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Upcycling nitrogen and curbing greenhouse gas emissions from wastewater through H_2-driven assimilatory mixotrophic metabolism

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ABSTRACT

Biological nitrogen assimilation is an emerging technique for the removal and upcycling of nitrogen from wastewater in the form of microbial protein (MP). For this purpose, a mixed hydrogen-oxidizing bacteria (HOB) culture capable of growing mixotrophically was evaluated for the treatment of synthetic and real wastewater under different carbon-to-nitrogen (C/N) ratios. The same mixed HOB culture was grown under heterotrophic conditions to compare treatment and process performances in terms of ammonium nitrogen (N-NH_4^+) removal and assimilation, chemical oxygen demand (COD) removal, CO_2 release, biomass concentration, yield and protein content. Under mixotrophic conditions, the highest biomass concentration was 342.5 mg VSS•L^{-1}, doubling that obtained under heterotrophic conditions (161.2 mg VSS•L^{-1}). Discharge limits for both COD and total N were met under mixotrophic conditions, with nitrogen removal and assimilation into protein-rich biomass achieving up to 99 %. On the contrary, under heterotrophic conditions, the high content of residual nitrogen as nitrite (up to 26 mg•L^{-1} of N-NO_2^-) did not allow to meet discharge limits for total N. The mixed HOB culture gave promising results in terms of biomass yield (0.32 g VSS·g $COD^{-1}_{H2+acetate}$) and protein content (up to 56 % of VSS) when grown mixotrophically. Under heterotrophic conditions, instead, the biomass yield (0.25 g VSS·g $COD^{-1}_{acetate}$) and protein content (35 %) were substantially lower. This study suggests that the H_2-driven assimilatory mixotrophic metabolism can be successfully applied for wastewater treatment to produce effluents that meet discharge limits while mitigating greenhouse gas emissions (CO_2 and N_2O) and upcycling nitrogen into MP.

1. Introduction

Nitrogen (N) in its reactive forms (ammonium, nitrite, nitrate, etc.) is crucial for life as it constitutes an essential nutrient for the production of amino acids and proteins [1,2]. The application of the Haber-Bosch process for the synthetic production of reactive nitrogen enabled a tremendous increase in agricultural production, yet the same process is currently responsible for 2 % of the total world energy consumption and 1.6 % of global CO_2 emissions [3]. The extensive use of nitrogen fertilizers also leads to a highly inefficient anthropogenic nitrogen cycle, due to the significant N-losses occurring along the feed and food chain (such as leaching, ammonia volatilization, denitrification and runoff during crop production) as well as the limited recovery of reactive nitrogen from waste [4,5]. Among the various solutions available to improve the efficiency of the anthropogenic N-cycle and limit greenhouse gasses (GHGs) emissions, the recovery of reactive nitrogen from urban wastewater, accounting for up to 15 % of the artificially produced nitrogen, could represent a valid alternative [6].

In conventional wastewater treatment systems, biological oxidation combined with nitrification–denitrification processes are employed for organic matter and nutrient removal. Despite the effectiveness in meeting the regulatory discharge limits, such a process is highly energy demanding and does not align with emerging resource recovery practices [7]. Indeed, relevant GHGs emissions, such as CO_2 and N_2O (produced during nitrification–denitrification), derive directly from the biological treatment units, due to the biodegradation of organic matter under aerobic conditions and the dissipation of reactive N-NH_4^+ into inert N_2 [8]. Moreover, since the amount of primary energy involved in the nitrification–denitrification process (~45 MJ•kgN^{-1}) is comparable to that of the Harber-Bosch process (~37–45 MJ•kgN^{-1}) [9], the current way of dealing with N-laden wastewaters contributes to the so-called N-paradox, where the same amount of primary energy is used first to fix

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and then to dissipate reactive nitrogen, thereby missing potential recovery opportunities [6]. Valid alternatives for a more energy efficient and effective nitrogen removal have been proposed, including anaerobic ammonium oxidation (AnAmmOx) and other short cut nitrification–denitrification processes, yet all of them are based on the same dissipative approach, and lead to the production of GHGs (e.g., CO_2, CH_4, N_2O) [10,11]. Hence, recent research efforts are focusing on biological nitrogen assimilation to remove and recover nitrogen from wastewater as an economically sustainable and environmentally friendly alternative to conventional wastewater treatment [12,13].

In biological assimilation processes, nitrogen is removed from wastewater and assimilated for anabolic purposes inside microbial biomass in the form of amino acids, which are ultimately converted into proteins. A wide range of microbial species of bacteria, yeasts, fungi and algae can be used for biological nitrogen assimilation [14]. In comparison to other microorganisms, bacteria present advantages such as a shorter generation time, a high protein content (up to 70–80 %), and the potential to utilize a broad spectrum of substrates [15]. Among the various bacterial groups, hydrogen-oxidizing bacteria (HOB) are highly effective in assimilating nitrogen into a protein-rich biomass, also known as microbial protein (MP), with potential applications spanning from animal and human nutrition to techno-functional polymers [16]. In the nitrogen assimilation process, HOB utilize hydrogen (H_2) as electron donor and oxygen (O_2) as electron acceptor to fix carbon dioxide (CO_2) and ammonium nitrogen (N-NH_4^+) [13]. In addition to the most commonly studied autotrophic metabolism, HOB can also grow heterotrophically by using various organic substrates such as glucose, acetate and lactate [17]. The autotrophic and heterotrophic growth mode can concomitantly take place, resulting in the mixotrophic growth mode [18]. For instance, HOB can grow on organic substrates which oxidation produces CO_2 that can be subsequently fixed into additional biomass in the presence of H_2, resulting in a symbiotic relationship between the two metabolic pathways. The same relationship can be established in a mixed microbial culture in the presence of heterotrophic bacteria. In this scenario, the biodegradation of organic matter by heterotrophs produces CO_2, which is used by autotrophic HOB for their growth.

During the anabolic processes for microbial biomass synthesis, the C/N ratio can be considered as an important metabolic parameter, representing the organic uptake rate over the NH_4^+ uptake rate, which is regulated by the expenditure for catabolic energy. To optimize the nitrogen assimilation process and obtain a good protein content, a C/N ratio of about 10 should be maintained in the growth medium [19]. For instance, higher C/N ratios may lead to an increase in carbohydrates and lipids over proteins, meanwhile lower values might limit the extent of NH_4^+ uptake [15,19].

In view of the above, this study aims to assess the performances of a mixed HOB culture grown under both heterotrophic and mixotrophic conditions, and further benchmarked against conventional autotrophic growth, for the treatment of synthetic and real wastewater and the upcycling of waste reactive nitrogen. More in detail, different C/N ratios were tested to investigate the feasibility of the process in relation to urban wastewater characterized by low C/N ratios (<8), which usually limit nitrogen removal efficiency in conventional nitrification–denitrification systems while not allowing to perform assimilatory nitrogen removal [20]. The ability of HOB to assimilate nitrogen and also capture CO_2 under different trophic conditions and C/N ratios was evaluated in terms of ammonium nitrogen (N-NH_4^+) removal and assimilation, chemical oxygen demand (COD) removal, CO_2 release, biomass concentration and yield, and protein content.

2. Materials and methods

2.1. Source and activation of hydrogen-oxidizing bacteria

A mixed HOB culture, previously enriched from active compost and tested for the aerobic conversion of syngas into microbial protein, was used as source of inoculum [21]. The inoculum was dominated by the *Paracoccus* genus and hosted other HOB as well as heterotrophic genera and species such as *Candidatus Kapabacteria, Flavobacteriia bacterium, Hydrogenophaga, Ancylobacter, Terrimonas uncult* and *Advenella kashmirensis* [21]. The composition and relative abundance of the inoculum is available in Table S1 of the Supplementary Information. Prior to being used for the tests, the inoculum, which was stored at 4 °C, was first activated for 20 days. The activation process was carried out in 250 mL serum bottles and consisted in diluting 7 mL of HOB inoculum in a volume of 25 mL of mineral medium (see Section 2.2), thereby leaving a headspace of 225 mL for the gas mixture. The bottles were purged with H_2, O_2 and CO_2 with the following composition: 65:20:15 (v/v) [2]. Subsequently, the bottles were kept at a controlled temperature of 30 ± 1 °C and shaken at 85 rpm. Biomass growth and gas consumption analyses were used to assess the reactivation of the mixed HOB culture.

2.2. Composition of mineral medium and of synthetic and real wastewater

The composition of the mineral medium, prepared according to Yu et al [22] and used to perform the reactivation of the HOB inoculum as well as stimulate synthetic wastewater is presented in Table S2 of the Supplementary Information. The synthetic wastewater for mixotrophic and heterotrophic growth conditions was prepared by adding acetate, as $CH_3COONa{\cdot}3H_2O$ (ITW Reagents, Milan, Italy) in the above mentioned mineral medium at the concentration of 625 and 325 mg·L^{-1} as shown in Table 2.

The final validation of the process was carried out by using the influent wastewater collected from an urban WWTP located in Angri (Campania region, Italy). The wastewater composition is presented in Table 1. The real wastewater was amended with acetate and NH_4Cl (Chem-Lab, Zedelgem, Belgium) to achieve final COD and N-NH_4^+ concentrations of 347 and 32.5 mg·L^{-1}, respectively.

2.3. Experimental design of biological nitrogen assimilation tests

2.3.1. Evaluation of different trophic modes

Batch biological nitrogen assimilation experiments were performed using 250 mL serum bottles with a working volume of 25 mL. The bottles were incubated at 30 ± 1 °C and placed on a shaker set at 85 rpm. The bottles headspace refreshment, sampling and pressure measurement were done every 48 h. The hydraulic retention time (HRT) and solid retention time (SRT) were set at 3.33 days by refreshing 15 out of 25 mL of medium every 48 h.

The batch tests were performed under three different experimental conditions defined by three trophic modes: mixotrophic, heterotrophic and autotrophic. Under mixotrophic conditions, acetate was supplied together with H_2, with the latter being provided through a gas mixture composed of H_2 and O_2 at a ratio of 68:32 (v/v) (Table 2). Under heterotrophic conditions, acetate dosed as $CH_3COONa{\cdot}3H_2O$ was supplied as the sole carbon source together with argon (replacing H_2) and O_2 in the ratio of 68:32 (v/v). The autotrophic batch test was performed for 28 days during phase IV to compare biomass growth, protein content and biomass yield with mixotrophic and heterotrophic conditions. The gas composition used for the autotrophic growth mode was H_2: O_2: CO_2 = 67: 30: 3, where CO_2 was calculated by considering that all the acetate

Table 1
Composition of the real urban wastewater used in this study.

Parameter	Units	Value
Total COD	mg COD·L^{-1}	134.2 ± 4.9
Soluble COD	mg COD·L^{-1}	70.1 ± 1.4
N-NH_4^+	mg N·L^{-1}	5.4 ± 0.3
N-NO_2^-	mg N·L^{-1}	1.2 ± 0.2
N-NO_3^-	mg N·L^{-1}	5.5 ± 0.1
N-PO_4^{3-}	mg P·L^{-1}	0.4 ± 0.2
Alkalinity	mg $CaCO_3$·L^{-1}	425 ± 0

Table 2
Experimental conditions tested under mixotrophic, heterotrophic and autotrophic modes.

Mode	Experimental phases	Days	Feed $N\text{-}NH_4^+$ concentration ($mg{\cdot}L^{-1}$)	Feed acetate concentration ($mg{\cdot}L^{-1}$)	COD concentration ($mg{\cdot}L^{-1}$)	C/N	Gas composition (v/v)
Mixotrophic	I	1–14	130	650	692	2.5	H_2: O_2 = 68: 32
	II	15–28	65			5	
	III	29–56	32.5			10	
	IV	57–98	32.5	325	347	5	
	V	99–133	32.5			5	
	VI	134–154	32.5			5	
Heterotrophic	I	1–14	130	650	692	2.5	Ar: O_2 = 68: 32
	II	15–28	65			5	
	III	29–42	32.5			10	
	IV	43–84	32.5	325	347	5	
	V	85–120	32.5			5	
	VI	121–142	32.5			5	
Autotrophic	IV	1–28	32.5	0	–	–	H_2: O_2: CO_2 = 67: 30: 3

present in the other conditions (325 $mg{\cdot}L^{-1}$) would be oxidized to CO_2. The gas composition and acetate concentration used in the batch tests are reported in Table 2.

2.3.2. Evaluation of different carbon to nitrogen ratios

Different carbon to nitrogen (C/N) ratios were examined under both mixotrophic and heterotrophic conditions (Table 2). During phases I to III, C/N ratios of 2.5, 5.0 and 10.0 were tested by changing the $N\text{-}NH_4^+$ concentration to 130, 65, 32.5 $mg{\cdot}L^{-1}$, respectively, while keeping an acetate concentration of 650 $mg{\cdot}L^{-1}$ constant (equivalent to 692 mg $COD{\cdot}L^{-1}$, by considering a conversion factor between COD and acetate of 1.07). The amount of acetate to be dosed was initially selected based on the residual $N\text{-}NH_4^+$ concentration (124 $mg{\cdot}L^{-1}$) obtained after the activation phase of HOB. The experiments were started with a low C/N of 2.5 to simulate urban wastewaters particularly rich in nitrogen. In phase IV, a C/N of 5.0 was obtained by halving the acetate concentration to 325 $mg{\cdot}L^{-1}$ (equivalent to 347 mg $COD{\cdot}L^{-1}$) and keeping a constant $N\text{-}NH_4^+$ of 32.5 $mg{\cdot}L^{-1}$ in order to evaluate the process in the presence of COD and ammonium concentrations typical of urban wastewater.

In phase V, the process was tested by using real wastewater (Table 1) amended with micronutrients, while keeping a C/N ratio of 5.0. The micronutrients were used to favor the acclimation of the mixed HOB culture within wastewater and to further evaluate their influence on the performances of the process. In phase VI, the real wastewater was used without the addition of micronutrients at the same C/N ratio of the previous phase to validate the potential of the mixed HOB culture to treat real wastewater through biological nitrogen assimilation. Since the collected wastewater was rather diluted and showed low $N\text{-}NH_4^+$ and total COD concentrations of 5.4 and 134.2 $mg{\cdot}L^{-1}$, respectively, in both phases V and VI the wastewater was amended with acetate and ammonium to maintain constant feed COD and $N\text{-}NH_4^+$ concentrations of 347 and 32.5 $mg{\cdot}L^{-1}$, respectively. All the batch experiments were conducted in triplicate and results are reported as the mean ± standard deviation.

2.4. Samples preparation and analytical methods

Prior to analysis, liquid samples were centrifuged with a Mikro 22R centrifuge (Hettich, Manchester, UK) at 14,000 rpm for 10 min, followed by filtration of the supernatant using 0.45 and 0.22 μm cellulose syringe filters (VWR, Milan, Italy). The $N\text{-}NH_4^+$ concentration was measured spectrophotometrically using the indophenol blue method [23] while ion chromatography was used to determine nitrate and nitrite concentrations as described by Di Capua et al [24]. Chemical oxygen demand (COD) was determined as total COD (tCOD) and soluble COD (sCOD) using the closed reflux colorimetric method [25]. The tCOD concentration was determined on uncentrifuged and unfiltered samples. Gas samples collected from the headspace of the serum bottles were analyzed for mixed gases (H_2, O_2, CO_2) composition with a Star 3400 gas chromatograph (Varian, USA) equipped with a thermal conductivity detector and a ShinCarbon ST80/100 column (Restek, USA). Total protein analysis was performed through the Lowry method [26] using liquid samples taken at the end of each test, stored at −20 °C and then thawed at 50 °C to promote cell lysis for the subsequent protein determination [27]. The pH of the medium was measured using a WTW Multi 3410 instrument equipped with a SenTix® 940 pH electrode (WTW, Germany).

2.5. Calculations

The difference between tCOD and sCOD was used as a proxy of biomass growth within the tests:

$$\text{Biomass concentration}\,(\text{mg VSS}\bullet\text{L}^{-1}) = \frac{\text{tCOD}\,(\text{mg}\bullet\text{L}^{-1}) - \text{sCOD}\,(\text{mg}\bullet\text{L}^{-1})}{1.42\,(\text{mg COD}\hat{\text{A}}\cdot\text{mg VSS}^{-1})} \quad (1)$$

where 1.42 mg $COD{\cdot}mg\ VSS^{-1}$ represents a conversion factor commonly employed to convert the COD fraction associated with microbial biomass into volatile suspended solids (VSS) [27].

The gas consumption efficiency was calculated as described in Eq. (2):

$$H_2\ \text{consumption}\,(\%)\ \frac{H_2\ \text{initial}\,(\text{mmol}) - H_2\text{final}\,(\text{mmol})}{H_2\ \text{initial}\,(\text{mmol})} \times 100 \quad (2)$$

Under mixotrophic conditions, the combined biomass yield on H_2 and acetate was estimated as described in Eq. (3):

$$Y_{H_2+\text{Acetate}}\left(\frac{g_{\text{VSS}}}{g\,\text{COD}_{H2} - \text{Acetate}}\right) = \frac{\text{Net biomass growth}\,(g\,\text{VSS}\bullet\text{L}^{-1})}{[(\text{COD}_{H2} - \text{consumed}) + (\text{COD}_{\text{Acetate}} - \text{consumed})](g\,\text{COD}_{H2+\text{Acetate}}\bullet\text{L}^{-1})} \quad (3)$$

where the net biomass growth was calculated as difference between the final and initial biomass content, while COD_{H2}-consumed was calculated

by considering a conversion factor of 16 g COD per mol of H_2 consumed [13].

Under heterotrophic conditions, the biomass yield on acetate, was calculated as follows:

$$Y_{Acetate}\left(\frac{g_{VSS}}{g\,COD_{Acetate}}\right)=\frac{\text{Net biomass growth}\,(g\,VSS\bullet L^{-1})}{[COD_{acetate-}\text{consumed}](g_{Acetate-COD}\bullet L^{-1})} \quad (4)$$

Under autotrophic conditions, the biomass yield on H_2, was calculated as follows:

$$Y_{H_2}\left(\frac{g_{VSS}}{g\,COD_{H2}}\right)=\frac{\text{Net biomass growth}\,(g\,VSS\bullet L^{-1})}{[(COD_{H2}-\text{consumed})](g\,COD_{H2}\bullet L^{-1})} \quad (5)$$

Biomass protein content was estimated as a percentage of total protein in biomass expressed as VSS:

$$\text{Protein content}\,(\%)=\frac{\text{Protein concentration}\,(mg.L^{-1})}{\text{Biomass growth}\,(mg\,VSS.L^{-1})}\times 100 \quad (6)$$

The nitrogen balance was used to calculate the fate of nitrogen during the process. The ammonium nitrogen ($N\text{-}NH_4^+$) fed to the system was either assimilated as microbial protein or present as nitrate nitrogen ($N\text{-}NO_3^-$), nitrite nitrogen ($N\text{-}NO_2^-$), ammonium nitrogen ($N\text{-}NH_4^+$) in the liquid phase. The percentage of nitrogen removal/assimilation was calculated as follows:

$$N_{removal/assimilation}\,(\%)=\frac{(N)_i-(N)_f}{(N)_i}\times 100 \quad (7)$$

where $(N)_i$ and $(N)_f$ represent, respectively, the initial and residual total inorganic nitrogen expressed as sum of nitrate ($N\text{-}NO_3^-$), nitrite ($N\text{-}NO_2^-$) and ammonium ($N\text{-}NH_4^+$) nitrogen concentrations.

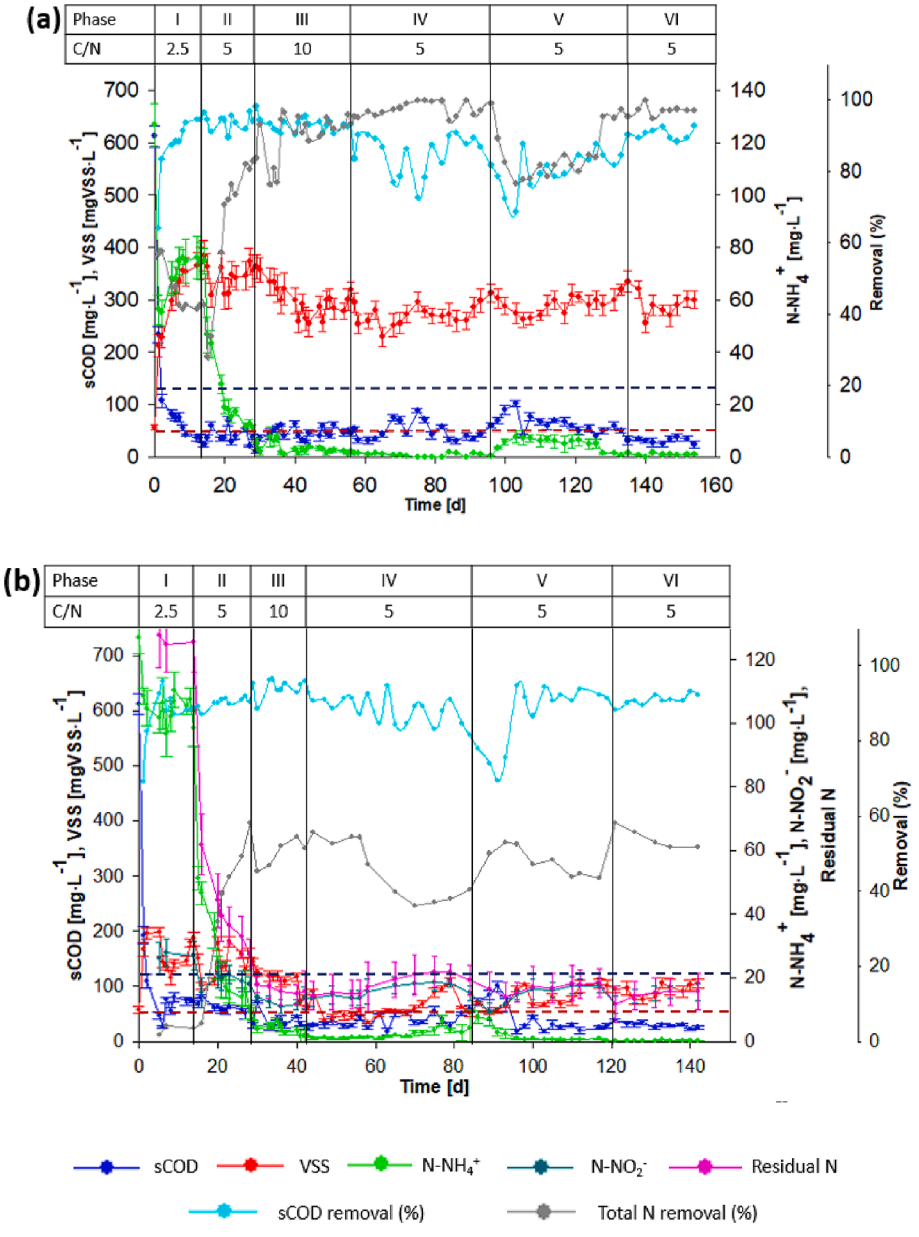

Fig.1. VSS, sCOD, ammonium ($N\text{-}NH_4^+$) and nitrite ($N\text{-}NO_2^-$) concentrations as well as sCOD and ammonium nitrogen removal efficiencies (%) under (a) mixotrophic (H_2 + acetate) and (b) heterotrophic (only acetate) conditions. Error bars refer to the standard deviation calculated from the triplicates of each test. Note: $N\text{-}NO_2^-$ was not reported in panel A since it was not detected in the experiments carried out mixotrophically. The blue and red dotted lines indicate the total N (10 mg•L^{-1}) and COD (125 mg•L^{-1}) discharge limits.

The amount of nitrogen assimilated as biomass protein was calculated as follows:

$$N_{assimilated}\,(mg) = \text{Protein content}\,(mg)\hat{A}\cdot 0.16 \quad (8)$$

The residual nitrogen in the process was calculated as follows:

$$N_{residual}(mg) = N_{supplemented}(mg) - N_{assimilated}(mg) - N_{unaccounted} \quad (9)$$

where $N_{supplemented}$ is the initial concentration of N supplied, $N_{assimilated}$ is the amount of N assimilated in the form of biomass protein and $N_{residual}$ is the amount of N left in the liquid phase in the form of nitrate ($N\text{-}NO_3^-$), nitrite ($N\text{-}NO_2^-$) and/or ammonium ($N\text{-}NH_4^+$) nitrogen. $N_{unaccounted}$ is the amount of nitrogen which could not be identified through the nitrogen balance.

3. Results and discussion

3.1. Biomass growth and treatment performances under different trophic modes

The performance of the mixed HOB culture during nitrogen assimilation under mixotrophic conditions was evaluated and compared with that of the same culture grown under heterotrophic conditions, being the latter those normally encountered in the biological oxidation step in WWTPs. The biomass growth and the $N\text{-}NH_4^+$ concentration observed under mixotrophic and heterotrophic conditions across the different experimental phases are shown in Fig. 1.

In phase I, where the C/N ratio was kept at 2.5, the average biomass growth achieved in the mixotrophic mode was 321.4 mg VSS•L^{-1}, with average residual sCOD and $N\text{-}NH_4^+$ of 77.1 and 69.9 mg·L^{-1}, respectively. Under heterotrophic conditions, instead, the average biomass growth obtained was much lower reaching 161.2 mgVSS•L^{-1}. The average $N\text{-}NH_4^+$ and sCOD concentrations were 104.2 and 80.3 mg·L^{-1}, respectively. The sCOD values were below the discharge limit for total COD (125 mg·L^{-1}) while the $N\text{-}NH_4^+$ was above the most stringent total N discharge limit (10 mg·L^{-1}) for Italian legislation concerning urban wastewater under both mixotrophic and heterotrophic conditions (D.Lgs 152/2006) [28]. The high feed $N\text{-}NH_4^+$ concentration used in this period, calculated based on a high strength wastewater with low C/N, explains why $N\text{-}NH_4^+$ levels were above the discharge limits. During phase I, while no nitrite nitrogen ($N\text{-}NO_2^-$) was observed in the presence of H_2, under strictly heterotrophic conditions the average $N\text{-}NO_2^-$ concentration was 48 mg·L^{-1}, thereby indicating the potential predominance of dissimilatory nitrogen metabolisms such as partial nitrification. Similarly, Kang et al [28] observed how the direct addition of acetate (above 1000 mg·L^{-1}) resulted in a partial-nitrification due to an inhibitory effect of acetate on nitrite-oxidizing bacteria (NOB) activity, thus causing an accumulation of NO_2^- and an overall lower nitrification rate.

During phase II, the C/N ratio was increased to 5.0. This resulted in an increased biomass concentration (342.5 mg VSS•L^{-1}) under mixotrophic conditions, while the latter decreased further under heterotrophic conditions (143.8 mg VSS•L^{-1}) with respect to the previous phase. The final $N\text{-}NH_4^+$ concentration under mixotrophic conditions reached an average value of 11.1 mg·L^{-1}, which was significantly lower than that obtained in phase I. Heterotrophically, the $N\text{-}NH_4^+$ concentration was higher (averagely equal to 16.5 mg·L^{-1}) and a considerable $N\text{-}NO_2^-$ accumulation of up to 27.5 mg·L^{-1} was observed. The sCOD value further decreased to 41.9 and 61.5 mg· L^{-1} in the case of mixotrophic and heterotrophic conditions, respectively.

The residual $N\text{-}NH_4^+$ concentration kept a decreasing trend in phase III, reaching a value of 3.2 and 4.3 mg·L^{-1} under mixotrophic and heterotrophic conditions, respectively, while the biomass concentration decreased to 304.3 (−11 %) and 110.9 (–23 %) mg VSS•L^{-1}, respectively. As for phase III, a decrease in the residual sCOD concentration was observed, achieving average values of 45.4 and 36.6 mg·L^{-1} under mixotrophic and heterotrophic conditions, respectively. The total residual N (as sum of $N\text{-}NH_4^+$ and $N\text{-}NO_2^-$) concentration observed under heterotrophic mode was 16.8 mg·L^{-1}, thus failing to meet the discharge limits. The decrease in VSS can be potentially attributed to the lower inlet $N\text{-}NH_4^+$ concentration, which decreased from 130 mg·L^{-1} in phase I to 32.5 mg·L^{-1} in phase III, thereby limiting the biomass growth under mixotrophic conditions. Despite the lower biomass concentration, in the subsequent phases, the inlet $N\text{-}NH_4^+$ concentration was kept constant at 32.5 mg·L^{-1}, while the inlet acetate concentration was decreased to 325 mg·L^{-1} to maintain the C/N ratio at 5.0 and to resemble more realistic wastewater compositions.

In phase IV, the biomass growth decreased under both conditions and the biomass concentration reached average values of 273.9 (−10 %) mg VSS•L^{-1} mixotrophically and 61.5 (−44.5 %) mg VSS•L^{-1} heterotrophically. The high VSS drop (44.5 %) under heterotrophic conditions could be ascribed to the decreased feed acetate concentration, being acetate the only energy source used by the mixed HOB consortium. A stable sCOD (50.3 and 37.6 mg·L^{-1}) and lower $N\text{-}NH_4^+$ (1.0 and 2.1 mg·L^{-1}) concentrations were observed under mixotrophic and heterotrophic conditions. The low $N\text{-}NH_4^+$ concentrations were probably due to a longer exposure of the HOB in the system that allowed a faster and more complete assimilatory microbial activity.

With the aim to evaluate the performances of the process under conditions similar to those found in WWTP, a real wastewater was used in the last two experimental phases V and VI. As mentioned in Section 2.3.2, the real wastewater was first amended with micronutrients to favor the acclimation of the mixed HOB culture, while additional acetate and $N\text{-}NH_4^+$ were used to keep the same sCOD, $N\text{-}NH_4^+$ and C/N ratio used in the previous phase. In phase V, the biomass concentration initially showed a decreasing trend under both conditions, probably due to the presence of different and more complex organic compounds in the wastewater. Yet, at the end of phase V, the biomass growth allowed to reach a biomass concentration of 290 (+6%) and 85.0 (+38 %) mg VSS•L^{-1} under mixotrophic and heterotrophic conditions, respectively. The average residual sCOD concentration increased to 62.0 mg·L^{-1} under mixotrophic conditions, while it decreased to 28.2 mg•L^{-1} heterotrophically. The lower sCOD observed during the heterotrophic growth, as compared to mixotrophic conditions, hints towards the fact that under the former conditions the utilization of the more complex organic matter present in wastewater was favored. Moreover, the $N\text{-}NH_4^+$ concentration was below the discharge limit under both mixotrophic (5.2 mg·L^{-1}) and heterotrophic (1.8 mg·L^{-1}) conditions. However, residual N under heterotrophic mode was found to be higher (18.6 mg·L^{-1}) than the discharge limit due to presence of $N\text{-}NO_2^-$.

In phase VI, the real urban wastewater was used without the addition of micronutrients to assess the feasibility of nitrogen assimilation via mixed HOB culture under more realistic conditions. In this phase, a slight decrease in the biomass growth (−2%) as well as in the residual sCOD (31.3 mg·L^{-1}) was observed under mixotrophic conditions. On the contrary, the biomass concentration slightly increased to 88.0 (+4%) mg VSS•L^{-1} and sCOD was further reduced to 20.2 mg•L^{-1} in the heterotrophic mode. The residual $N\text{-}NH_4^+$ decreased to 0.9 and 0.2 mg•L^{-1} under mixotrophic and heterotrophic growth conditions, respectively, while the total residual N of 14.6 mg•L^{-1} was still above discharge limits in the heterotrophic mode.

In a recent study on $N\text{-}NH_4^+$ removal through assimilation from liquid anaerobic digestate using a pure culture of *Paracoccus denitrificans* Y5 under diverse trophic conditions, Dou et al [18] reported that the highest $N\text{-}NH_4^+$ removal efficiency was 35.4 and 95.5 % under mixotrophic and heterotrophic conditions, respectively. The low $N\text{-}NH_4^+$ removal efficiency under mixotrophic conditions observed by the authors could be due to the use of biogas slurry of chicken manure as substrate instead of glucose, which was used as the only carbon source under heterotrophic conditions. In addition, under mixotrophic conditions, the strain of HOB did not show any gas consumption in the first 3 days of experiments, indicating that the heterotrophic pathway dominated under mixotrophic conditions. Differently from the study of Dou et

al [18], in the present study the highest biomass growth and N-NH_4^+ removal efficiency of up to 99.7 % were obtained under mixotrophic conditions. The N-NH_4^+ removal efficiency was lower than 60 % in all six phases under heterotrophic conditions in the present study. The obtained results, thus, confirm that a mixed HOB culture harboring a set of multiple potential metabolic pathways including autotrophic, heterotrophic and mixotrophic metabolism, could enable to simultaneously use gaseous substrates (H_2) and organic and inorganic carbon (e.g., acetate and CO_2 obtained by its oxidation) sources to grow and assimilate N-NH_4^+ into cell biomass. The use of both synthetic and real wastewater gave promising results in terms of biomass growth, sCOD and N-NH_4^+ removal efficiency under mixotrophic conditions, thereby showing a high potential of the H_2-driven assimilatory metabolism for wastewater treatment and resource recovery.

3.2. Biomass yield

Fig. 2 shows the biomass yield under mixotrophic and heterotrophic conditions. Under mixotrophic conditions, the lowest average biomass yield (0.18 g VSS·g COD $^{-1}_{H2+acetate}$) was obtained in phase I, with the same which kept increasing in the subsequent phases. The highest yield obtained under mixotrophic conditions was 0.32 g VSS·g $COD^{-1}_{H2+acetate}$, being 28 % higher than that achieved in the heterotrophic mode (0.25 g VSS·g $COD^{-1}_{acetate}$) in phase V. The use of unconditioned wastewater in phase VI did not significantly affect the yield, and values of 0.29 g VSS·g $COD^{-1}_{acetate+H2}$ and 0.22 g VSS·g $COD^{-1}_{acetate}$ were obtained in the mixotrophic and heterotrophic growth modes, respectively.

The average biomass yield obtained in the autotrophic growth mode, tested during period IV to have a mean of comparison, was 0.13 g VSS·g COD^{-1}_{H2} (Figure S1 in Supplementary Information). Christiaens et al [29], who used an autotrophic HOB culture in a bubble column reactor for microbial protein production, obtained yields of 0.17 g of biomass (expressed as cell dry weight, CDW) per gram of COD_{H2}. Similarly, Dou et al [18] obtained a biomass yield of 0.13 g CDW·g COD^{-1}_{H2} under autotrophic conditions using *Paracoccus denitrificans* Y5. In an another study, Zhang et al [14] used a continuous process for nitrogen assimilation using H_2 and CO_2 as sole energy and carbon sources, respectively, obtaining a yield of 0.18 g VSS·g COD^{-1}_{H2}. Therefore, the biomass yield here obtained with the mixed HOB culture under autotrophic conditions was comparable with the values obtained in other studies.

Overall, the highest yield obtained under mixotrophic conditions was higher than those achieved under both heterotrophic and autotrophic conditions. The reason for such a higher yield could be found in the synergistic effect on biomass growth obtained by combining autotrophic and heterotrophic metabolisms under mixotrophic conditions. Indeed, Sakarika et al [30] observed a 20 % higher biomass production (3.7 g CDW•L^{-1}) compared to the case where H_2 was not provided (3.1 g CDW•L^{-1}) during a pH-stat cultivation of *Cupriavidus necator* on lactic acid.

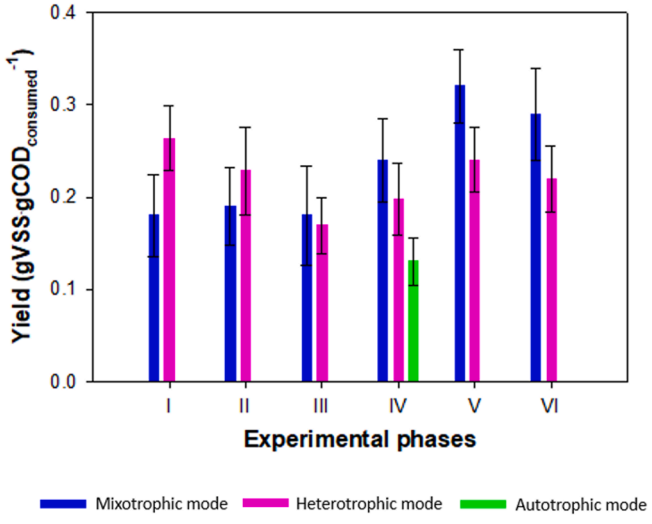

Fig.2. Biomass yield in case of mixotrophic, heterotrophic and autotrophic growth conditions. Error bars refer to the standard deviation calculated from the triplicates of each test.

3.3. Protein content in the produced microbial biomass

The C/N ratio represents a crucial parameter ruling nitrogen assimilation as protein-rich biomass. Fig. 3 shows the protein content of the microbial biomass and the protein concentration under mixotrophic and heterotrophic conditions. An average protein content of 56 and 35 % was obtained in Phase I at a C/N of 2.5 in the case of mixotrophic and heterotrophic growth, respectively. Such protein content corresponds to an average protein concentration of 179.6 and 58.6 mg·L^{-1} in the case of mixotrophic and heterotrophic growth, respectively. Indeed, under heterotrophic conditions, most of the residual nitrogen was present as nitrite, which is rarely utilized as a nitrogen source for protein accumulation [31]. Thus, the prevalence of nitrite under heterotrophic conditions could have contributed to the observed lower protein content. The increase of the C/N ratio to 5.0 (phase II) led to rather stable average protein contents of 52 and 32 % and average protein concentrations of 192.5 and 49.0 mg·L^{-1} in the case of mixotrophic and heterotrophic growth, respectively.

The lowest protein content (37.3 %) and protein concentration (106.2 mg·L^{-1}) were observed with a C/N of 10 under mixotrophic conditions. In agreement, Schryver et al [32] observed a decrease in the protein content from 23 to 20 % while increasing the C/N ratio from 2.5 to 5.0 using sequencing batch reactors (SBRs) fed with acetate as the sole carbon source. In an another study, Van Peteghem et al [33] observed a decrease in protein content from 63 to 25 % while increasing the C/N ratio from 5 to 20 using bioethanol as carbon source and a pure culture of *Corynebacterium glutamicum*. Hence, the negative effect of the increasing C/N ratio from 2.5 to 10 observed in this study is generally in line with the results from previous studies. A partial recovery in the protein content was observed in phase IV when the C/N ratio was again decreased to 5.0 in the mixotrophic growth mode, while the protein content remained comparable to that observed in the previous phase under heterotrophic conditions. In a similar study, Wang et al. [34] obtained a MP content of 41.8 and 26.7 % under mixrotrophic and heterotrophic conditions, respectively, using a mixed HOB culture and glucose as organic carbon source.

The use of real wastewater along with micronutrients in phase V did not affect the protein concentration and protein content as compared to the previous phase. The protein content was 42.5 and 27.3 % under mixotrophic and heterotrophic conditions, respectively. Also in phase VI, when the unconditioned real wastewater was used, the protein content was basically identical to the value obtained in phase V. The protein concentration ranged between 120–130 mg·L^{-1} and 20–30 mg·L^{-1} in phase V and VI under mixotrophic and heterotrophic conditions, respectively.

The average protein concentration observed under autotrophic mode in this study was 118 mg·L^{-1}, corresponding to a protein content of 60 % (Fig. 3). Dou et al [18] obtained a 64 % crude protein content in MP produced by an axenic culture of *Paracoccus denitrificans* Y5 using CO_2 as sole carbon source under similar conditions. Zhang et al [14] observed a slightly lower protein content (54.3 %) while removing ammonium under similar autotrophic conditions by means of the hydrogen-oxidizing bacterium *Ideonella* sp. TH17.

The mixed HOB culture used in this study showed a strong adaptivity to a wide range of C/N ratios in terms of protein content. The highest performance in terms of N-NH_4^+ removal efficiency, biomass growth, protein concentration and protein content was achieved with a C/N ratio of 5.0, under mixotrophic conditions. This result showed the synergistic

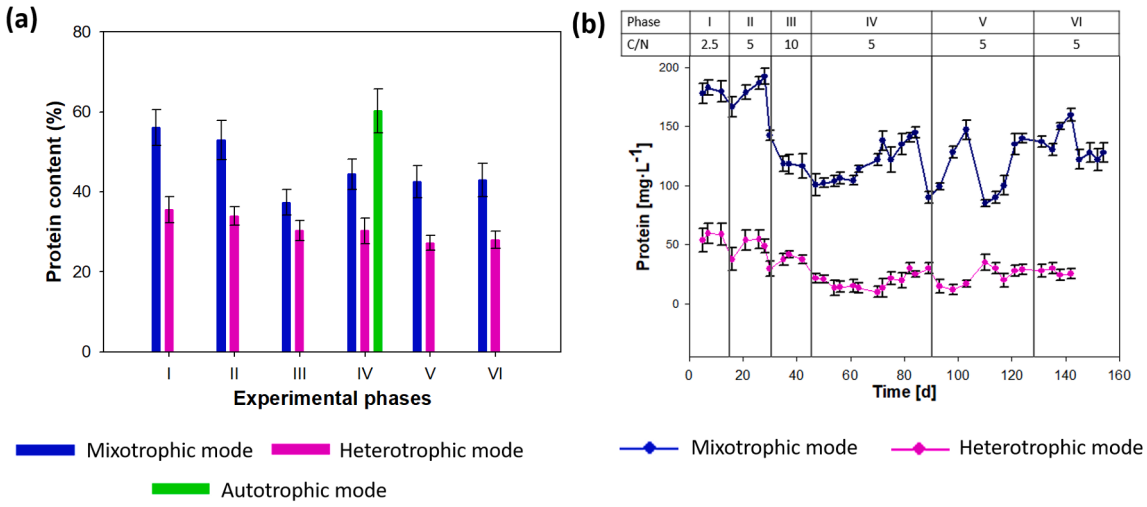

Fig.3. (a) Protein content and (b) protein concentration under mixotrophic and heterotrophic conditions. Error bars refer to the standard deviation calculated from the triplicates of each test.

relationship between autotrophic and heterotrophic pathways under mixotrophic mode. Overall, the growth of the mixed HOB culture, under mixotrophic conditions was advantageous over the other trophic modes in terms of both VSS concentration and protein content. The latter result is in agreement with other studies on HOB for MP production [18], and confirms that HOB might be suitable as MP-producing microorganisms, both as pure or mixed cultures.

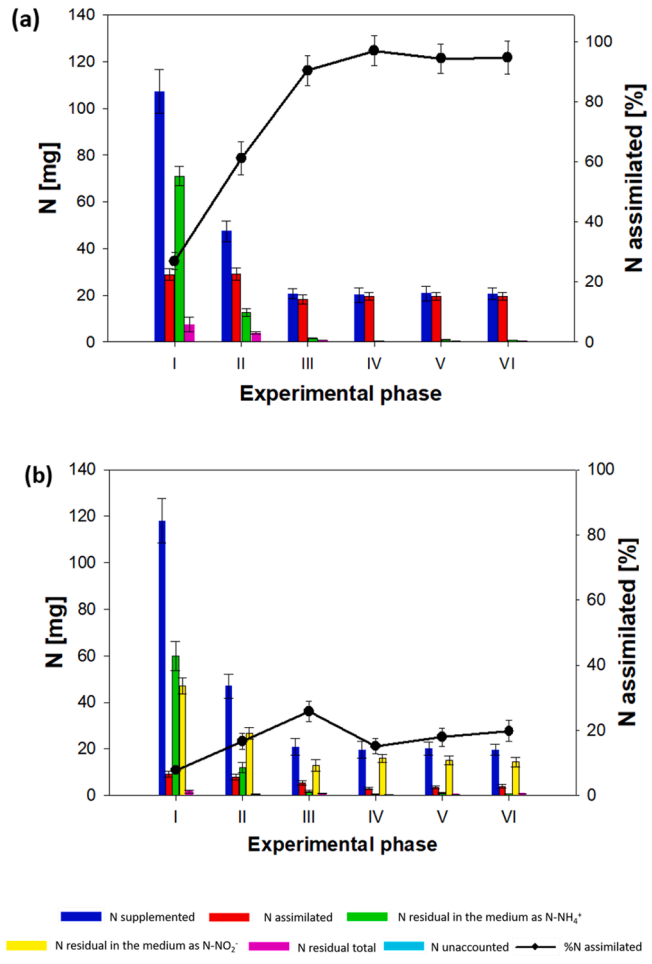

Fig.4. Nitrogen balance in the case of (a) mixotrophic and (b) heterotrophic growth mode. Error bars refer to the standard deviation calculated from the triplicates of each test.

3.4. *Fate of nitrogen under mixotrophic and heterotrophic growth conditions*

The analysis of the dynamics linked to nitrogen evolution observed during the bioprocess driven by the mixed HOB culture allowed to evaluate the fate of the N-NH_4^+ supplemented and to draw an overall nitrogen balance. Fig. 4a shows the concentration of the various nitrogen forms observed under the six different experimental phases in the case of mixotrophic growth conditions. In phase I, about 26.9 % of the added nitrogen was assimilated as protein, while most of the nitrogen was present as residual N-NH_4^+. In phase II, instead, nitrogen assimilation increased to 61.2 % and kept increasing up to 90.4 % in phase III when the C/N ratio was increased to 10. The highest nitrogen assimilation into proteins of 97 % was obtained in phase V. Similar results of more than 90 % of NH_4^+-N assimilated in the biomass was obtained by Zhang et al [14], who used *Ideonella* sp. TH17. Unaccounted nitrogen was less than 10 % in phases I and II, and decreased to less than 5 % in the subsequent phases. The high value of unaccounted nitrogen in first two phases could be due to the high inlet N-NH_4^+ concentration.

Fig. 4b shows the nitrogen supplemented, nitrogen assimilated, and nitrogen unaccounted in the case of heterotrophic growth. The low nitrogen assimilation (7.7 %) obtained in phase I increased to 16.6 and 25.8 % in the subsequent phases II and III, respectively, likely due to the increased C/N ratio. Such low assimilation efficiencies resulted in a high residual N-NH_4^+ concentration. Most of the N under heterotrophic conditions was present as N-NH_4^+ as well as N-NO_2^- in phases I and II, while in the subsequent phases (from III to VI) residual nitrogen was mostly present as N-NO_2^-. Nitrite, which was not assimilated into the biomass, remained in the medium and peaked at 48 mg·L^{-1} in phase I. In phase II and III, the residual N-NO_2^- concentration decreased to 26.7 and 12.8 mg·L^{-1} and remained almost constant at around 15.0 mg·L^{-1} in the subsequent phases (IV, V and VI). Unaccounted N was below 5 % in phases I and II and further decreased in the subsequent phases.

The higher residual nitrogen concentration as N-NO_2^- under heterotrophic conditions can be most likely attributed to a partial nitrification of N-NH_4^+. Multiple studies have shown that high concentrations of VFAs (e.g. acetate) (>1200 mg/L) can inhibit the nitrite-oxidizing bacteria (NOB) activity by up to 100 % [28,35]. A reduction of 89 % was observed in nitrite oxidation when acetate concentration was increased from 200 to 1000 mg·L^{-1} confirming that acetate could cause a direct inhibition on the nitrification rate, and the inhibition was more severe

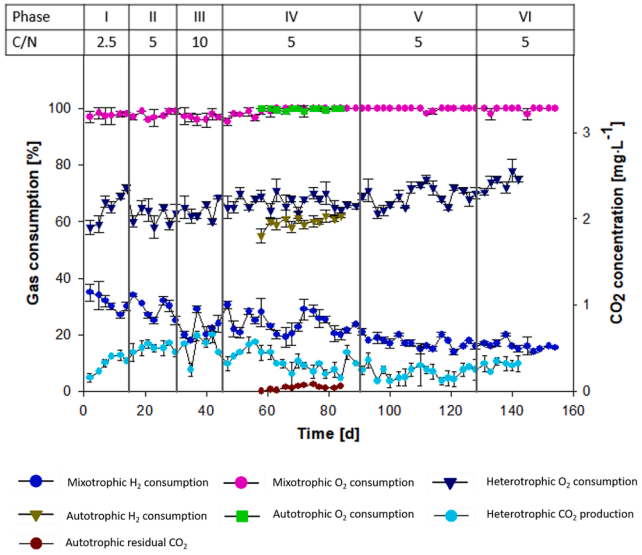

Fig.5. H_2 and O_2 consumption under mixotrophic, heterotrophic and autotrophic conditions. The graph also shows the production of CO_2 under heterotrophic conditions.

on NOB compared to ammonium oxidizing bacteria [28].

3.5. Utilization of gases and fate of carbon dioxide

The concentration of hydrogen (H_2), carbon dioxide (CO_2), and oxygen (O_2) in the headspace was measured to monitor and evaluate gas consumption and potential GHG emissions during biological nitrogen assimilation. Fig. 5 shows gas consumption under mixotrophic, heterotrophic and autotrophic conditions through the different experimental phases.

Under mixotrophic conditions, the average H_2 consumption was 31 and 28 % during phase I and II, indicating a higher demand for H_2 during the adaptation phase, which was further confirmed by the high biomass growth (321 and 342 mg VSS•L^{-1}) (Fig. 1a). The average H_2 consumption constantly decreased along the following phases, being 24 and 21 % in phase III and IV, respectively, and reaching 17 % in Phase V when the micronutrient amended wastewater was used. In phase VI, when the raw wastewater was used, the average H_2 consumption was 16 %. The supplementation of the raw influent wastewater might have potentially impacted negatively the metabolism of the mixed HOB culture, which might have decreased the activity linked to H_2 oxidation to focus more on the removal of more complex organic carbon compounds.

O_2 consumption was above 95 % throughout all experimental phases, and no residual CO_2 was observed under mixotrophic conditions, suggesting that all the CO_2 produced after the oxidation of acetate, or of other organic compounds in the real wastewater used in phase V and VI, was fixed into microbial biomass by the HOB mixed community, thereby promoting full carbon capture and avoiding potential and uncontrolled GHG emissions. The latter means that, according to Eq. (10), a theoretical CO_2 production from acetate (650 and 325 mg·L^{-1}) of nearly 0.52 (Phases I, II and III) and 0.26 mmol (Phases IV, V and VI) was entirely used by the enriched HOB culture as carbon source. The higher availability of CO_2 derived from acetate oxidation production in phases I, II and III, could justify the oxidation of more H_2, resulting in a higher H_2 consumption as compared to phases IV, V and VI, where the lower CO_2 production possibly limited H_2 consumption through the autotrophic pathway. Thus, heterotrophic and autotrophic pathways could form mutualistic relationships (from the perspective of CO_2) under mixotrophic conditions.

$$CH_3COOH + 2O_2 \rightarrow 2CO_2 + 2H_2O \quad (10)$$

For a better understanding of the results, the H_2/CO_2 uptake ratio, calculated for each experimental phase by considering that all organic carbon present would be oxidized to CO_2, was equal to 4.0, 3.9, 3.1, 5.8, 4.4 and 4.2 in phases I, II, III, IV, V and VI, respectively (Table S3 of the Supplementary Information). All H_2/CO_2 uptake ratios were lower than the stoichiometric H_2/CO_2 ratio estimated for HOB (8.5) during autotrophic growth, meaning that enough CO_2 was present to fulfill the stoichiometric demand. Similar results in terms of high biomass yield at lower H_2/CO_2 ratio (2.0) were obtained by Pelagalli et al [21], who used mixed HOB culture to directly upcycle municipal sewage sludge derived syngas into MP. In the present study, the actual amount of the CO_2 available was likely lower than that previously calculated, since part of the organic carbon was directly assimilated into biomass. Hence, the actual H_2/CO_2 uptake ratio during mixotrophic growth might be different from that typically reported for autotrophic growth. Further studies are thus warranted to gain better insights into the H_2/CO_2 uptake ratio established during mixotrophic growth.

In this study, three different C/N ratios were tested being C/N a crucial parameter for biomass growth and nitrogen assimilation. The decrease in acetate concentration caused a quick lowering in terms of CO_2 generation rate from acetate oxidation. These results indicated that, with the lack of organic carbon, the autotrophic pathway steadily strengthened and developed as secondary metabolic mode. This observation supports the hypothesis that key enzymes in autotrophic mode could be formed under heterotrophic conditions [36]. Moreover, the absence of CO_2 in the headspace under mixotrophic conditions suggests that the addition of H_2 stimulated CO_2 consumption produced via acetate oxidation. However, further research is needed to understand these mechanisms in more detail.

The H_2 and O_2 consumption, together with the CO_2 concentration in the case of autotrophic mode are presented in Fig. 5. The H_2 consumption obtained under autotrophic mode was around 60 %, whereas all O_2 was consumed. The final CO_2 concentration observed was almost zero, showing that CO_2, which was the only carbon source present, was almost completely assimilated by the mixed HOB culture also under autotrophic conditions.

Under heterotrophic conditions, O_2 consumption fluctuated between 60 and 80 % during the whole experimentation. The incomplete O_2 consumption under heterotrophic conditions could be related to the fact that enough O_2 was present to oxidize acetate, since only acetate was present as electron donor in phases I, II, III and IV, while also other electron donors were available in the real wastewater in phases V and VI. Meanwhile in the autotrophic and mixotrophic conditions, where H_2 was provided, the oxygen demand driven by H_2 oxidation was markedly higher (Fig. 5). A measurable and constant CO_2 production was observed under heterotrophic conditions. The detected CO_2 concentration was below 1 mg·L^{-1} during the entire experimentation, regardless of the tested C/N ratio.

3.6. Application potential

The current findings clearly show the potential of the H_2-driven mixotrophic nitrogen assimilation process to remove and upcycle ammonium nitrogen as MP while concomitantly treating wastewater below discharge limits for COD and total inorganic nitrogen. Fig. 6 shows a possible scheme to implement this process within an urban WWTP. Under mixotrophic conditions, the process allows assimilative N-NH_4^+ and COD removal while limiting GHG emissions, therefore representing a suitable and more compact alternative to the conventional nitrification–denitrification scheme. Indeed, by including this technology in the WWTP scheme, N_2O emissions deriving from the nitrification–denitrification process would be avoided and the anoxic compartments required to perform denitrification would not be needed, thus reducing the WWTP carbon footprint, land use and investment costs.

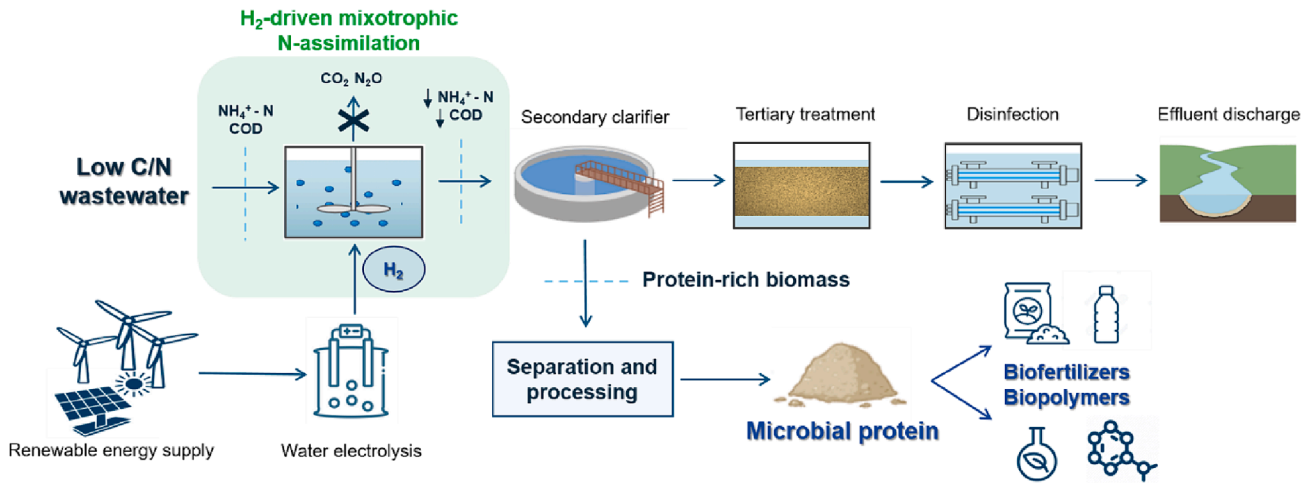

Fig.6. Potential process scheme for the implementation of the H_2-driven mixotrophic N- assimilation in WWTPs.

In practical applications, gaseous substrates (e.g., H_2 and O_2) would need to be produced by green techniques, such as water electrolysis powered by renewable energy (e.g., wind turbines, photovoltaic panels, etc.). By curbing GHG emissions and N-losses, and by enabling more compact WWTPs, this process can thus contribute to the sustainability of the wastewater treatment sector. Further research is needed to implement and optimize this technology in continuous reactors, to scale up the process to an industrial level, and to address current major challenges, such as the low solubility of gaseous substrates and the formation of an explosive atmosphere composed of H_2 and O_2.

The harvested protein rich biomass during the wastewater treatment process (Fig. 6) has multiple marketable applications. Among them, the most suitable is the production of bio-polymers and biofertilizers rather than products for the feed and food sector. This is due to the still high commercial value as compared to MP in feed/food, as well as to the absence of stringent quality standards that are instead required for the quality applications (e.g. absence of biological and chemical contaminants) [36].

Based on the above considerations, and leveraging the experimental findings from this study, a preliminary economic assessment was conducted to evaluate the feasibility of the H_2-driven mixotrophic wastewater treatment and nitrogen upcycling into MP. On average, the collected data indicate that 2.5 kg of H_2 is needed to remove and assimilate 1 kg of nitrogen under the form of MP (Supplementary Table S4). Based on an average production cost of H_2 of 3 Euro per kg [37], this would translate into a cost of 7.5 Euro per Kg of N removed and assimilated in MP. The latter value is higher but comparable to the costs incurred in N removal and dissipation into N_2 through conventional biological nitrification–denitrification using external organic electron donors (3 to 5 Euro/kg N). Moreover, if a net profit of about 0.5 Euro per kg MP could be attained by selling the produced MP e.g. as biofertilizer or as building block for protein-based bioplastics [6], the assimilation of 1 kg of N, resulting in about 10 kg of MP, would result into a net income of 5 Euro. This implies that the overall cost of N removal and assimilation, in net terms, would be around 2.5 Euro per kilogram N, potentially making this process even more economical compared to conventional nitrification and denitrification. Concerning these preliminary estimates, it is essential to acknowledge that the final cost and economic balance of the process can vary greatly, mostly depending on the actual H_2 production costs and potentially attainable MP profits, with the latter being influenced by the final quality and market potential of the derived MP products.

4. Conclusions

Sustainable nitrogen removal and recovery, as well as COD removal, through biological assimilation driven by H_2 was achieved by growing a mixed HOB culture under mixotrophic conditions. As a typical nitrogen assimilator and MP producer, the mixed HOB culture showed better growth performances under mixotrophic conditions, with a biomass concentration (342.5 mg VSS•L^{-1}) that was more than double and 52 % higher than those achieved under heterotrophic (161.2 mg VSS•L^{-1}) and autotrophic (225.0 mg VSS•L^{-1}) conditions. Indeed, under mixotrophic conditions, the mixed HOB culture could grow by utilizing acetate or the organic carbon in the wastewater as energy and carbon source, and the addition of H_2 stimulated the capture of all CO_2 produced from the oxidation of organic carbon, thereby avoiding potential diffused GHG emissions. Under mixotrophic conditions, neither nitrite nor nitrate were detected, confirming the absence of nitrification–denitrification dynamics and, consequently, avoiding potential N_2O emissions. In view of optimizing operational conditions and testing the efficiency of the process, different C/N ratios were investigated, with the highest $N\text{-}NH_4^+$ removal efficiency (more than 99 %), biomass yield (0.32 g VSS·g COD $^{-1}_{H2+acetate}$) and protein content (54 %) obtained at a C/N ratio of 5.0 under mixotrophic conditions. On the contrary, under heterotrophic conditions, most of the nitrogen was probably subjected to dissimilatory partial nitrification and accumulated as nitrite. The use of real wastewater gave promising results in terms of $N\text{-}NH_4^+$ (97 %) and COD (91 %) removal as well as biomass yield (0.29 g VSS·g COD $^{-1}_{H2+acetate}$) under mixotrophic conditions. Overall, the present study indicates how the H_2-driven mixotrophic nitrogen assimilation holds a high potential for improving anthropogenic nitrogen management by curbing GHGs emissions and valorizing waste resources from WWTPs.

CRediT authorship contribution statement

Manoj Kumar: Writing – review & editing, Writing – original draft, Validation, Methodology, Data curation. **Silvio Matassa:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Methodology, Investigation, Data curation, Conceptualization. **Chiara Belloni:** Writing – review & editing, Writing – original draft, Data curation. **Francesco Pirozzi:** Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition. **Giovanni Esposito:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Methodology, Conceptualization. **Stefano Papirio:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Methodology, 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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cej.2024.151207.

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