed to accumulate a protein content of only 42.1 ± 3.6 % and 35.6 ± 10.3 % in condition 1.3 (HOB alone with 65 % H_2, 20 % O_2, 15 % CO_2) and 2.3 (HOB alone with 50 % H_2, 20 % O_2, 30 % CO_2), respectively, lower than what obtained by the microalgae culture alone (57.6 ± 10.8 % and 59.4 ± 13.6 % in condition 1.2 and 2.2, respectively). The high carbohydrate content of around 30 % in the HOB culture grown alone could be related to the sub-optimal growth conditions, which could also explain the low content of protein. This could also have negatively affected the accumulation of protein in the microalgae-HOB consortium (52.6 ± 10.2 % and 43.6 ± 6.5 % in condition 1.1 and 2.1, respectively), which was always between that of the microalgae culture and that of the HOB culture cultured individually.

Regarding the carbohydrates content, conditions 1.1, 2.1, and 4.1 (HOB + microalgae with external O_2) present carbohydrates content of about 30 %, while condition 3.1 (85 % H_2, 15 % CO_2, HOB + microalgae without external O_2) reached a content of 15.3 ± 1.9 %, a value similar to what was obtained in condition 2.2 (14.0 ± 0.8 %) and 3.2 (18.8 ± 0.7 %) (microalgae alone). These results could be explained considering that the accumulation of carbohydrates, similarly to lipids, is usually enhanced under nitrogen limiting conditions, which did not fully materialise in condition 3.1. However, since the biosynthetic pathways leading to the production of lipids and carbohydrates need the same precursor metabolites, it is likely that the lower amount of both protein and carbohydrates measured in condition 3.1 is an indication of an enhanced lipid accumulation (Debnath et al., 2021). Interestingly, the carbohydrates content of the microalgae-HOB mixed cultures and of the single HOB cultures in conditions 1.1, 1.3, 2.1, 2.3, and 4.1 were always higher than what reported in the literature by Volova and Barashkov (2010), but are similar to the results obtained from the aforementioned study of Nyyssölä et al. (2021), while still presenting higher protein contents compared to Nyyssölä et al. (2021).

3.7. Advantages and limitations of photo-chemoautotrophic microbial protein production

Microbial consortia are becoming an interesting alternative to monocultures thanks to the multiple advantages that they offer, including a higher capability to metabolize more complex substrates and good resilience when facing external disturbances (Areniello et al., 2023). Microalgae are often paired with other microorganisms to improve their biomass yield, their carbon capture and nutrient recovery potential, as well as their protein, carbohydrates, or fatty acids content (Chia et al., 2021; Satpati et al., 2023). Compared to the microalgae-HOB consortium, in which the main mutualistic relationship in terms of gaseous exchange regards only O_2, which is produced by the microalgae and consumed by the HOB, when pairing microalgae with heterotrophs, such as fungi or heterotrophic bacteria, the gaseous exchange includes both O_2 and CO_2. On the one hand, O_2 is still produced by the microalgae and consumed by the heterotrophs, which in turn produce CO_2 to be consumed by the microalgae (Chia et al., 2021; Flores-Salgado et al., 2021; Satpati et al., 2023). On the other hand, in the microalgae-HOB consortia both the microbial groups need CO_2 as a carbon source. Consortia of microalgae and heterotrophic bacteria have been widely applied to the treatment of wastewater, which is used as a low-cost organic carbon and nutrients source (Flores-Salgado et al., 2021), even though it can easily lead to contamination by other microbial species and can severely limit light penetration due to its turbidity (Chia et al., 2021; Satpati et al., 2023). On the contrary, Microalgae-HOB grown on gas substrates face limited contamination issues. Gases rich in H_2 and CO_2 such as syngas, biogas produced through dark fermentation, or a mixture of flue gas and green hydrogen can be used to fuel the growth of the microalgae-HOB consortium. Using syngas or flue gas can bring the disadvantage of high levels of CO, NOx and SOx, yet several HOB (e.g. from the *Pseudomonas, Stenotrophomonas, and Sphingopyxis* genera (Brown et al., 2012)), and microalgae belonging to the *Scenedesmus* sp. and *Chlorella* sp., have been found as capable of tolerating high concentrations of these gases (Nagappan et al., 2020), thereby potentially enabling CCU for MP production from a wide array of waste and residues.

An interesting comparison could be done with consortia of microalgae and methane oxidizing bacteria (MOB). As heterotrophs, the MOB consume O_2 and produce CO_2 through CH_4 oxidation (Li et al., 2022). Also, both HOB and MOB are known to produce organic acids that could promote the growth of microalgae thanks to their mixotrophic behaviour (Li et al., 2022; Matassa et al., 2015). An advantage potentially

offered by the presence of H_2 rather than CH_4 in the headspace could be identified in the stimulation of the growth of the microalgae through the metabolic path of photoreduction (Sleutels et al., 2020). Moreover, despite working in similar ways and producing the same kind of bio-products, HOB could be an overall better option since they are generally reported to have higher biomass yields, growth rates, and protein content over dry weight than MOB (Matassa et al., 2015). A shared bottleneck to the cultivation of microalgae-HOB and microalgae-MOB consortia is the need to supply flammable gaseous streams. According to Zlochower and Green (2009), the minimum O_2 concentration needed to propagate flames in either an atmosphere of CH_4 or H_2 is around 5. Therefore, for both microbial consortia it could be possible to exploit a mutual gas exchange to limit the presence of O_2 gas below the lower flammability limit. As already mentioned above, avoiding O_2 accumulation brings also other benefits such as preventing the microalgae from triggering the metabolic path of photorespiration and that of photo-oxidation of chlorophyll (Hong et al., 2020; Kliphuis et al., 2011). Furthermore, according to the results from the present study, the highest values of biomass concentrations were observed when additional O_2 for HOB growth was supplied through atmospheric air and in concentrations below the flammability threshold. This approach would be advantageous when considering process upscaling, as it would avoid the injection of pure O_2 gas. Also, as seen in Fig. 4d, CO_2 and H_2 were usually completely consumed within 48 h, requiring the supply of fresh gas. Having a continuous flow of gases could thus support a higher biomass production mainly through HOB growth, which would further increase O_2 uptake and avoid the necessity of outgassing O_2 from the photobioreactor.

Despite the potential advantages, the viability of this photochemoautotrophic system critically depends on the availability and cost-effectiveness of hydrogen. Green hydrogen, in particular, must be supplied in stoichiometric quantities to sustain HOB metabolism. Given hydrogen's low solubility in water, its efficient use requires specialized reactor configurations, such as systems equipped with micro- or nano-spargers and gas recirculation units, to ensure adequate mass transfer. These adaptations, while necessary, significantly increase both the capital expenditures (CAPEX) and operational expenditures (OPEX) associated with the process.

Compared to conventional open raceway systems, which are relatively low-cost and yield average productivities of 9–15 g biomass/m^2/day, closed photobioreactors offer greater control over cultivation conditions and the potential for higher productivity. Tubular photobioreactors, for instance, can reach 18 g biomass/m^2/day, with values up to 50 g biomass/m^2/day for certain strains such as *Scenedesmus*, while flat panel systems typically produce between 5–35 g biomass/m^2/day (Fernández et al., 2020). However, these gains come with trade-offs: investment costs for closed photobioreactors are approximately 0.51 M€/ha at the 100-ha scale, roughly double the 0.13–0.37 M€/ha required for open raceways (Fernández et al., 2020).

Overall, thus, due to the high infrastructure and energy costs associated with photobioreactors and hydrogen handling, this approach is most likely to be viable only in large-scale facilities or in regions where low-cost, renewable hydrogen is readily available, such as countries with high shares of renewable energy and surplus electricity during off-peak periods. Finally, to ensure the economic feasibility of such more energy-intensive CCU processes, higher-value applications in the food, cosmetics and pharmaceutical sector should be targeted and prioritized.

4. Conclusions

The obtained results highlight how using a microalgae-HOB consortium allows to reach higher concentrations of biomass than the single microalgae and HOB cultures, a high uptake of CO_2, and an average accumulation of protein compared to the literature. Also, by decreasing the external O_2 supply below flammability limits, a higher production of photosynthetic O_2, and overall higher biomass concentrations compared to the other tested conditions were observed. An almost complete depletion of N-NH_4 within about 16 days occurred under all conditions, indicating that potentially higher biomass concentrations and protein contents could be achieved in the future by avoiding nitrogen limitation.

CRediT authorship contribution statement

Raffaele Manzo: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Stefano Papirio:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Luca d'Antonio:** Writing – review & editing, Supervision, Conceptualization. **Giovanni Esposito:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Silvio Matassa:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Funding acquisition, 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.

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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.2025.132792.

Data availability

The raw sequencing line regarding the microbial community analyses reported in this study can be found in online repositories. The accession number(s) of these repositories can be found below: https://www.ncbi.nlm.nih.gov/nuccore/?term=PQ472147:PQ472406[accn]. https://www.ncbi.nlm.nih.gov/nuccore/?term=PQ471684:PQ472078[accn].

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