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From waste to high-value nitrogen: optimizing and validating direct nitrogen stripping from anaerobic digestate for sustainable microbial protein production

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Abstract:	In the quest for sustainable and circular alternative protein sources, low-cost and contaminant-safe resource recovery techniques are urged. This study investigated and optimized a zero-chemical, low-temperature direct air stripping process from anaerobic digestate for clean nitrogen (N) recovery and its upcycling into microbial protein (MP) produced from cheese whey permeate (CWP). Key operational parameters, including air-to-digestate (A:D) ratio, temperature, initial nitrogen concentration, and batch vs. continuous operation, were assessed. Stripping efficiencies of up to 100% were achieved within 24 hours under batch conditions with A:D ratios of 2:1 and 4:1 at mesophilic (25, 35, 45 °C) and thermophilic (55 °C) temperatures. Under continuous operation, up to 61% stripping efficiency was obtained at 45 °C with an A:D ratio of 4:1. The latter operating condition was selected to integrate the direct air stripping process with the aerobic MP production step, leveraging the aeration flow of the latter. This N-recovery strategy was compared to other N-supply routes such as the direct addition of N-rich digestate or of NH_4Cl to CWP. The impact of heavy metals in digestate on biomass growth and MP quality was also investigated. Biomass concentrations reached approximately 17 g TSS·L-1 when nitrogen was supplied through digestate, while using stripped ammonia from digestate led to a lower biomass growth, reaching 4.78 g TSS·L-1. Although heavy metals did not inhibit biomass growth, they compromised the quality of the final MP product when digestate was supplied directly as nitrogen source.

Title: From waste to high-value nitrogen: optimizing and validating direct nitrogen stripping from anaerobic digestate for sustainable microbial protein production

Authors:

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Naples, March 3rd, 2025

To the Editorial Board of *Journal of Environmental Management*

Cover letter – Manuscript submission

Dear Editors,
We are pleased to submit the attached research article entitled "From waste to high-value nitrogen: optimizing and validating direct nitrogen stripping from anaerobic digestate for sustainable microbial protein production" authored by Antonella Scotto di Uccio, Silvio Matassa, Alessandra Cesaro, Francesco Pirozzi, Giovanni Esposito and Stefano Papirio, to be considered for publication in *Journal of Environmental Management*.

The rising global protein demand is straining traditional agricultural and animal farming methods, which are resource-intensive and vulnerable to disruptions. Microbial protein (MP) production offers a promising alternative due to its efficiency and sustainability. However, sourcing nitrogen (N) for bioprocessing remains costly and environmentally impactful. Utilizing waste-derived N, such as that from anaerobic digestate, could address these challenges by providing nutrients essential for microbial growth. The direct use of digestate faces hurdles, including pathogen contamination and the presence of organic and inorganic pollutants. Ammonia stripping is a viable solution, separating ammonia gas (NH_3) from digestate, reducing contamination risks and improving the MP production quality.

This study evaluated first the ammonia stripping efficiency under various conditions (different operational modes, temperature and air to digestate (A:D) ratio) and then evaluated the recovered N as a substrate for MP production using cheese whey permeate (CWP) as a carbon-rich substrate. The tests compared the effectiveness of using N recovered through stripping or through direct digestate feeding, as well as through the addition of mineral N (NH_4Cl), in terms of biomass yield, protein content, and ammonium-nitrogen removal. Additionally, the potential transfer of heavy metals from digestate to the final product was assessed under the main tested scenarios.

Findings highlight the potential of optimized ammonia stripping as a sustainable N recovery strategy, enabling efficient and safe MP production while addressing environmental and economic concerns.
The direct air stripping process effectively removed ammonia nitrogen ($N\text{-}NH_4^+$) from digestate at temperatures ≤55 °C without chemical pH adjustment, achieving up to 100% efficiency in batch conditions (55 °C, A:D=4:1) and 61% in continuous conditions (45 °C, A:D=4:1).
Nitrogen feeding strategies significantly impacted MP production. When N was supplied via NH_4Cl or direct digestate addition, biomass concentrations reached 10.08–17.06 g $TSS \cdot L^{-1}$. In contrast, the ammonia stripped from digestate yielded a lower biomass (4.78 g $TSS \cdot L^{-1}$), likely due to an insufficient N amount during the early biomass growth phases.

Depending on how the digestate was used as N source, the MP quality was differently affected in terms of heavy metals content. Ammonia stripping from the digestate prevented metal transfer, preserving the MP biomass quality. Conversely, directly feeding the digestate to the MP production reactor resulted in an elevated heavy metal content in the biomass, lowering protein levels and making it unsuitable for human consumption and potentially problematic for the use as animal feed or fertilizers.

The present manuscript is an original work of the authors, which has not been previously submitted to the *Journal of Environmental Management*, nor is any part of it under consideration for publication in another journal. All authors have approved the manuscript and mutually agreed to the submission to *Journal of Environmental Management*. The article has been prepared according to the Guide for Authors and in compliance with the Ethics in Publishing Policy.

On behalf of the co-authors, I thank you for receiving our manuscript and for considering it for review. We appreciate your time and look forward to your response.

Sincerely,

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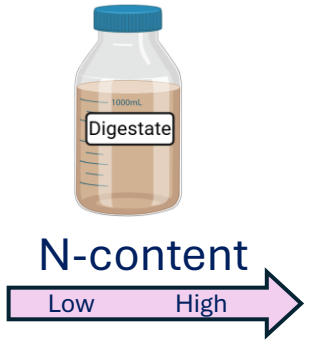
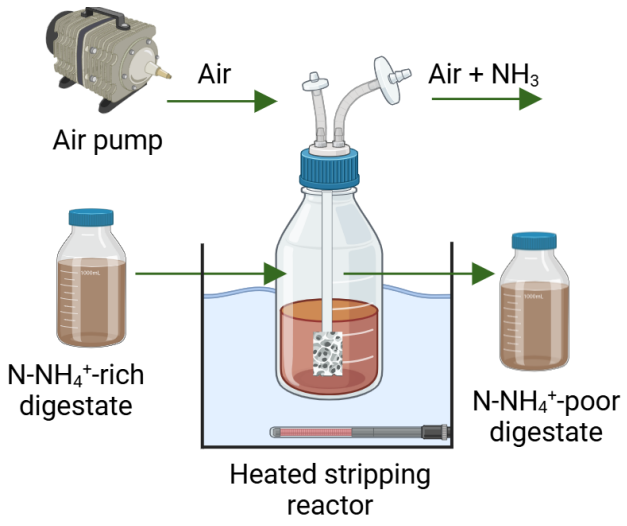
N-source

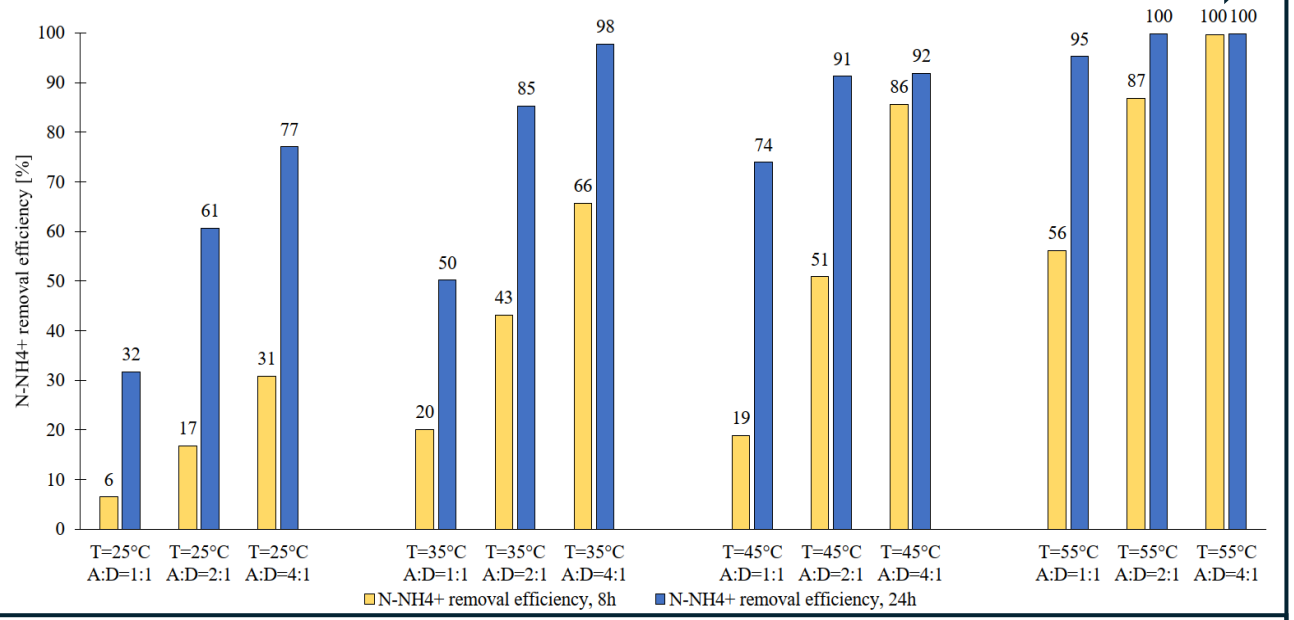
Experimental set up

Results

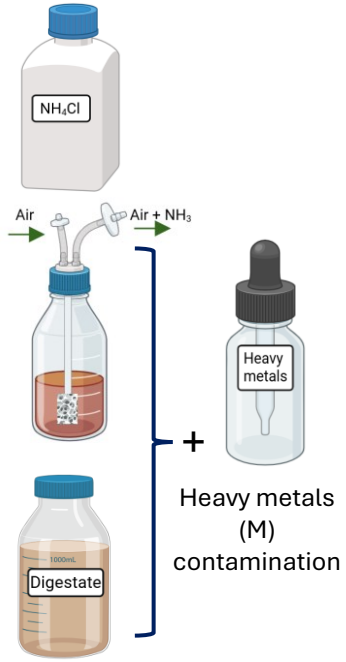
Direct N stripping

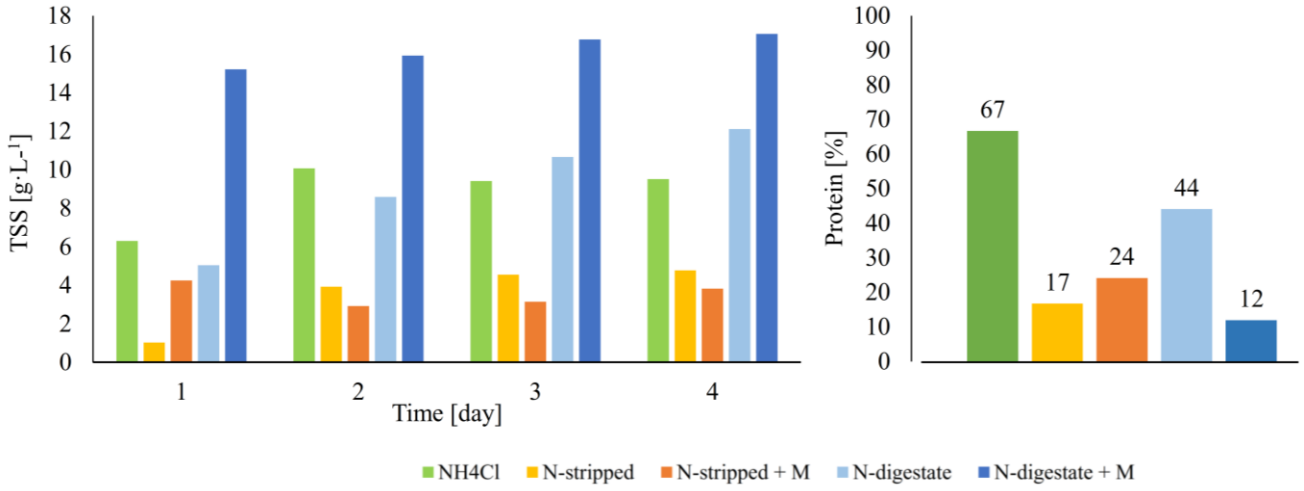






Aerobic microbial protein (MP) production





- Ammonia stripping was 100% in batch at 55°C and with an air to digestate ratio of 4:1
- Stripped N vs. direct N feeding led to a lower biomass growth (5 vs. 17 g $TSS \cdot L^{-1}$)
- An initial low N availability through N stripping limited the final protein content
- Heavy metals in digestate increased biomass concentration but lowered protein content
- N stripping prevented metal transfer from digestate improving final biomass quality

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ABSTRACT

In the quest for sustainable and circular alternative protein sources, low-cost and contaminant-safe resource recovery techniques are urged. This study investigated and optimized a zero-chemical, low-temperature direct air stripping process from anaerobic digestate for clean nitrogen (N) recovery and its upcycling into microbial protein (MP) produced from cheese whey permeate (CWP). Key operational parameters, including air-to-digestate (A:D) ratio, temperature, initial nitrogen concentration, and batch vs. continuous operation, were assessed. Stripping efficiencies of up to 100% were achieved within 24 hours under batch conditions with A:D ratios of 2:1 and 4:1 at mesophilic (25, 35, 45 °C) and thermophilic (55 °C) temperatures. Under continuous operation, up to 61% stripping efficiency was obtained at 45 °C with an A:D ratio of 4:1. The latter operating condition was selected to integrate the direct air stripping

process with the aerobic MP production step, leveraging the aeration flow of the latter. This N-recovery strategy was compared to other N-supply routes such as the direct addition of N-rich digestate or of NH_4Cl to CWP. The impact of heavy metals in digestate on biomass growth and MP quality was also investigated. Biomass concentrations reached approximately 17 g $TSS \cdot L^{-1}$ when nitrogen was supplied through digestate, while using stripped ammonia from digestate led to a lower biomass growth, reaching 4.78 g $TSS \cdot L^{-1}$. Although heavy metals did not inhibit biomass growth, they compromised the quality of the final MP product when digestate was supplied directly as nitrogen source.

Keywords: ammonia stripping; anaerobic digestate; cheese whey; microbial protein; resource recovery; heavy metals.

1. Introduction

The global protein demand is rising unsustainably, projected to increase by 78% by 2050 (Van Peteghem et al., 2022). Traditional protein sources, reliant on resource-intensive farming and vulnerable to disruptions like disease and overfishing, are no longer viable (Mohammad et al., 2023; Tubb and Seba, 2021). To ensure food security amidst climate change and population growth, efficient protein alternatives such as plant-based proteins, cultured meat, and microbial protein (MP) are essential (Aiking and de Boer, 2020).

MP synthesis, utilizing microorganisms like bacteria and microalgae in closed bioreactors, offers high resource efficiency, including waste nitrogen (N) recycling, reducing environmental impact (Matassa et al., 2016; Pikaar et al., 2017). As a matter of fact, MP production relies heavily on the availability of reactive N, the building block of biomass and protein synthesis. However, the cost and environmental impact of sourcing N for bioprocessing remain substantial, influencing both the economic and ecological footprint of MP production (Van Peteghem et al., 2023). Consequently, growing interest is being directed towards the utilization of waste-derived N sources, which can both reduce operational costs and contribute to more circular and sustainable bioproduction systems (Pikaar et al., 2017).

One of the most abundant N-rich wastes from livestock farms is represented by anaerobic digestate (Scotto di Perta et al., 2023). Anaerobic digestate is a valuable source of organic matter, amino acids, vitamins and nutrients such as phosphorus, potassium, ammonium nitrogen ($N\text{-}NH_4^+$) and several trace elements such as copper and zinc (Di Costanzo et al., 2023; Jin and Chang, 2011) . With $N\text{-}NH_4^+$ concentration in a digestate ranging between 0.8-6.0 $g \cdot L^{-1}$ (Möller and Müller, 2012; Shi et al., 2018), various processes have been developed to N recovery from digestate for microbial growth (Bertasini et al., 2022; Kovačić et al., 2022). However, the direct utilization of digestate poses several challenges. One significant concern is the presence of

pathogens, including bacteria, viruses and parasites, which could contaminate the resulting MP if adequate treatment is not applied (Di Costanzo et al., 2024; Kovačić et al., 2022). Additionally, digestate may contain organic pollutants (e.g., pharmaceutical and pesticides) or inorganic contaminants (e.g., heavy metals) which could interfere with microbial cultivation or result in unsafe MP products (Golovko et al., 2022).

To address these challenges, ammonia stripping emerges as a viable alternative to recover N from digestate in a suitable form for MP production. The stripping process facilitates the transition of ammonium ions (NH_4^+) to ammonia gas (NH_3), effectively separating nitrogen from the contaminated liquid digestate (Matassa et al., 2022; Scotto di Perta et al., 2023). Ammonia stripping efficiency depends on pH, temperature, mass transfer area, and dissolved CO_2 in the liquid matrix (Limoli et al., 2016). Despite its attractiveness, the need for dedicated equipment (Kar et al., 2023), chemical additives to adjust pH (Alitalo et al., 2012; Bonmatí and Flotats, 2003), and significant thermal energy to heat digestate to 70-90 °C (Matassa et al., 2015) lead to high operational and capital costs. Recent studies (Matassa et al., 2022; Zhao et al., 2015) explored the possibility of operating the direct air stripping process at milder temperatures and without chemical pH adjustments by using aeration flows to strip CO_2 from digestate, as its buffer capacity is mainly due to the bicarbonate/carbonate equilibrium. The latter process not only recovers N but also minimizes contamination risks, as ammonia gas is free from non-volatile pathogens and pollutants remaining in the liquid fraction. The air-ammonia-rich output gas stream can be exploited to provide oxygen and nitrogen for MP production by promoting the solubilization of N in growth substrates characterized by acidic pH such as cheese whey permeate (CWP) (pH 4.0-6.5, Carvalho et al., (2013)). The acidic environment promotes the absorption of ammonia as $N\text{-}NH_4^+$ without the need of pH adjustment, providing a readily available N source for microbial growth.

Data about the actual potential of the direct air stripping process are scarce, and comparative evaluations with other N recovery and feeding strategies for MP production are lacking. In light of this, the present study first investigated experimentally the influence of mesophilic and thermophilic temperatures and air to digestate (A:D) ratios during the direct air stripping of ammonia from the liquid fraction of anaerobic digestate under batch conditions, and then evaluated the same process under continuous reactor settings and with different initial $N\text{-}NH_4^+$ concentrations. The process performances were evaluated in terms of $N\text{-}NH_4^+$ stripping efficiency and rates. Subsequently, MP production from CWP using N recovered through the optimised direct air stripping process was investigated and compared with MP production using direct addition of digestate and mineral N (NH_4Cl) as N sources. The MP production process was evaluated with respect to key process performance indicators such as biomass concentration, protein content, sCOD removal and residual $N\text{-}NH_4^+$. Finally, considering the potential presence of high heavy metals content in the digestate, which could hinder biomass growth as well as compromise product quality (Dragicevic et al., 2017), the work evaluated how the latter may be transferred and/or accumulated in the final product when different N recovery and feeding strategies are applied.

2. Materials and methods

2.1 Source, characterization and pretreatment of anaerobic digestate

The anaerobic digestate was sourced from anaerobic digesters located in the Campania region (Italy) treating buffalo manure and agricultural residues. Digestates were stored at 4 °C prior to physic-chemical characterization (Table S1). A high-N digestate was used for batch air stripping, while a low-N digestate was used for continuous air stripping. The low-N digestate, amended with NH_4Cl to achieve different N concentrations, was used for other continuous air stripping tests and as direct N source for the aerobic MP production.

Before being used, the digestate was filtered through a 1 mm stainless steel filter mesh to separate the liquid from the solids, then centrifuged at 6000 rpm for 10 minutes to remove residual solids, improving liquid-to-gas mass transfer in air stripping and nutrient assimilation in MP production.

When used as N source directly supplemented in the synthetic C-rich CWP, the liquid fraction was pasteurized (70 °C for 1 hour) and diluted to achieve 1.3 $g \cdot L^{-1}$ $N\text{-}NH_4^+$, ensuring a C/N ratio of 15. After pasteurization and dilution, the dissolved organic carbon (DOC) of the digestate, used as indicator of the dissolved organic matter, was equal to 1.40 $g \cdot L^{-1}$. This allowed to consider the DOC fed through the digestate as negligible, as the DOC provided through the lactose of the CWP used was 14.40 $g \cdot L^{-1}$ (Table S2).

Furthermore, to investigate the effect on biomass of the heavy metals potentially present in the digestate, the anaerobic digestate was chemically amended with metals (Table S3) to reach typical concentration values of digestate from animal manure (Dragicevic et al., 2017; Jin and Chang, 2011; Li et al., 2018).

2.2 Composition of the synthetic cheese whey permeate

A synthetic lactose-based solution, adapted from Dubois Frigon (2020) and reported in Table S2, was used to simulate a CW-based organic substrate for MP production. The nitrogen concentration provided either through mineral nitrogen (NH_4Cl) or through direct digestate supply was calculated to achieve a C/N ratio equal to 15.

2.3 Direct air stripping tests

Direct air stripping tests were performed under both batch and continuous modes in 250 mL Schott bottles with 150 mL of liquid digestate in a water bath at 25, 35, 45 and 55 °C. Air was

provided as stripping agent by a stainless steel air sparger (bubble size 0.5 – 2.0 mm) connected to a 520S peristaltic pump (Watson Marlow, United Kingdom). The sparger was placed 5 cm below the surface to enhance pH elevation and ammonia desorption (Zhao et al., 2015). A:D ratios of 1:1, 2:1 and 4:1, were guaranteed by providing airflows of 150, 300 and 600 mL·min^{-1}, respectively. To avoid foaming, a volume of 1 mL of antifoaming agent (Arauner defoamer, Germany) was added to 150 mL of digestate.

2.3.1 Batch tests

Batch tests lasting 24 hours, performed with high-N digestate (Table S1), screened temperature and A:D combinations. Gas and liquid samples were taken periodically to monitor the time-dependent evolution of gas composition, pH and N-NH_4^+ concentration.

2.3.2 Continuous tests

Continuous tests, performed with low-N digestate (Table S1), were conducted with an A:D ratio of 4:1 at temperatures of 25, 35 and 45 °C and with an A:D ratio of 2:1 at temperature of 55 °C. Continuous tests were carried out with a hydraulic retention time (HRT) equal to 8 hours and lasted 48 hours. Furthermore, additional continuous tests were performed using digestate amended with NH_4Cl (ITW Reagents, Italy) to evaluate the effects of different initial N-NH_4^+ concentrations of 1.5, 2.5, 3.5 and 4.5 g·L^{-1}. Liquid samples were collected periodically to monitor pH and N-NH_4^+ concentration.

2.4 Aerobic MP production

2.4.1 Source and activation of the microbial inoculum

A commercial lyophilized mixed culture of yeasts and bacteria (Genesis Laboratories, Bulgaria) for kefir production served as microbial inoculum. Prior to its use for the aerobic MP production process, the inoculum was activated under batch conditions. Activation was performed in a 500

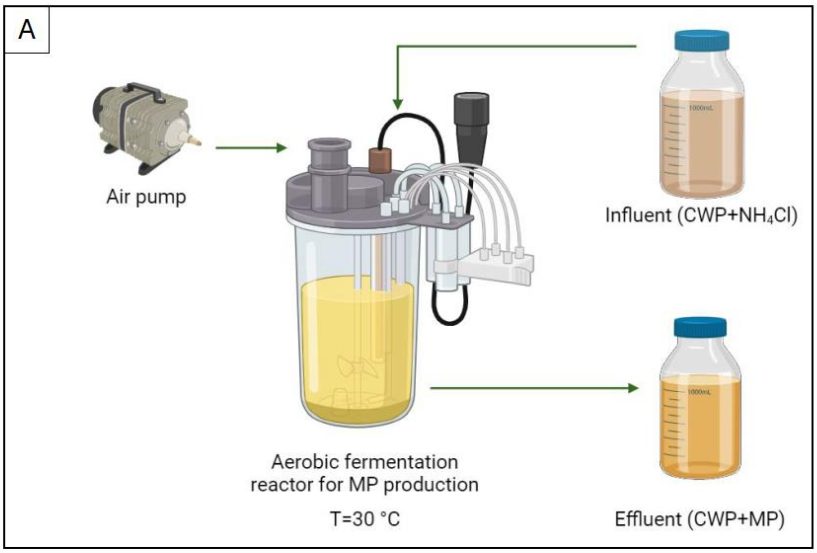
mL Schott bottle with 200 mL synthetic CWP, incubated at 30 °C and stirred at 500 rpm for 48 hours. Aeration was supplied through a porous stone connected to a 520S peristaltic pump (Watson Marlow, United Kingdom).

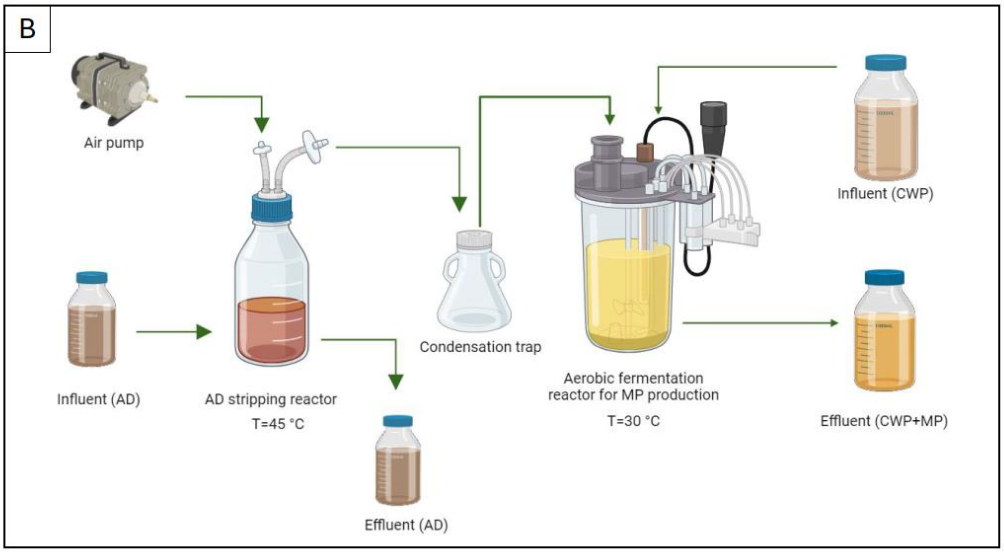
2.4.2 Experimental setup of the aerobic MP production process under different N recovery/feeding strategies

Microbial biomass growth was tested under different N feeding strategies (Figure 1). While the C source was always provided by lactose contained in synthetic CWP, N was provided either through NH_4Cl addition in the synthetic CWP medium fed in the bioreactor (Figure 1 panel A), or by direct air stripping of the digestate connected to the aerobic reactor (Figure 1 panel B), or by directly mixing the digestate with the synthetic CWP free of NH_4Cl (Figure 1 panel C). The first N feeding strategy, involving the addition of chemical N, was used as control condition since it reproduces the typical MP production scenario from CWP (Scotto di Uccio et al., 2023). The latter two conditions relating to waste N recovery and valorisation were also tested using a heavy metal-contaminated digestate as N source.

The continuous MP production setup consisted of a Biostat B Plus (Sartorius, Germany) with a 2 L vessel operated as continuous stirred-tank reactor (CSTR) with a 1-day HRT. The working volume of 1 L was inoculated at 10% of the volume with the activated inoculum. Aerobic conditions (>2 $mgO_2 \cdot L^{-1}$) were guaranteed by sparging air with a flowrate of 600 $mL \cdot min^{-1}$ and by stirring at 600-800 rpm. The process was maintained at 30 °C, with pH controlled at 4.5 ± 0.1 using 1 M NaOH or HCl.

Based on initial results, air stripping was performed in 500 mL Schott bottles filled with 250 mL of liquid digestate at 45 °C with an A:D ratio of 4:1. The air stripping process was performed in continuous mode under the same experimental set up described in Section 2.3 under a 8 h HRT.


A
Air pump
Influent (CWP+NH4Cl)
Aerobic fermentation
reactor for MP production
T=30 °C
Effluent (CWP+MP)


B
Air pump
Influent (CWP)
Influent (AD)
AD stripping reactor
T=45 °C
Condensation trap
Aerobic fermentation
reactor for MP production
T=30 °C
Effluent (CWP+MP)
Effluent (AD)

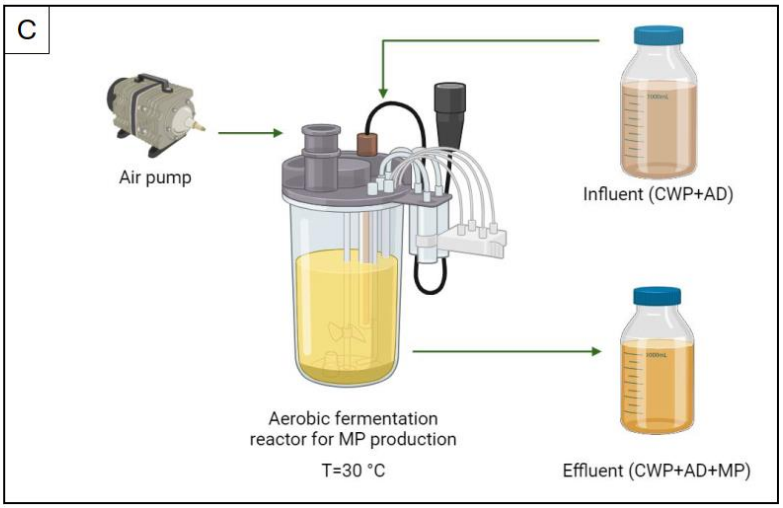

C
Air pump
Influent (CWP+AD)
Aerobic fermentation
reactor for MP production
T=30 °C
Effluent (CWP+AD+MP)

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Figure 1. Overview of the experimental conditions and setup. Panel A: MP production using synthetic CWP medium including NH_4Cl as N source. Panel B: MP production using synthetic CWP medium (free of NH_4Cl) and ammonia from the digestate stripping process as N source. Panel C: MP production using synthetic CWP medium (free of NH_4Cl) mixed with digestate as N source. AD=anaerobic digestate.

2.5 Analytical procedures

Total (TS) and volatile solids (VS) concentrations were analyzed according to the Standard Methods (APHA, 2007). Total suspended solids (TSS) concentrations, analyzed according to the Standard Methods (APHA, 2007), were used as indicator of the biomass growth. When digestate was directly supplied to the aerobic reactor as N-source, the TSS related to the biomass concentration were calculated excluding the TSS contribution from the digestate. The pH was measured by means of a HI98100 pH-meter (Hanna Instruments, Italy). $N\text{-}NH_4^+$ concentration in the digestate was detected spectrophotometrically, through the indophenol blue method (Aminot et al., 1997), on the liquid fraction obtained by centrifuging the digestate at 1000 rpm for 10 min and filtering it through 0.45 μm polypropylene membranes (VWR, Italy). The same concentration in the CWP medium was analysed through a steam distillation unit (Behr Labor-Technik, Germany) according to Standard Methods (APHA, 2007). Soluble (sCOD) and total chemical oxygen demand (tCOD) were determined through the closed reflux colorimetric method (APHA, 2007), while DOC was determined through a TOC-L analyser (Shimadzu, Japan). Protein content in the biomass was evaluated spectrophotometrically by the Folin method (Lowry et al., 1951). At the end of each experiment, the biomass was harvested through centrifugation at 4000 rpm for 10 min, frozen at −20 °C and then dried at 70 °C for 24 h. Then, the extraction of heavy metals was performed on grinded biomass thorough a microwave assisted acid digestion with a mixture 9:1 of HNO_3 and H_2O_2 according to the EPA 3051A (EPA, 2007) method. The extracted heavy metals were analysed through an atomic absorption spectrophotometer (GBC Avanta, Germany).

2.6 Calculations

The N-NH_4^+ removal efficiencies along the tests were calculated according to Equation 1:

$$\text{N-NH}_4^+ \text{ removal efficiency} = \frac{\text{N}-\text{NH}_4^{+\,in} - \text{N}-\text{NH}_4^{+\,\text{i}}}{\text{N}-\text{NH}_4^{+\,in}} \cdot 100\ [\%] \quad (1)$$

where $\text{N}-\text{NH}_4^{+\,\text{in}}$ indicates the initial ammonium nitrogen concentration and $\text{N}-\text{NH}_4^{+\,\text{i}}$ indicates the ammonium nitrogen concentration at a given time or at the end of the test. Since water evaporation occurred during the batch tests, the N-NH_4^+ concentration values were calculated by taking into account the change in volume and were always referred to the initial digestate volume.

The N-NH_4^+ stripping rate (SR), which represents the ammonium nitrogen removed per unit of time per unit of volume ($g \cdot L^{-1} \cdot h^{-1}$), was calculated, for batch tests, based on Equation 2:

$$\text{SR} = \frac{\text{N}-\text{NH}_{4\,i-1}^{+} - \text{N}-\text{NH}_{4\,i}^{+}}{\Delta i} \left[\frac{\text{gN}-\text{NH4}+}{\text{L}\cdot\text{h}}\right] \quad (2)$$

where the subscripts *i* and *i-1* indicate the values of ammonium nitrogen concentration at the chosen sampling time and at the previous one, respectively, and Δi indicates the interval time between the two moments considered.

The difference between N-NH_4^+ at 0 and 24th hour was used to calculate the daily SR value in batch mode.

With reference to continuous test, the SR was calculated based on Equation 3:

$$\text{SR} = \frac{\text{N}-\text{NH}_{4\,i-1}^{+} + \left(\text{N}-\text{NH}_{4\,IN}^{+} \cdot \Delta i\right) - \text{N}-\text{NH}_{4\,i}^{+} - \left(\frac{\text{N}-\text{NH}_{4\,i-1}^{+} + \text{N}-\text{NH}_{4\,i}^{+}}{2} \cdot \Delta i\right)}{\Delta i} \left[\frac{\text{gN}-\text{NH4}+}{\text{L}\cdot\text{h}}\right] \quad (3)$$

Where $\text{N}-\text{NH}_{4\,IN}^{+}$ indicates the N-NH_4^+ concentration in the influent digestate.

The average hourly SR, calculated over the 48-hours test and multiplied by 24 hours, was used to calculate the daily SR in continuous mode.

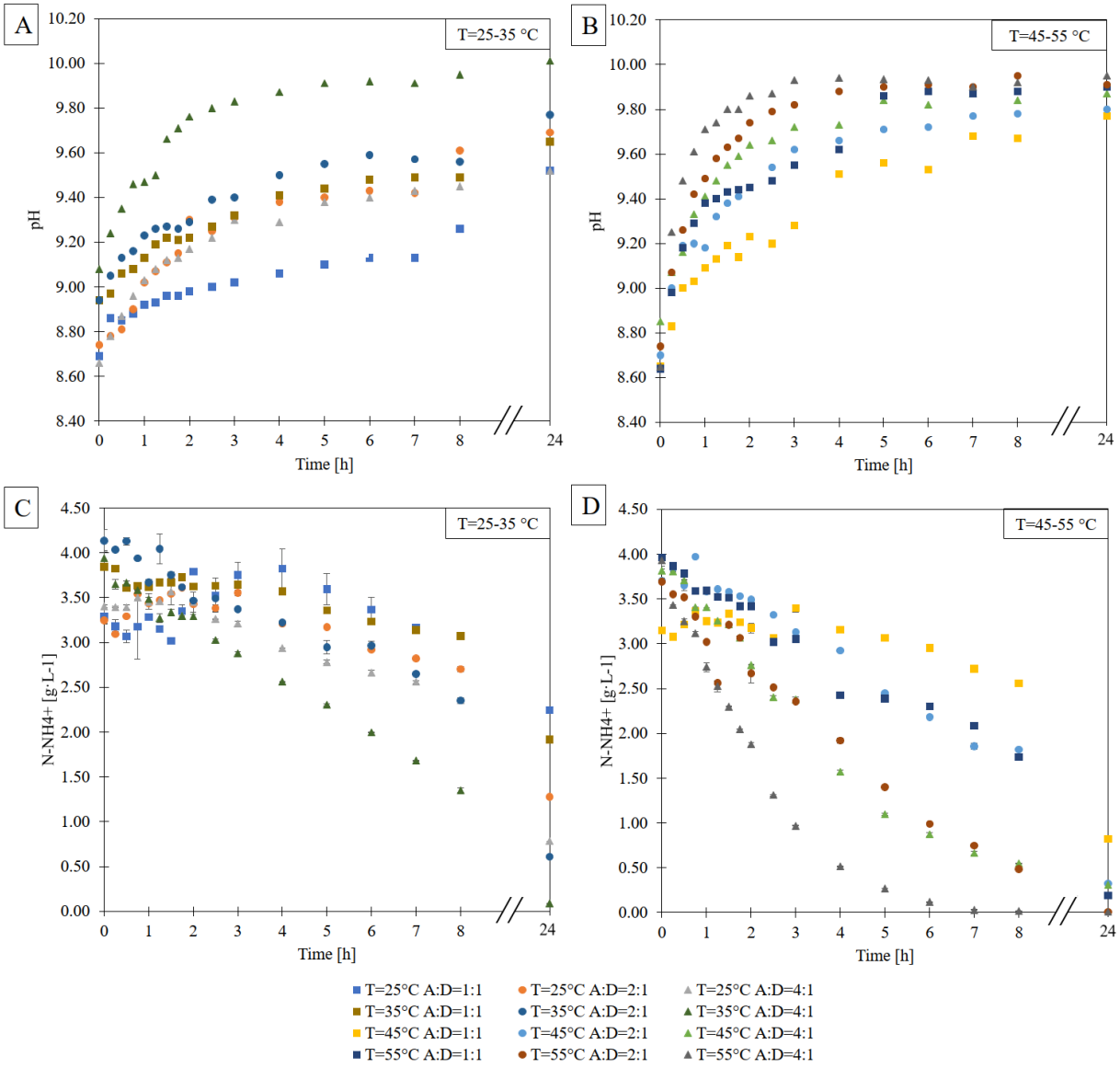
3. Results and discussion

3.1 *Effect of temperature and air to digestate ratio on direct air stripping under batch and continuous conditions*

Figure 2 shows the evolution of pH and N-NH_4^+ concentration along the 24 hours of batch experiments under different temperatures and A:D ratios. As expected, the pH increase was positively correlated with the increase in temperature. As clearly visible in Figure 2, panels A and B, the increase in pH mainly occurred in the first few hours of the stripping process, regardless of the operating condition adopted. However, such an increase in pH did not immediately correspond to a decrease in ammonium nitrogen (Figure 2 panels C and D). Indeed, the temporal evolution of N-NH_4^+ shows an initial lag phase associated with CO_2 volatilization, being the latter more volatile than ammonia due to its lower solubility (1.7 g CO_2 L^{-1} compared with 535 g NH_3 L^{-1} in pure water at 20 °C) (Alitalo et al., 2012; Laureni et al., 2013). CO_2 volatilization is needed to increase the pH up to a value sufficient to favor the dissociation of NH_4^+ into NH_3 and prompt ammonia volatilization.

At A:D ratios of 1:1 and 2:1 and with a temperature of 25 °C, the process showed low ammonia stripping performances since, after 24 hours of treatment, a N-NH_4^+ removal efficiency of only 32 and 61%, respectively, was obtained (Figure 2 panel E). When increasing the temperature to 45 °C, as shown in Figure 2, the A:D ratio increase from 2:1 to 4:1 did not have relevant effects on the N-NH_4^+ removal efficiency, which achieved values of 91 and 92%, respectively. Similar values higher than 90% were obtained by Laureni et al. (2013) by performing an air stripping process on liquid digestate at 50 °C for 225 minutes with an A:D ratio equal to 10:1, thus much higher than the values used in this study. By further increasing the temperature at 55

°C, a complete N stripping (100% efficiency) was obtained with both A:D ratios of 2:1 and 4:1. Along with the increasing temperature, not only the percentage of free ammonia increases, but also the molecular diffusion coefficient of ammonia in both liquid and gas films is enhanced, while the viscosity, the surface tension of the liquid phase and the liquid-gas distribution ratio of ammonia are reduced (Zhao et al., 2015).


A
T=25-35 °C
pH
Time [h]
B
T=45-55 °C
pH
Time [h]
C
T=25-35 °C
N-NH4+ [g·L-1]
Time [h]
D
T=45-55 °C
N-NH4+ [g·L-1]
Time [h]
T=25°C A:D=1:1
T=25°C A:D=2:1
T=25°C A:D=4:1
T=35°C A:D=1:1
T=35°C A:D=2:1
T=35°C A:D=4:1
T=45°C A:D=1:1
T=45°C A:D=2:1
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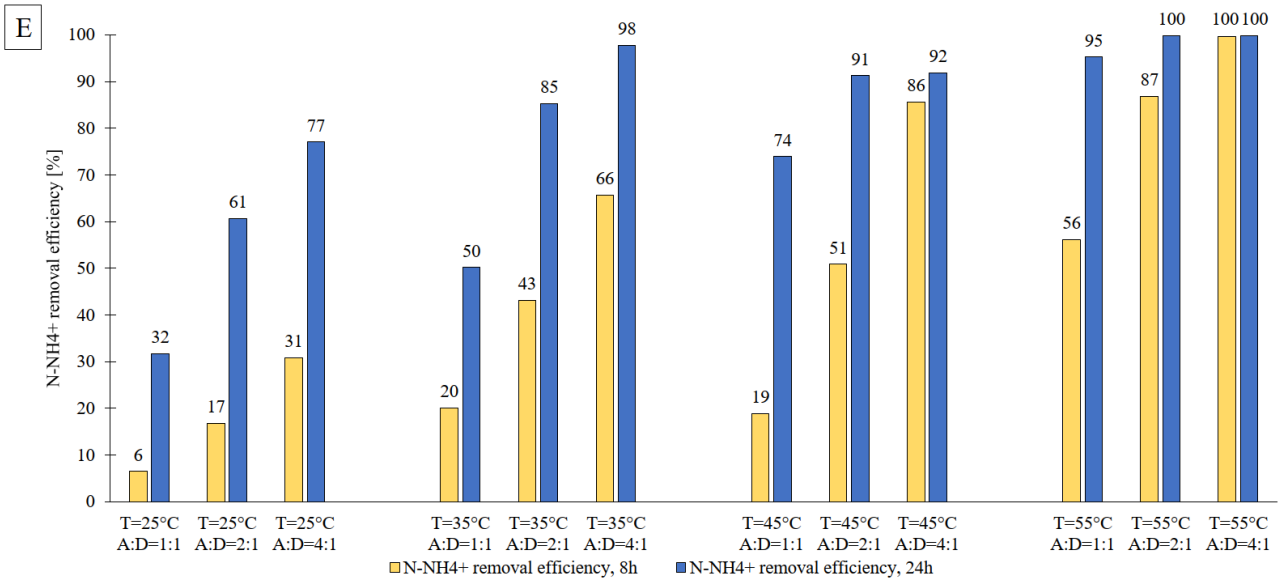
T=55°C A:D=2:1
T=55°C A:D=4:1


E
N-NH4+ removal efficiency [%]
6
32
17
61
31
77
20
50
43
85
66
98
19
74
51
91
86
92
56
95
87
100
100
100
T=25°C A:D=1:1
T=25°C A:D=2:1
T=25°C A:D=4:1
T=35°C A:D=1:1
T=35°C A:D=2:1
T=35°C A:D=4:1
T=45°C A:D=1:1
T=45°C A:D=2:1
T=45°C A:D=4:1
T=55°C A:D=1:1
T=55°C A:D=2:1
T=55°C A:D=4:1
N-NH4+ removal efficiency, 8h
N-NH4+ removal efficiency, 24h

255

Figure 2. Evolution of pH (Panels A, B), residual N-NH_4^+ concentrations in the liquid fraction of digestate (Panels C, D) and N-NH_4^+ removal efficiencies (Panel E) along the 24 h batch mode direct air stripping process of ammonia under different temperature and air-to-digestate (A:D) conditions.

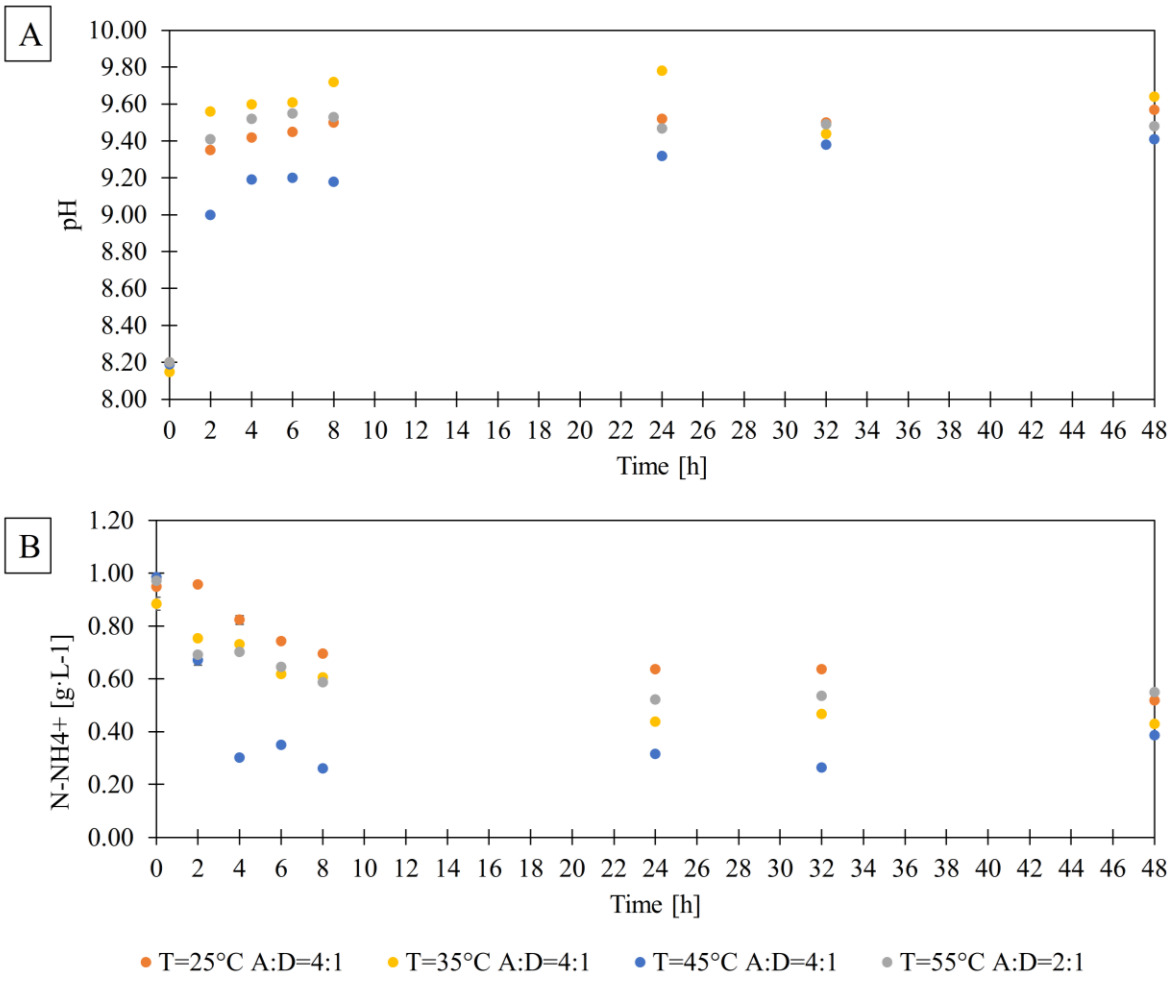
The SR values obtained during the ammonia stripping process in batch (reported in supplementary information in Table S4) were strongly dependent on both the temperature and air-to-digestate (A:D) ratio. The results are in good agreement with those referred to ammonia removal from the digestate at higher A:D ratios (2:1 or 4:1), with increased temperatures enhancing ammonia stripping and achieving the maximum SR values earlier. At 35 °C with A:D ratios of 2:1 and 4:1, or at 55 °C with similar ratios, SR values of up to 1.19 g N-$NH_4^+ \cdot L^{-1} \cdot h^{-1}$ were reached within the first hour. In general, the values reported in Table S4 highlight that, given a specific temperature, the maximum stripping rate is reached earlier or achieves higher values when a higher A:D ratio is applied. Daily SR values peaked at nearly 4 g N-$NH_4^+ \cdot L^{-1} \cdot d^{-1}$ at 55°C with an A:D ratio of 4:1 being only slightly lower (3.85 g N-$NH_4^+ \cdot L^{-1} \cdot d^{-1}$) under milder process conditions, such as 35°C and A:D of 4:1.

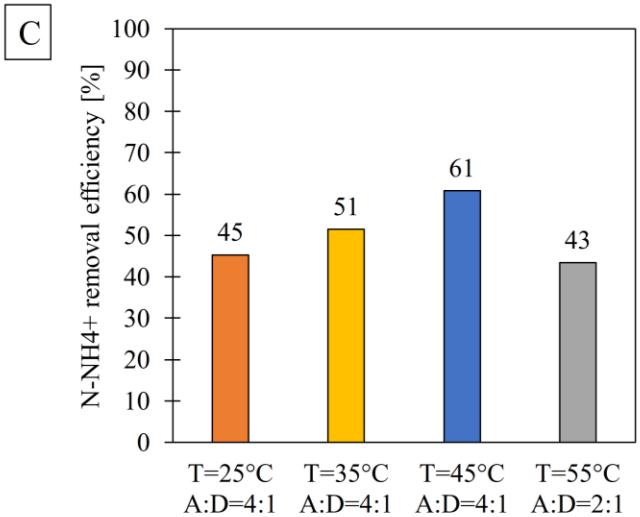
Figure 3 shows the evolution of pH and N-NH_4^+ concentration along the 48 hours of direct ammonia stripping in continuous experiments at different temperatures and A:D ratios. The results highlight how the most significant increase in pH (panel A) and the lowest residual N-NH_4^+ concentration (panel B) were achieved already within the first 8 hours of operation. Subsequently, stationary conditions appeared to be established in terms of both pH value and residual N-NH_4^+ concentration. Such stationary conditions were reached earlier, after 4 hours of operation, when both a temperature of 45 °C and an A:D ratio of 4:1 were applied.

At the same A:D ratio of 4:1, the N-NH_4^+ removal efficiency increased along with temperature, reaching values of 45, 51 and 61% when moving from 25 to 35 and 45 °C, respectively (Figure

3 panel C). Increasing the temperature by an additional 10 °C, thereby reaching 55 °C, while halving the A:D ratio from 4:1 to 2:1, did not improve the stripping process. As shown in panel B of Figure 3, after eight hours of stripping process at 45 °C and with an A:D ratio of 4:1, the residual N-NH_4^+ concentration was about 0.26 g·L^{-1} while, at 55 °C and with an A:D ratio of 2:1, the latter was about 0.55 g·L^{-1}, i.e. approximately twice as much. In terms of stripping efficiency, the process operated with a temperature of 55 °C and an A:D ratio equal to 2:1 performed worse than the process carried out at a temperature of 25 °C and an A:D ratio equal to 4:1, reaching a value of only 43% N-NH_4^+ removal efficiency. The latter results show that, also under continuous stripping conditions, the effect caused by the increased driving force of the stripping flow is predominant over the effect of temperature. This agrees with Laureni et al. (2013), who obtained ammonia removal efficiencies higher than 90% by performing an air stripping process at 50 °C for 225 minutes with an A:D ratio equal to 10:1.

By comparing the results reported in panel E of Figure 2 with those presented in panel C of Figure 3, it is clearly visible that the values of ammonia removal efficiency, at the same temperature and applied A:D ratio, are higher in the batch operational mode. In particular, operating in batch, an ammonia removal efficiency of up to 87% (with a temperature of 55 °C and an A:D ratio of 2:1) was achieved, while operating continuously under the same conditions the highest ammonia removal efficiency was 43%. Such a difference can be explained by the higher initial N-NH_4^+ concentration in the digestate used in batch tests compared to the digestate used in the continuous tests, which affects the rate of diffusion of dissolved liquid ammonia into stripped ammonia gas.


A
pH
Time [h]
B
N-NH4+ [g·L-1]
Time [h]
T=25°C A:D=4:1
T=35°C A:D=4:1
T=45°C A:D=4:1
T=55°C A:D=2:1


C
N-NH4+ removal efficiency [%]
45
51
61
43
T=25°C A:D=4:1
T=35°C A:D=4:1
T=45°C A:D=4:1
T=55°C A:D=2:1

302

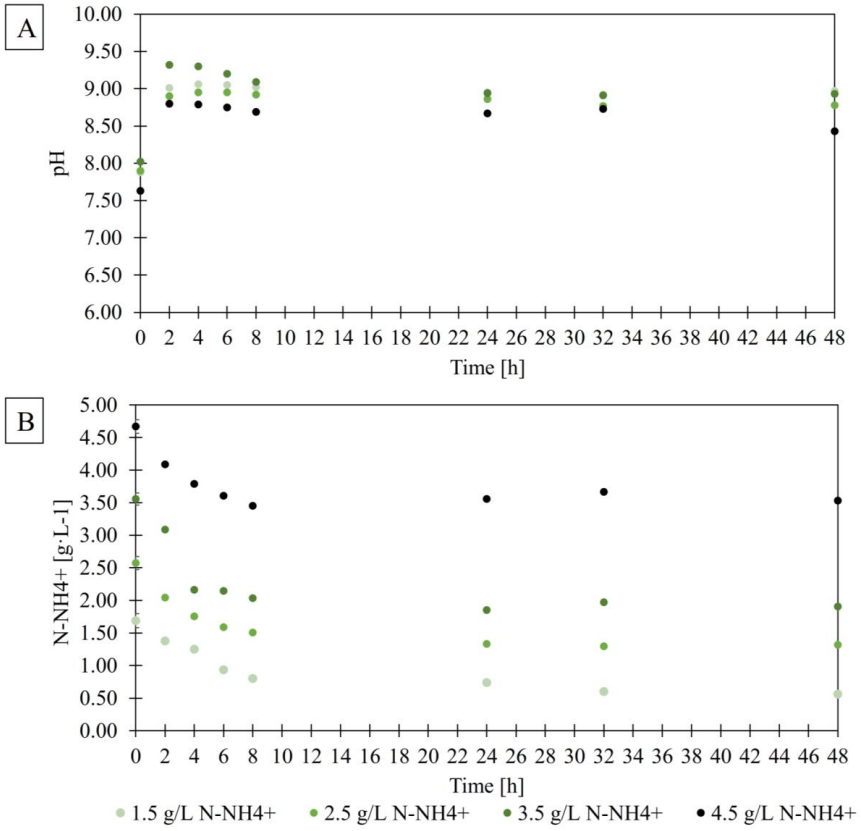
Figure 3. Evolution of pH (Panel A), residual N-NH_4^+ concentrations in the liquid fraction of digestate (Panel B) and N-NH_4^+ removal efficiencies (Panel C) along the 48-h continuous direct air stripping process of ammonia under different temperature and air-to-digestate conditions.

The SR values obtained during the continuous stripping process under the tested experimental conditions (see supplementary information Table S5) increased with the temperature at constant A:D ratio. At 25 °C and 35 °C with an A:D of 4:1, maximum hourly SR were 0.07 and 0.08 g N-$NH_4^+ \cdot L^{-1} \cdot h^{-1}$, respectively. At 45 °C, the SR reached 0.17 g N-$NH_4^+ \cdot L^{-1} \cdot h^{-1}$ within 2 hours and peaked at 0.24 g N-$NH_4^+ \cdot L^{-1} \cdot h^{-1}$ after 4 hours. At 55 °C and an A:D of 2:1, the SR peaked at 0.15 g N-$NH_4^+ \cdot L^{-1} \cdot h^{-1}$ before stabilizing at lower values. Thus, given a specific A:D ratio, the maximum stripping rate is reached earlier or achieves higher values when a higher temperature is applied. The daily SR varied with temperature and A:D ratio. At 25 °C and 35 °C with an A:D of 4:1, SR were 1.12 and 1.36 g N-$NH_4^+ \cdot L^{-1} \cdot d^{-1}$, respectively. Increasing the temperature to 45 °C at the same A:D ratio significantly raised SR to 2.69 g N-$NH_4^+ \cdot L^{-1} \cdot d^{-1}$. However, raising the temperature to 55°C while reducing the A:D ratio to 2:1 led to a decrease in SR to 1.44 g N-$NH_4^+ \cdot L^{-1} \cdot d^{-1}$. Also for the SR, lower values were observed for the stripping process operated in continuous vs. batch mode. The limitation was likely due to the lower initial concentration of dissolved ammonium nitrogen present in the continuous tests, which limited the driving force of the overall stripping process.

3.2 Effect of initial nitrogen content on continuous direct air stripping of ammonia

As previously discussed, under continuous conditions, the highest N-NH_4^+ removal efficiency was obtained at a temperature of 45 °C and an A:D ratio of 4:1. Therefore, additional continuous stripping tests were performed under these operating conditions using digestate amended with

NH_4Cl to evaluate the effects of different initial ammonium nitrogen contents of 1.5, 2.5, 3.5 and 4.5 $g \cdot L^{-1}$.



C

Time of operation [h]	Initial $N\text{-}NH_4^+$ concentration $[g \cdot L^{-1}]$			
	1.5	2.5	3.5	4.5
2	18	21	13	12
4	26	32	39	19
6	45	38	40	23
8	53	41	43	26
24	56	48	48	24
32	65	50	45	21
48	67	49	46	24

Figure 4. Evolution of pH (Panel A), residual $N\text{-}NH_4^+$ concentrations in the liquid fraction of digestate (Panel B) and $N\text{-}NH_4^+$ removal efficiency [%] (Panel C) along the 48-h continuous direct air stripping process of ammonia from digestate characterized by initial $N\text{-}NH_4^+$ concentrations of 1.5, 2.5, 3.5 and 4.5 $g \cdot L^{-1}$. In panel C, different

colors have been used to indicate how values appear over time: variable (red), almost stable (orange) and stable (green) values.

As already observed with the lower N concentrations, pH and residual ammonium nitrogen concentration values tend to stabilize after four to eight hours of operation (Figure 4 panels A, B). Figure 4 panel C shows the average efficiency of continuous stripping, calculated with reference to the initial $N\text{-}NH_4^+$ concentration to evaluate its effect on the process. The latter results show that, under each tested condition, the $N\text{-}NH_4^+$ removal efficiencies tend to stabilize and be constant over time. Such a stabilization in terms of $N\text{-}NH_4^+$ removal efficiency occurs faster at higher initial $N\text{-}NH_4^+$ loading. Nevertheless, the value of the final stripping efficiency decreases from 67 to 49, 46 and 24% as the initial ammonium nitrogen concentration increases from 1.5 to 2.5, 3.5 and 4.5 $g \cdot L^{-1}$, respectively. However, despite such differences in stripping efficiency, the total mass of nitrogen stripped is approximately the same under all tested conditions. In particular, a very similar decrease of ammonium-nitrogen concentration of 1.12 and 1.14 $g \cdot L^{-1}$ was obtained at an initial nitrogen concentration of both 1.5 and 4.5 $g \cdot L^{-1}$. Therefore, the initial concentration of ammonium nitrogen in the digestate does not seem to affect the total amount of N removed under continuous process conditions.

The SR values obtained during the continuous stripping process of ammonia from the digestate characterized by initial $N\text{-}NH_4^+$ concentrations of 1.5, 2.5, 3.5 and 4.5 $g \cdot L^{-1}$ under the different experimental conditions (see supplementary information Table S6) stabilized between 0.14 and 0.21 g $N\text{-}NH_4^+ \cdot L^{-1} \cdot h^{-1}$ regardless of the initial $N\text{-}NH_4^+$ concentration. In all cases, the stabilization of the SR value occurred after the first 8 hours, which corresponds to one HRT. Under continuous conditions, daily SR values increased with the initial $N\text{-}NH_4^+$ concentration of the digestate. At 45°C and an A:D ratio of 4:1, SR values were 3.74, 4.68, 4.96, and 4.80 g $N\text{-}NH_4^+ \cdot L^{-1} \cdot d^{-1}$ for initial concentrations of 1.5, 2.5, 3.5, and 4.5 $g \cdot L^{-1}$, respectively, while the

one obtained with the digestate characterized by an initial N-NH_4^+ concentration of 0.89 g·L^{-1} was 2.69 g N-NH_4^+·L^{-1}·d^{-1}. These results confirm that higher initial concentrations of dissolved ammonium nitrogen in the digestate increase the driving force of the stripping process.

3.3 From waste-N to protein-N: evaluating the potential of direct air nitrogen stripping for microbial protein production from digestate and C-rich side streams

As previously discussed in Section 3.1, the highest N-NH_4^+ removal efficiency in continuous mode was obtained at a temperature of 45 °C and an A:D ratio of 4:1. The latter allows to recover up to 4.96 g N-NH_4^+·L^{-1}·d^{-1} from the digestate, which would be sufficient to support a microbial growth during aerobic fermentation of 50 g TSS L^{-1}·d^{-1} (Scotto di Uccio et al., 2023). Based on the obtained results, the direct air stripping of ammonia from digestate was combined with an aerobic fermentation process aimed at MP production. The final MP product obtained was compared with those obtained by operating aerobic fermentation with either an external mineral nitrogen source or through direct digestate supply, with and without heavy metals addition. The latter comparison was aimed at evaluating how N recovery from digestate through stripping process can minimize the transfer and accumulation of heavy metals in the final MP product, thereby enhancing its quality.

3.3.1 Effect of different N sources on the aerobic MP production process and protein content

Figure 5 shows the daily biomass concentrations measured during the tests carried out with the different N sources. In the control, when N was provided by NH_4Cl, the biomass concentration rapidly increased during the first day of operation until stabilising at a value close to 10 g TSS·L^{-1}. The latter was similar to the value of 10.55 g TSS·L^{-1} obtained from the cultivation of the same inoculum under the conditions previously investigated by the authors (Scotto di Uccio et al., 2023).

When the N source was the N stripped from digestate and then solubilised in the aerobic reactor, a lower biomass concentration was observed and a TSS concentration of 4.78 g TSS·L^{-1} (Figure 5) was achieved. The highest biomass concentration here obtained from the combined stripping-aerobic process was similar to the average concentration equal to 4.05 g TSS·L^{-1} obtained by Matassa et al. (2022), who used a similar experimental configuration but performed the stripping process at 30 °C and under batch conditions. Furthermore, when N was provided through the direct mixing of digestate in the aerobic reactor, the biomass concentration rapidly increased until reaching a value of approximately 12 g TSS·L^{-1} (Figure 5).

The latter observation suggests that N provided through NH_4Cl and digestate favoured the biomass growth. Indeed, NH_4Cl provided a readily available and efficiently metabolizable form of N, while the digestate may have provided not only N but also a wide range of other nutrients to the microorganisms. The biomass concentrations obtained in the present study when using digestate as N-source directly supplied in the reactor are higher than those obtained in similar studies. Bertasini et al. (2022) reached concentrations up to 1.95 g TSS·L^{-1} by performing aerobic MP production process of *Saccharomyces cerevisiae* using a candies production effluent as C source (70 g sCOD·L^{-1}) and the liquid fraction of agricultural digestate as N source (0.44 g Total Kjeldal Nitrogen·L^{-1}). Qin et al. (2019) obtained biomass concentrations up to 1.15 g TSS·L^{-1} while treating the liquid fraction of digestate from the yeast production industry with a pure culture of the *Yarrowia Lipolytica* yeast. The biomass concentration increased up 1.53 g TSS·L^{-1} by co-culturing *Yarrowia Lipolytica* with the *Chlorella Vulgaris* microalga. Conversely, when N was in this study provided from the stripping and solubilization processes, the low availability of N in the initial exponential growth phase (the phase in which the highest N consumption occurs, see panel 6 of Figure 6) likely led to a limitation of the biomass growth.

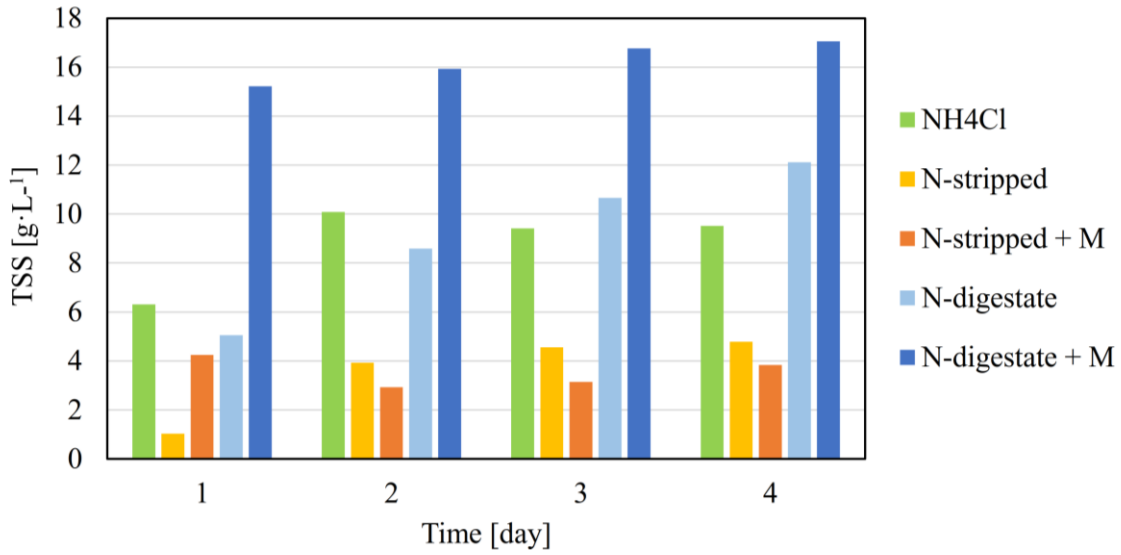


Figure 5. Performance of the aerobic microbial protein production process in terms of daily biomass concentration under the different experimental conditions. NH_4Cl: N supplied as a chemical, N-stripped: N supplied through the stripping process, N-stripped + M: N supplied through the stripping process using digestate contaminated with heavy metals, N-digestate: N supplied through digestate in the aerobic reactor, N-digestate + M: N supplied through digestate contaminated with heavy metals directly fed in the aerobic reactor.

The daily DOC and N-NH_4^+ concentrations detected in the reactor under the different experimental conditions are reported in Figure 6. Except in the case where N was provided through the stripping process from the digestate contaminated with heavy metals, the DOC consumption mainly occurred during the first day of operation, while its concentration remained relatively constant afterwards. The difference in N-NH_4^+ concentration visible in Figure 6 (panel B) at the time zero is related to the different N-supply strategies, as the test using stripped N was characterized by an initial negligible nitrogen concentration. In all cases, in the first day of reactor operation, almost all available N-NH_4^+ was consumed, while in the subsequent days either a decrease in consumption or even an accumulation of N-NH_4^+ was observed. These observations are in line with the biomass growth trends previously discussed (Figure 5).

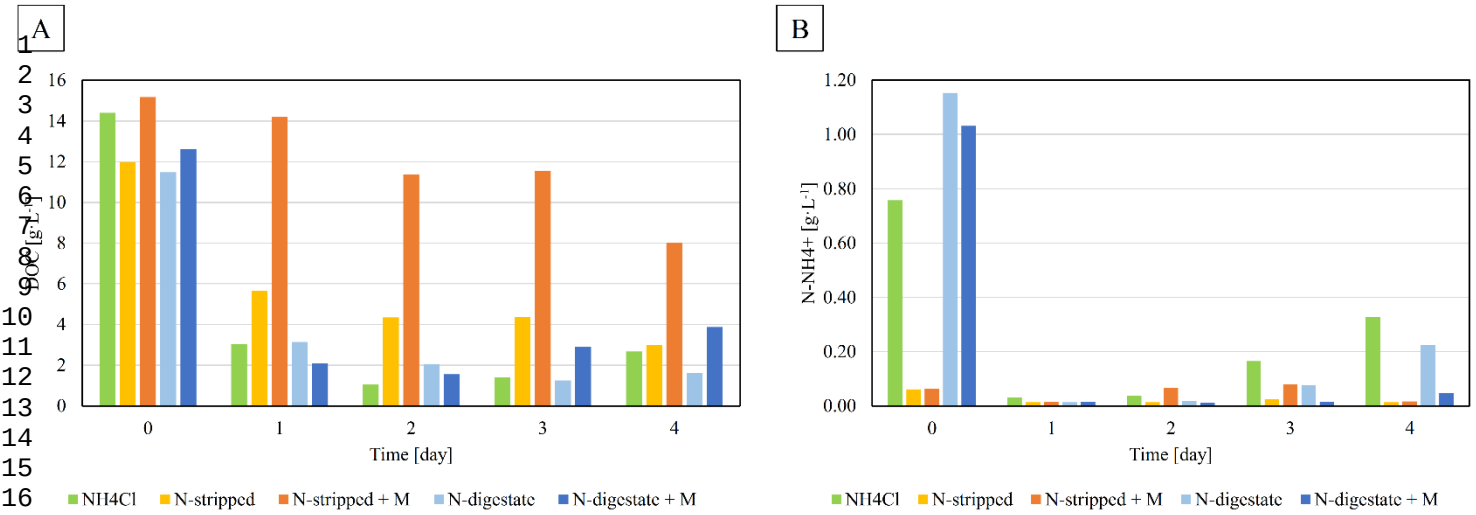


Figure 6. Performance of the aerobic microbial protein production process in terms of daily DOC and N-NH_4^+ concentrations under the different experimental conditions. NH_4Cl: N supplied as a chemical, N-stripped: N supplied through the stripping process, N-stripped + M: N supplied through the stripping process using digestate contaminated with heavy metals, N-digestate: N supplied through digestate in the aerobic reactor, N-digestate + M: N supplied through digestate contaminated with heavy metals directly fed in the aerobic reactor.

Figure 7 shows the protein content of the biomass produced under different N sources. In the control, when N was provided by NH_4Cl, a high protein content of 67% was detected. As NH_4Cl provides an easily assimilable form of nitrogen, it likely allowed an efficient protein synthesis leading to a biomass characterized by a protein content similar to those reported in other studies on MP production through aerobic production process from CWP (Matassa et al., 2022; Scotto di Uccio et al., 2023). When the N was stripped from digestate and then solubilised in the aerobic reactor, the protein content decreased to 17%, suggesting that the stripping process led to the microbial growth under N-limiting conditions. The issue primarily revolves around the low initial availability of N for microorganisms when provided with stripped N. This limitation, which significantly restricted both the final biomass concentration and the protein content of the resulting MP, might be overcome by inoculating the reactor only once a sufficient initial N concentration is established, or by directly adding mineral N in the growth substrate to support

the process in the initial phase. The latter was already demonstrated by Matassa et al. (2022), who produced biomass with a protein content as high as 75% by performing a process similar to that adopted in the present study combining direct nitrogen stripping and aerobic fermentation of cheese whey amended with NH_4Cl. Directly feeding the digestate into the reactor led to a biomass characterized by a 44% protein content. In this case, the raw digestate likely provided a more complete nutrient supply, including also organic nitrogen under the form of amino acids and peptides, better supporting the microbial growth and protein production compared to stripped N.

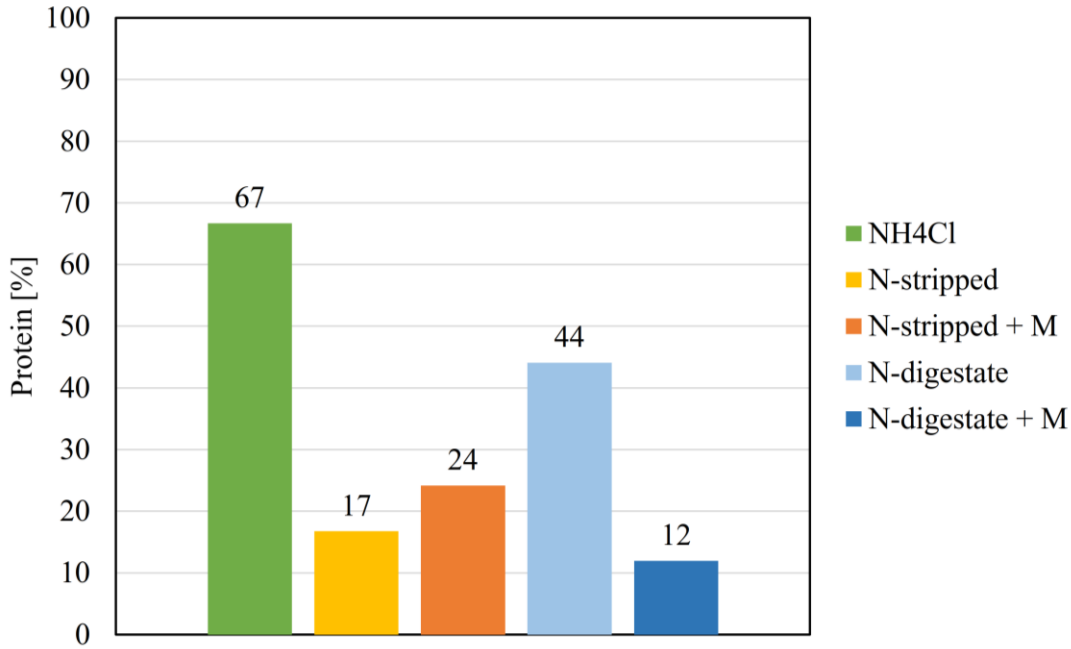


Figure 7. Protein content of the biomass produced under the different experimental conditions. NH_4Cl: N supplied as a chemical, N-stripped: N supplied through the stripping process, N-stripped + M: N supplied through the stripping process using digestate contaminated with heavy metals, N-digestate: N supplied through digestate in the aerobic reactor, N-digestate + M: N supplied through digestate contaminated with heavy metals directly fed in the aerobic reactor.

3.3.2 Effect of heavy metals in the digestate on the aerobic MP production process

As clearly visible in Figure 5, the presence of heavy metals contamination in the digestate used in the present study seems not to have affected the biomass growth when N was supplied through the stripping process. The TSS value varied from a minimum of 2.92 to a maximum of 4.24 g $TSS \cdot L^{-1}$, thus similar to those reported in the absence of added metals in the digestate. Conversely, the presence of heavy metals in the digestate, used as direct N source in the aerobic MP production reactor, resulted in an increase of the biomass concentration up to a value of 17.06 g $TSS \cdot L^{-1}$. The increase in biomass growth in kefir-based fermentation process when the digestate is contaminated with heavy metals may be linked to specific microbial responses (Mrvčić et al., 2013). Kefir microorganisms, including those belonging to the *Lactobacillus* and *Saccharomyces* genera, showed mechanisms helping them to resist and tolerate metal stress, such as producing metal-chelating substances or altering their metabolism to cope with oxidative stress induced by metals.

However, when the digestate was contaminated with heavy metals, the protein content decreased from 44 (in the absence of heavy metals) to 12%. The latter suggests that, while heavy metals can induce some beneficial stress responses for biomass growth, as already discussed in Section 3.3.1, an excessive presence of metals might inhibit protein synthesis, leading to a lower overall protein content. In particular, it has been shown that the high presence of metals such as Cu assimilated by biomass (as occurred in that obtained in this condition, see Section 3.3.3) leads to stress response that promotes the degradation of proteins and the repair of DNA to mitigate the toxic effects (Mrvčić et al., 2013).

3.3.3 Effect of heavy metals in the digestate on the quality of microbial protein

Table 1 reports the heavy metals content measured in the biomass produced by using as N source the digestate contaminated with heavy metals, and the respective regulatory limits

established at the European level. When the heavy metals-contaminated digestate was directly supplemented to the aerobic reactor, the produced biomass presented a higher heavy metal concentration than the biomass produced by feeding N through the stripping process.

The extent of the increase in heavy metal content in biomass varied significantly depending on the metal. While for Cd no accumulation was detected under both N-feeding strategies (i.e., N-stripping and N-digestate supply), for Ni, Pb and Zn a low to moderate metal accumulation was measured, achieving a 1.4, 2.0 and 4.6 higher concentration, respectively, in the biomass produced through N-digestate supply as compared to that produced through N-stripping. A very high increase of almost 66-fold was finally observed for Cu, the concentration of which varied from 5 to 329 $mg_{METAL} \cdot kg_{DRY\ BIOMASS}^{-1}$ in the biomass produced through N-stripping and N-digestate feeding, respectively). These results suggest that when metal-contaminated digestate is used as N source directly in the aerobic process, heavy metals are readily available and assimilable by biomass. On the contrary, removing and recovering the nitrogen through stripping allows to physically disconnect the contaminated waste matrix from the aerobic reactor, thereby avoiding the transfer and accumulation of contaminants such as heavy metals in the final MP product.

The MP produced in the bioreactor coupled with the stripping process from contaminated digestate meets all the standards to be valorised as organic fertiliser, while the MP produced using the N from the contaminated digestate slightly exceeded the standards for fertilisers use only for Cu (329 against 300 $mg_{METAL} \cdot kg_{DRY\ BIOMASS}^{-1}$ allowed). Furthermore, the biomass produced through both N-stripping and N-digestate addition exceeded the Pb limit of 10 $mg_{METAL} \cdot kg_{DRY\ BIOMASS}^{-1}$ in the case of use as feed, achieving values of 36 and 71 $mg_{METAL} \cdot kg_{DRY\ BIOMASS}^{-1}$, respectively. In the same way, being the latter Pb contents also higher than the limit of 3 $mg_{METAL} \cdot kg_{DRY\ BIOMASS}^{-1}$ allowed for food products, the biomass would not be suitable for human nutrition. Ni concentration values were equal to 11 and 15 $mg_{METAL} \cdot kg_{DRY}$

$_{BIOMASS}^{-1}$ in the present study under both experimental configurations, being comparable with the value of 8 $mg_{METAL} \cdot kg_{DRY\ BIOMASS}^{-1}$ obtained by Van Peteghem et al. (2023), who cultivated a mixed culture of *Metschnikowia pulcherrima* and *Corynebacterium glutamicum* using recovered N from pig manure and municipal organic waste.

Table 1. Heavy metals content in the biomass produced via aerobic process by providing N by means of contaminated digestate through stripping or by direct feeding. Maximum heavy metals content allowed for different purposes according to the European regulatory limits.

Metal	Metal content in MP from N-stripped	Metal content in MP from N-digestate	Maximum permitted content in food[a]	Maximum permitted content in organic fertilisers[b]	Maximum permitted content in feed[c]
	$mg_{METAL} \cdot kg_{DRY\ BIOMASS}^{-1}$	$mg_{METAL} \cdot kg_{DRY\ BIOMASS}^{-1}$	$mg_{METAL} \cdot kg_{DRY\ BIOMASS}^{-1}$	$mg_{METAL} \cdot kg_{DRY\ BIOMASS}^{-1}$	$mg_{METAL} \cdot kg_{DRY\ BIOMASS}^{-1}$
Ni	11	15	-	50	-
Pb	36	71	3	120	10
Cu	5	329	-	300	-
Zn	28	128	-	800	-
Cd	0	0	3	1.5	2

[a] (European Parliament and the Council of the EU, 2023)

[b] (European Parliament and the Council of the EU, 2019)

[c] (European Parliament and the Council of the EU, 2006)

4. Conclusions

The direct air stripping process of ammonia nitrogen from digestate showed promising performances at temperatures equal to or lower than 55 °C and without chemical pH adjustment, reaching efficiencies of up to 100% under batch conditions (55 °C, A:D=4:1) and up to 61% under continuous conditions (45 °C, A:D=4:1). The initial $N\text{-}NH_4^+$ concentration did not affect the amount of nitrogen recovered, as the mass of nitrogen stripped remained relatively constant, although the final stripping efficiency decreased from 67 to 24% along with the increasing initial nitrogen loading. The different N supply modes influenced the MP production process. When $N\text{-}NH_4^+$ was already present in the culture medium, either provided

by NH_4Cl or digestate addition, biomass concentrations up to 10.08 and 17.06 g $TSS \cdot L^{-1}$ were reached. Conversely, when N was provided by stripping and solubilising ammonia from digestate, a lower biomass concentration of 4.78 g $TSS \cdot L^{-1}$ was observed, likely due to the low availability of N in the early exponential growth phase. The presence of heavy metals in the digestate used as N source via stripping did not affect the biomass growth due to the gas phase transition of ammonia that avoided their transfer and accumulation in MP, while it led to a metal increase in biomass when the digestate was fed directly into the aerobic reactor. The latter also affected the quality of the final MP product. As a matter of fact, the biomass obtained from the aerobic MP production process fed with heavy metals-contaminated digestate presented a lower protein content and higher contents of each heavy metal tested with respect to the same biomass grown by using stripped nitrogen, making it unsuitable for human food while not completely suitable for animal feed and fertilizers production.

CRediT author contribution statement

Antonella Scotto di Uccio: Conceptualization, Data curation, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Silvio Matassa:** Project administration, Conceptualization, Supervision, Writing – review & editing. **Alessandra Cesaro:** Conceptualization, Supervision, Writing – review & editing. **Francesco Pirozzi:** Supervision, Writing – review & editing. **Giovanni Esposito:** Supervision, Writing – review & editing. **Stefano Papirio:** Conceptualization, Supervision, Writing – review & editing.

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Supplementary material

Supplementary data of this work can be found in online version of the paper.

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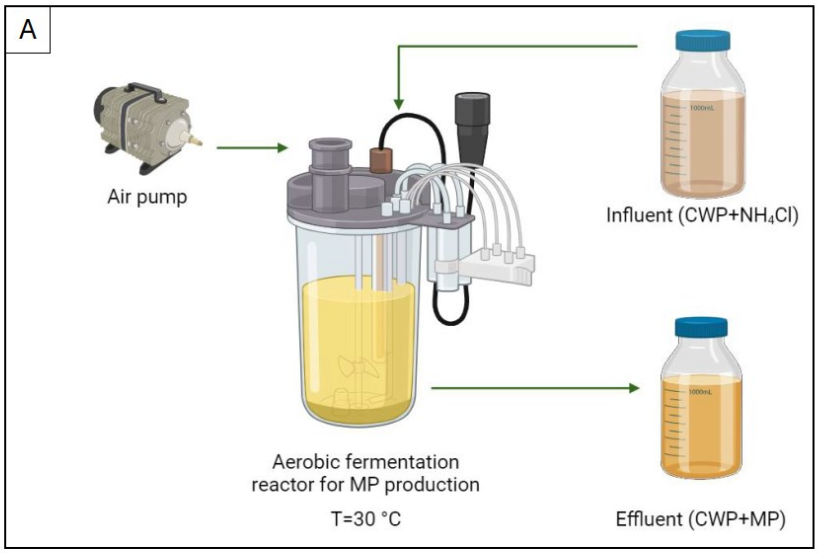
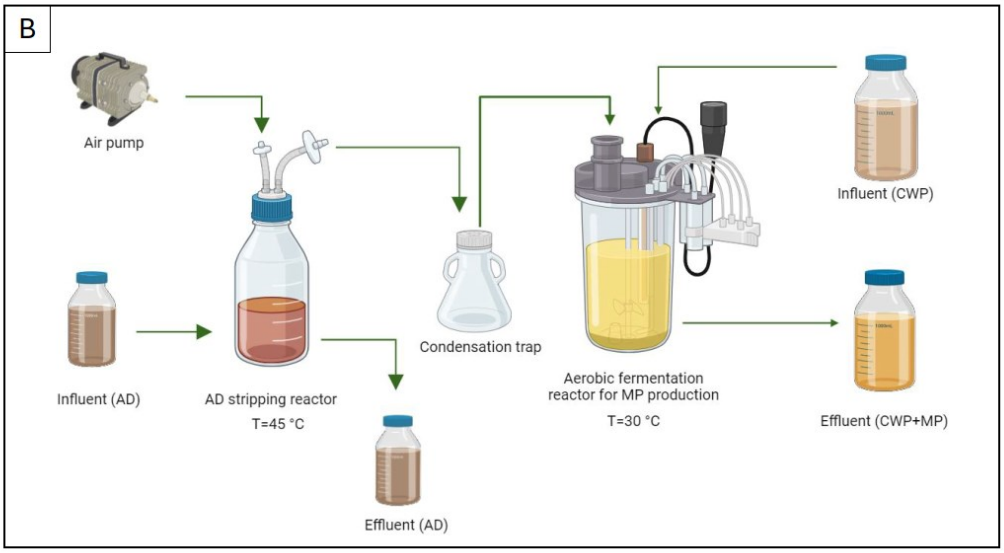
Figure 1





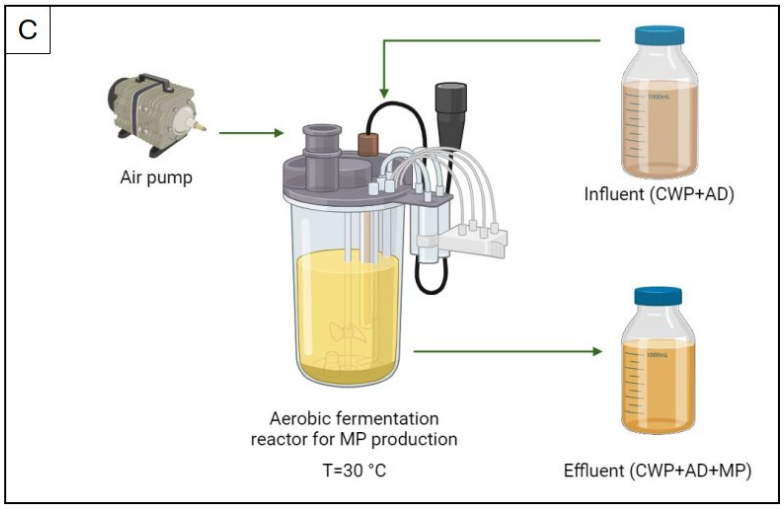


Figure 1. Overview of the experimental conditions and setup used in the present study. Panel A: MP production using synthetic CWP medium including NH_4Cl as N source. Panel B: MP production using synthetic CWP medium (free of NH_4Cl) and ammonia from the digestate stripping process as N source. Panel C: MP production using synthetic CWP medium (free of NH_4Cl) mixed with digestate as N source. AD=anaerobic digestate.

Figure 2

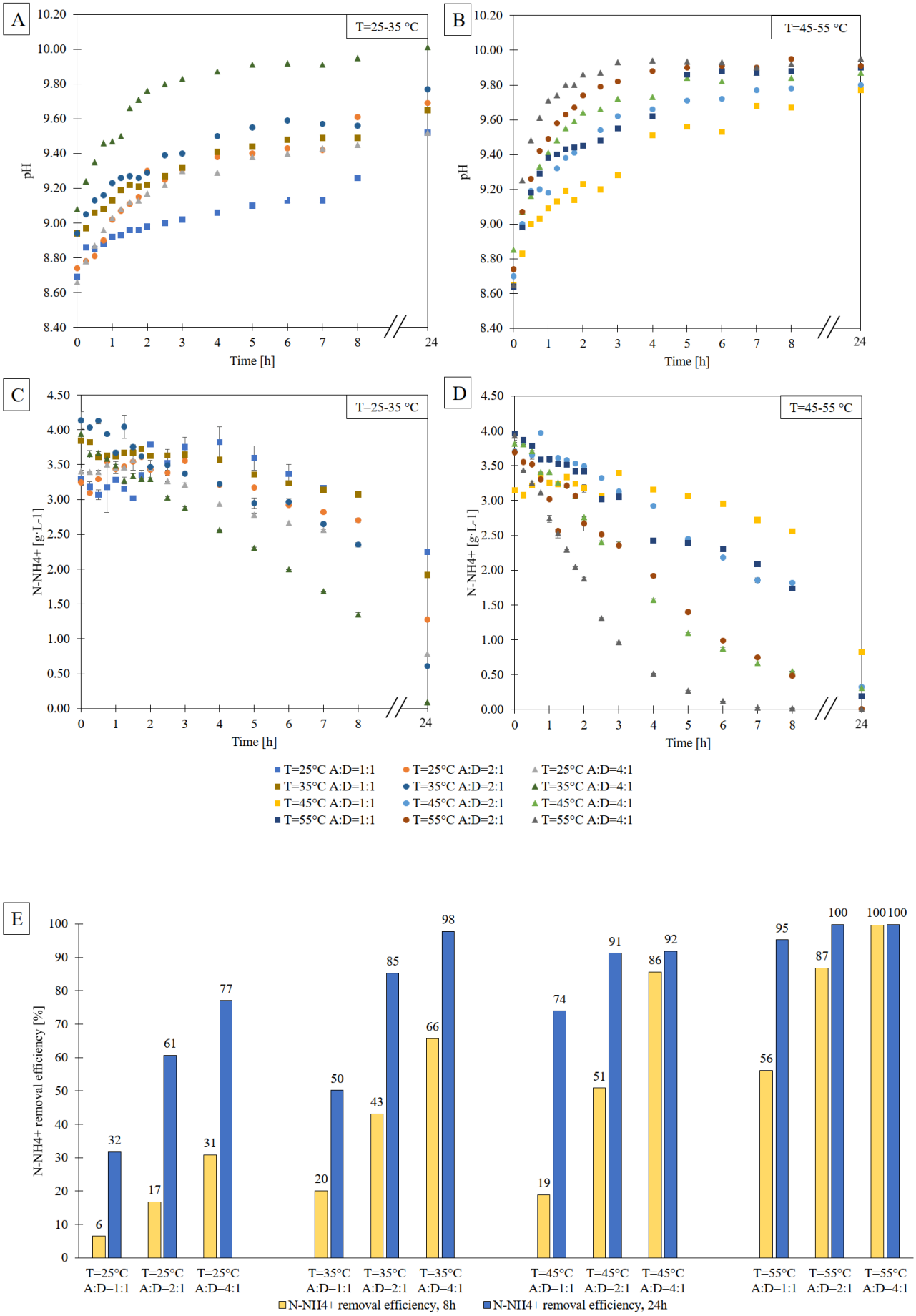
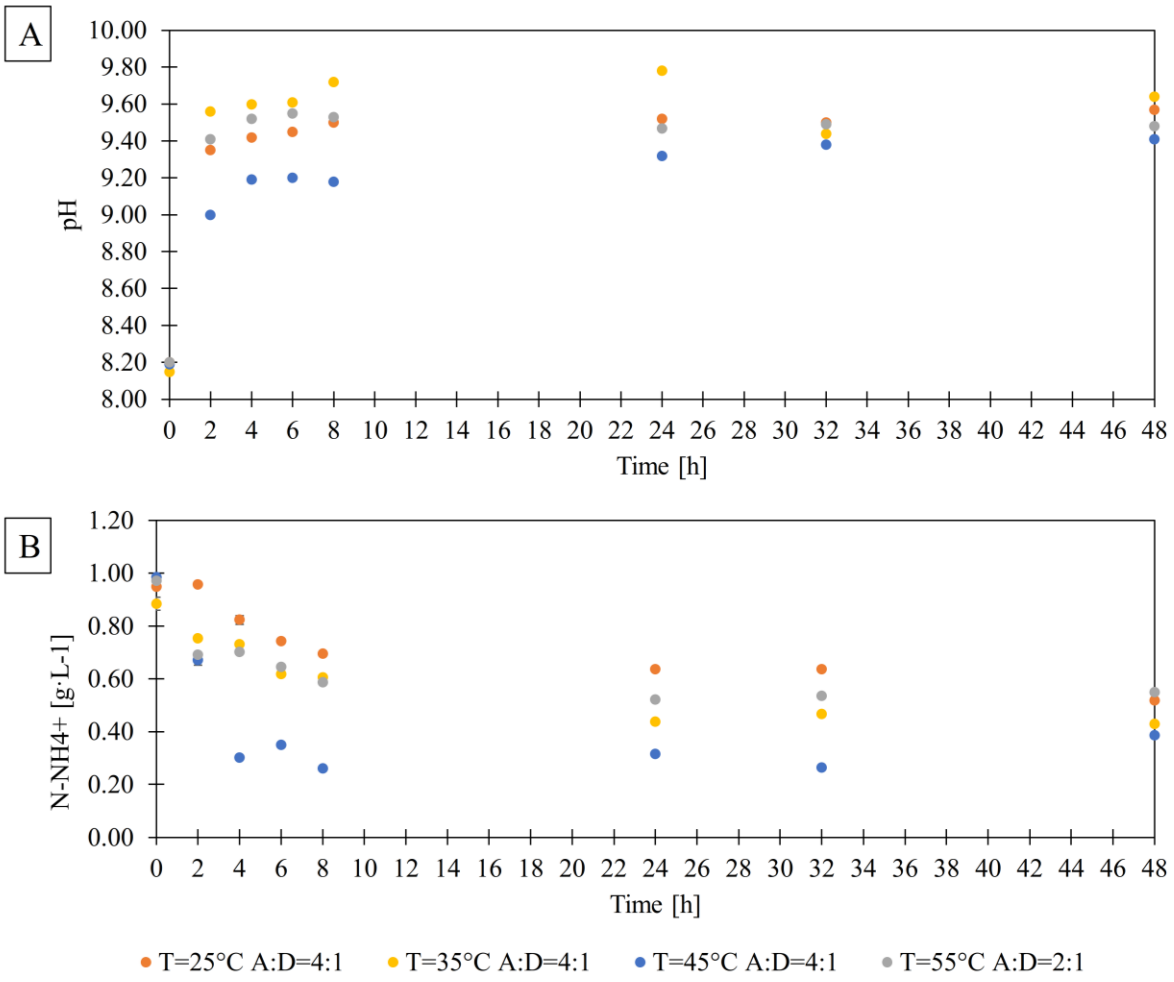


Figure 2. Evolution of pH (Panels A, B), residual N-NH_4^+ concentrations in the liquid fraction of digestate (Panels C, D) and N-NH_4^+ removal efficiencies (Panel E) along the 24 h batch mode direct air stripping process of ammonia under different temperature and air-to-digestate (A:D) conditions.



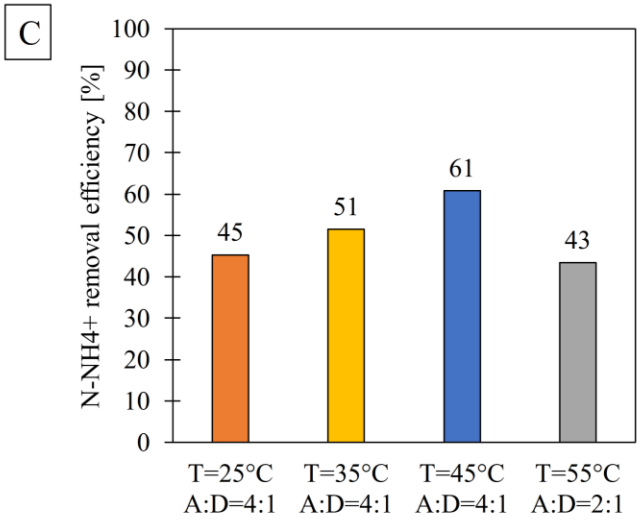
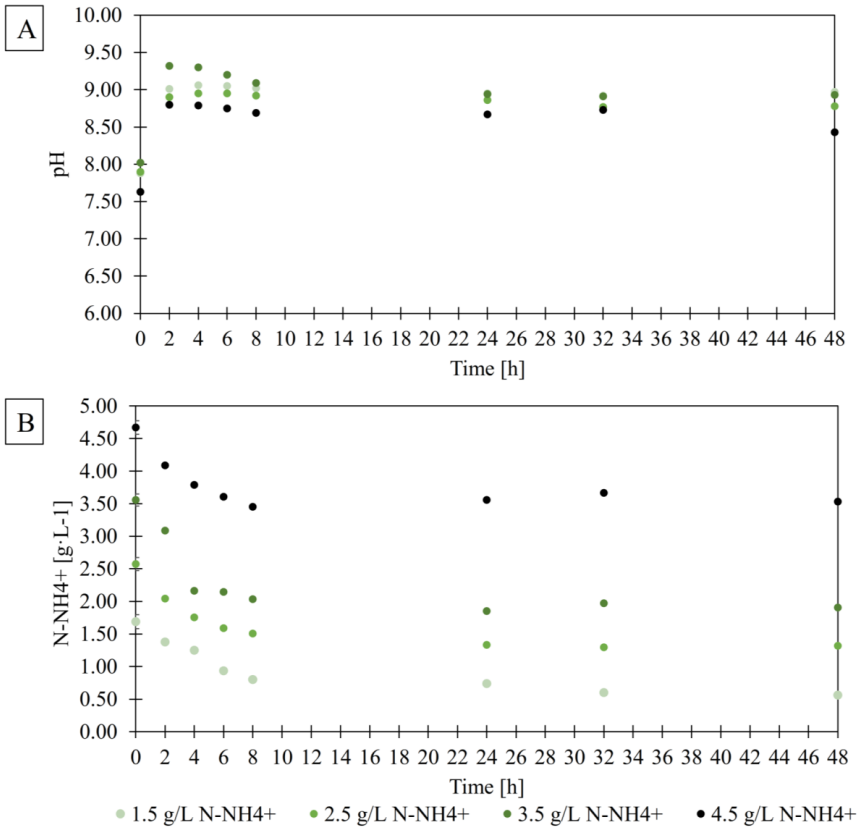


Figure 3. Evolution of pH (Panel A), residual N-NH_4^+ concentrations in the liquid fraction of digestate (Panel B) and N-NH_4^+ removal efficiencies (Panel C) along the 48-h continuous direct air stripping process of ammonia under different temperature and air-to-digestate conditions.



C

Time of operation [h]	Initial $N\text{-}NH_4^+$ concentration $[g \cdot L^{-1}]$			
	1.5	2.5	3.5	4.5
2	18	21	13	12
4	26	32	39	19
6	45	38	40	23
8	53	41	43	26
24	56	48	48	24
32	65	50	45	21
48	67	49	46	24

Figure 4. Evolution of pH (Panel A), residual $N\text{-}NH_4^+$ concentrations in the liquid fraction of digestate (Panel B) and $N\text{-}NH_4^+$ removal efficiency [%] (Panel C) along the 48-h continuous direct air stripping process of ammonia from digestate characterized by initial $N\text{-}NH_4^+$ concentrations of 1.5, 2.5, 3.5 and 4.5 $g \cdot L^{-1}$. In panel C, different colors have been used to indicate how values appear over time: variable (red), almost stable (orange) and stable (green) values.

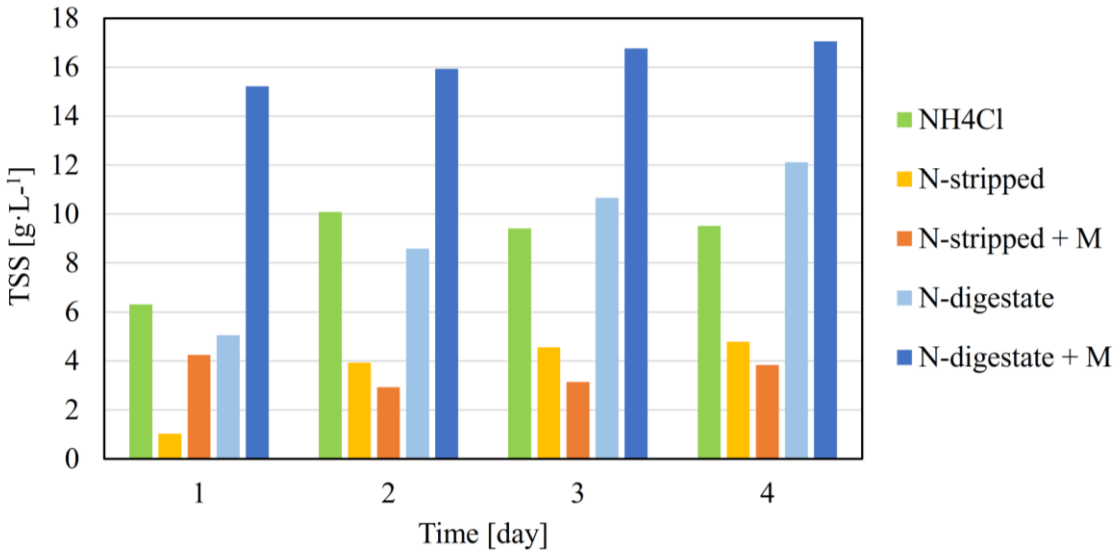


Figure 5. Performance of the aerobic microbial protein production process in terms of daily biomass concentration under the different experimental conditions. NH_4Cl: N supplied as a chemical, N-stripped: N supplied through the stripping process, N-stripped + M: N supplied through the stripping process using digestate contaminated with heavy metals, N-digestate: N supplied through digestate in the aerobic reactor, N-digestate + M: N supplied through digestate contaminated with heavy metals directly fed in the aerobic reactor.

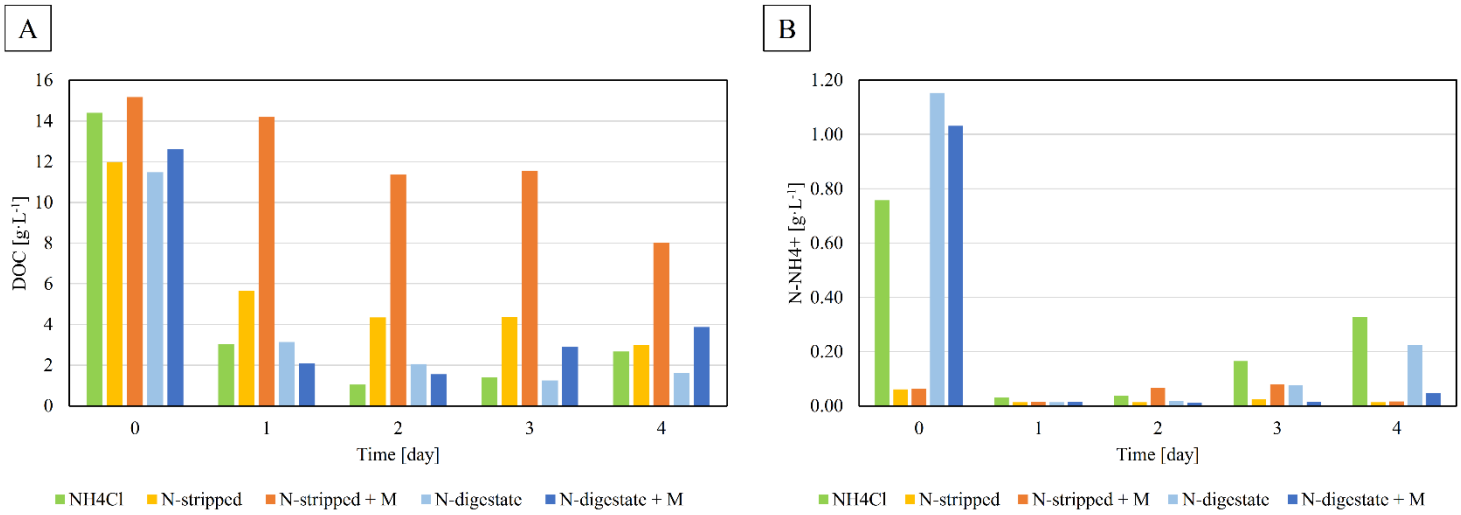


Figure 6. Performance of the aerobic microbial protein production process in terms of daily DOC and N-NH_4^+ concentrations under the different experimental conditions. NH_4Cl: N supplied as a chemical, N-stripped: N supplied through the stripping process, N-stripped + M: N supplied through the stripping process using digestate contaminated with heavy metals, N-digestate: N supplied through digestate in the aerobic reactor, N-digestate + M: N supplied through digestate contaminated with heavy metals directly fed in the aerobic reactor.

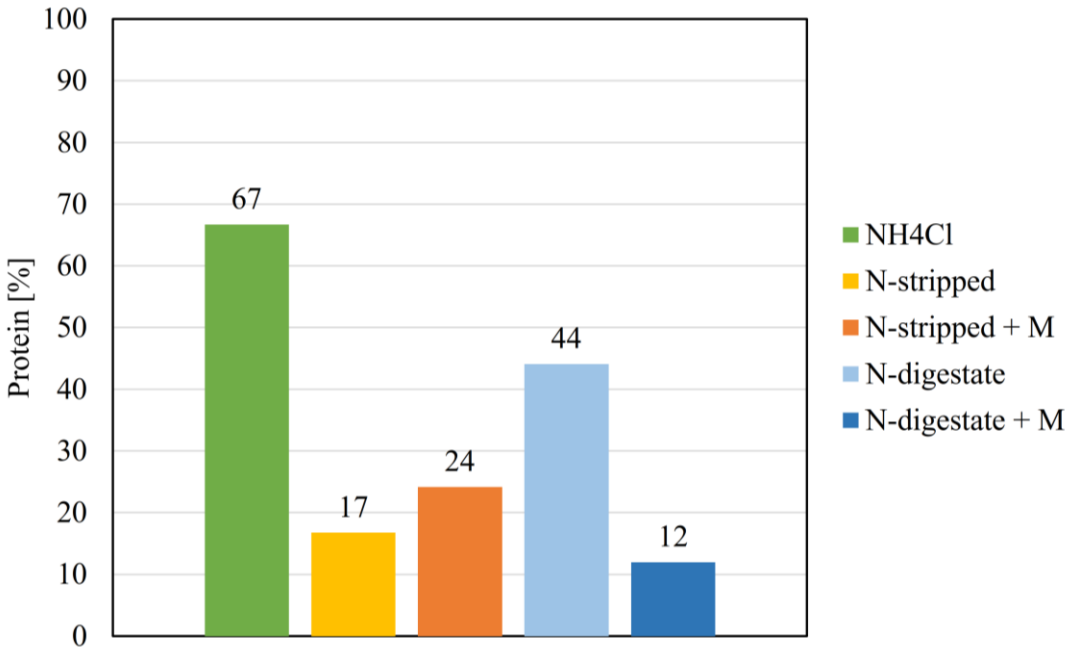


Figure 7. Protein content of the biomass produced under the different experimental conditions. NH_4Cl: N supplied as a chemical, N-stripped: N supplied through the stripping process, N-stripped + M: N supplied through the stripping process using digestate contaminated with heavy metals, N-digestate: N supplied through digestate in the aerobic reactor, N-digestate + M: N supplied through digestate contaminated with heavy metals directly fed in the aerobic reactor.

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Figure 5. Performance of the aerobic microbial protein production process in terms of daily biomass concentration under the different experimental conditions. NH_4Cl: N supplied as a chemical, N-stripped: N supplied through the stripping process, N-stripped + M: N supplied through the stripping process using digestate contaminated with heavy metals, N-digestate: N supplied through digestate in the aerobic reactor, N-digestate + M: N supplied through digestate contaminated with heavy metals directly fed in the aerobic reactor.

Figure 6. Performance of the aerobic microbial protein production process in terms of daily DOC and N-NH_4^+ concentrations under the different experimental conditions. NH_4Cl: N supplied as a chemical, N-stripped: N supplied through the stripping process, N-stripped + M: N supplied through the stripping process using digestate contaminated with heavy metals, N-digestate: N supplied through digestate in the aerobic reactor, N-digestate + M: N supplied through digestate contaminated with heavy metals directly fed in the aerobic reactor.

Figure 7. Protein content of the biomass produced under the different experimental conditions. NH_4Cl: N supplied as a chemical, N-stripped: N supplied through the stripping process, N-stripped + M: N supplied through the stripping process

using digestate contaminated with heavy metals, N-digestate: N supplied through digestate in the aerobic reactor, N-digestate + M: N supplied through digestate contaminated with heavy metals directly fed in the aerobic reactor.

Supplementary Information

From waste- to protein-nitrogen: optimizing and validating direct nitrogen stripping from anaerobic digestate for sustainable microbial protein production

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Table S1. Physic-chemical characterization of the anaerobic digestates used under batch (high-N digestate) and continuous (low-N digestate) conditions. High-N digestate was also used as direct N source supplied to the fermentation reactor. $N\text{-}NH_4^+$ = ammonium nitrogen; tCOD = total chemical oxygen demand; sCOD = soluble chemical oxygen demand; DOC = dissolved organic carbon; TS = total solids; VS = volatile solids.

Parameter	High-N digestate	Low-N digestate
pH	8.85 ± 0.02	8.20 ± 0.03
$N\text{-}NH_4^+ [g \cdot L^{-1}]$	3.78 ± 0.28	0.89 ± 0.05
tCOD $[g \cdot L^{-1}]$	56.53 ± 1.33	64.70 ± 1.97
sCOD $[g \cdot L^{-1}]$	18.53 ± 0.43	9.67 ± 0.66
DOC $[g \cdot L^{-1}]$	4.29 ± 0.33	2.25 ± 0.13
TS [%]	8.02 ± 0.09	6.41 ± 0.01
VS [%]	5.56 ± 0.06	4.00 ± 0.02
Ashes [%]	2.46 ±0.15	2.41 ± 0.03

Table S2. Composition of the synthetic CWP used in this study for microbial protein production.

Chemical	Concentration $[mg \cdot L^{-1}]$
$C_{12}H_{22}O_{11}$	40000
NH_4Cl	5000
KH_2PO_4	2820
$MgSO_4 \cdot 7H_2O$	2802
$ZnSO_4 \cdot 7H_2O$	3.0
$CaCl_2$	6.0

$MnCl_2 \cdot 2H_2O$	7.2
$FeCl_3 \cdot 6H_2O$	9.9
$(NH_4)_6Mo_7O_{24}$	1.2
$CuSO_4 \cdot 5H_2O$	1.2
$CoCl_2$	1.2

Table S3. Heavy metals concentration in the manually-contaminated digestate used as N source.

Metal	**Concentration [mg·L^{-1}]**
Nichel (Ni)	1
Lead (Pb)	27
Copper (Cu)	73
Zinc (Zn)	93
Cadmium (Cd)	3

Table S4. Stripping rate (SR) values obtained from the batch stripping process under the different experimental conditions calculated according to Equation 3 reported in Section 2.6 both hourly and daily. The null value was reported if SR assumed negative values due to initial process instability.

	SR [$\frac{g\ N-NH_4^+}{L \cdot h}$]											
T[°C]	25	25	25	35	35	35	45	45	45	55	55	55
A:D	1:1	2:1	4:1	1:1	2:1	4:1	1:1	2:1	4:1	1:1	2:1	4:1
Time [h]												
0	-	-	-	-	-	-	-	-	-	-	-	-
1	0.00	0.00	0.00	0.23	0.46	0.46	0.00	0.12	0.41	0.37	0.67	1.19

2	0.00	0.01	0.00	0.00	0.20	0.19	0.08	0.09	0.64	0.17	0.35	0.86
3	0.04	0.00	0.24	0.00	0.10	0.41	0.00	0.36	0.38	0.37	0.31	0.91
4	0.00	0.35	0.27	0.07	0.15	0.32	0.24	0.21	0.81	0.63	0.44	0.45
5	0.23	0.04	0.16	0.21	0.28	0.26	0.09	0.47	0.47	0.04	0.52	0.25
6	0.23	0.25	0.12	0.12	0.00	0.31	0.11	0.27	0.23	0.08	0.41	0.15
7	0.20	0.10	0.11	0.10	0.32	0.31	0.23	0.33	0.21	0.22	0.24	0.09
8	0.09	0.12	0.20	0.07	0.30	0.33	0.16	0.04	0.11	0.35	0.26	0.01
24	0.05	0.09	0.10	0.07	0.11	0.08	0.11	0.09	0.01	0.10	0.03	0.00

SR $[\frac{\mathbf{g\ N-NH_4^+}}{\mathbf{L \cdot d}}]$												
T[°C]	25	25	25	35	35	35	45	45	45	55	55	55
A:D	1:1	2:1	4:1	1:1	2:1	4:1	1:1	2:1	4:1	1:1	2:1	4:1
	1.04	1.97	2.63	1.93	3.53	3.85	2.33	3.38	3.50	3.77	3.69	3.93

Table S5. Stripping rate (SR) values obtained from the continuous stripping process under the different experimental conditions calculated according to Equation 3 reported in Section 2.6 both hourly and daily.

SR $[\frac{\mathbf{g\ N-NH_4^+}}{\mathbf{L \cdot h}}]$				
T[°C]	25	35	45	55
A:D	4:1	4:1	4:1	2:1
Time [h]				
0	-	-	-	-
2	0.00	0.08	0.17	0.15
4	0.07	0.03	0.24	0.02
6	0.05	0.08	0.05	0.06
8	0.05	0.04	0.12	0.06
24	0.03	0.06	0.07	0.05
32	0.03	0.05	0.08	0.04
48	0.05	0.06	0.06	0.04

SR $[\frac{\mathbf{g\ N-NH_4^+}}{\mathbf{L \cdot d}}]$				
T[°C]	25	35	45	55
A:D	4:1	4:1	4:1	2:1
	1.12	1.36	2.69	1.44

Table S6. Stripping rate (SR) values obtained from the continuous stripping process of ammonia from digestate characterized by initial N-NH_4^+ concentrations of 1.5, 2.5, 3.5 and 4.5 g·L^{-1} under the different experimental conditions calculated according to Equation 3 reported in Section 2.6 both hourly and daily.

	SR $[\frac{\mathbf{g\ N-NH_4^+}}{\mathbf{L \cdot h}}]$			
T[°C]	45	45	45	45
A:D	4:1	4:1	4:1	4:1
Initial N-NH_4^+ [g·L^{-1}]	1.5	2.5	3.5	4.5
Time [h]				
0	-	-	-	-
2	0.17	0.30	0.26	0.33
4	0.11	0.23	0.58	0.24
6	0.23	0.20	0.18	0.21
8	0.17	0.17	0.24	0.22
24	0.12	0.16	0.21	0.14
32	0.14	0.16	0.19	0.12
48	0.14	0.16	0.21	0.14

	SR $[\frac{\mathbf{g\ N-NH_4^+}}{\mathbf{L \cdot d}}]$			
T[°C]	45	45	45	45
A:D	4:1	4:1	4:1	4:1
Initial N-NH_4^+ [g·L^{-1}]	1.5	2.5	3.5	4.5
	3.74	4.68	4.96	4.80

Title: From waste to high-value nitrogen: optimizing and validating direct nitrogen stripping from anaerobic digestate for sustainable microbial protein production

ABSTRACT

In the quest for sustainable and circular alternative protein sources, low-cost and contaminant-safe resource recovery techniques are urged. This study investigated and optimized a zero-chemical, low-temperature direct air stripping process from anaerobic digestate for clean nitrogen (N) recovery and its upcycling into microbial protein (MP) produced from cheese whey permeate (CWP). Key operational parameters, including air-to-digestate (A:D) ratio, temperature, initial nitrogen concentration, and batch vs. continuous operation, were assessed. Stripping efficiencies of up to 100% were achieved within 24 hours under batch conditions with A:D ratios of 2:1 and 4:1 at mesophilic (25, 35, 45 °C) and thermophilic (55 °C) temperatures. Under continuous operation, up to 61% stripping efficiency was obtained at 45 °C with an A:D ratio of 4:1. The latter operating condition was selected to integrate the direct air stripping process with the aerobic MP production step, leveraging the aeration flow of the latter. This N-recovery strategy was compared to other N-supply routes such as the direct addition of N-rich digestate or of NH_4Cl to CWP. The impact of heavy metals in digestate on biomass growth and MP quality was also investigated. Biomass concentrations reached approximately 17 g $TSS \cdot L^{-1}$ when nitrogen was supplied through digestate, while using stripped ammonia from digestate led to a lower biomass growth, reaching 4.78 g $TSS \cdot L^{-1}$. Although heavy metals did not inhibit biomass growth, they compromised the quality of the final MP product when digestate was supplied directly as nitrogen source.

Keywords: ammonia stripping; anaerobic digestate; cheese whey; microbial protein; resource recovery; heavy metals.

1. Introduction

The global protein demand is rising unsustainably, projected to increase by 78% by 2050 (Van Peteghem et al., 2022). Traditional protein sources, reliant on resource-intensive farming and vulnerable to disruptions like disease and overfishing, are no longer viable (Mohammad et al., 2023; Tubb and Seba, 2021). To ensure food security amidst climate change and population growth, efficient protein alternatives such as plant-based proteins, cultured meat, and microbial protein (MP) are essential (Aiking and de Boer, 2020).

MP synthesis, utilizing microorganisms like bacteria and microalgae in closed bioreactors, offers high resource efficiency, including waste nitrogen (N) recycling, reducing environmental impact (Matassa et al., 2016; Pikaar et al., 2017). As a matter of fact, MP production relies heavily on the availability of reactive N, the building block of biomass and protein synthesis. However, the cost and environmental impact of sourcing N for bioprocessing remain substantial, influencing both the economic and ecological footprint of MP production (Van Peteghem et al., 2023). Consequently, growing interest is being directed towards the utilization of waste-derived N sources, which can both reduce operational costs and contribute to more circular and sustainable bioproduction systems (Pikaar et al., 2017).

One of the most abundant N-rich wastes from livestock farms is represented by anaerobic digestate (Scotto di Perta et al., 2023). Anaerobic digestate is a valuable source of organic matter, amino acids, vitamins and nutrients such as phosphorus, potassium, ammonium nitrogen ($N\text{-}NH_4^+$) and several trace elements such as copper and zinc (Di Costanzo et al., 2023; Jin and Chang, 2011) . With $N\text{-}NH_4^+$ concentration in a digestate ranging between 0.8-6.0 $g \cdot L^{-1}$ (Möller and Müller, 2012; Shi et al., 2018), various processes have been developed to N recovery from digestate for microbial growth (Bertasini et al., 2022; Kovačić et al., 2022). However, the direct utilization of digestate poses several challenges. One significant concern is the presence of

pathogens, including bacteria, viruses and parasites, which could contaminate the resulting MP if adequate treatment is not applied (Di Costanzo et al., 2024; Kovačić et al., 2022). Additionally, digestate may contain organic pollutants (e.g., pharmaceutical and pesticides) or inorganic contaminants (e.g., heavy metals) which could interfere with microbial cultivation or result in unsafe MP products (Golovko et al., 2022).

To address these challenges, ammonia stripping emerges as a viable alternative to recover N from digestate in a suitable form for MP production. The stripping process facilitates the transition of ammonium ions (NH_4^+) to ammonia gas (NH_3), effectively separating nitrogen from the contaminated liquid digestate (Matassa et al., 2022; Scotto di Perta et al., 2023). Ammonia stripping efficiency depends on pH, temperature, mass transfer area, and dissolved CO_2 in the liquid matrix (Limoli et al., 2016). Despite its attractiveness, the need for dedicated equipment (Kar et al., 2023), chemical additives to adjust pH (Alitalo et al., 2012; Bonmatí and Flotats, 2003), and significant thermal energy to heat digestate to 70-90 °C (Matassa et al., 2015) lead to high operational and capital costs. Recent studies (Matassa et al., 2022; Zhao et al., 2015) explored the possibility of operating the direct air stripping process at milder temperatures and without chemical pH adjustments by using aeration flows to strip CO_2 from digestate, as its buffer capacity is mainly due to the bicarbonate/carbonate equilibrium. The latter process not only recovers N but also minimizes contamination risks, as ammonia gas is free from non-volatile pathogens and pollutants remaining in the liquid fraction. The air-ammonia-rich output gas stream can be exploited to provide oxygen and nitrogen for MP production by promoting the solubilization of N in growth substrates characterized by acidic pH such as cheese whey permeate (CWP) (pH 4.0-6.5, Carvalho et al., (2013)). The acidic environment promotes the absorption of ammonia as $N\text{-}NH_4^+$ without the need of pH adjustment, providing a readily available N source for microbial growth.

Data about the actual potential of the direct air stripping process are scarce, and comparative evaluations with other N recovery and feeding strategies for MP production are lacking. In light of this, the present study first investigated experimentally the influence of mesophilic and thermophilic temperatures and air to digestate (A:D) ratios during the direct air stripping of ammonia from the liquid fraction of anaerobic digestate under batch conditions, and then evaluated the same process under continuous reactor settings and with different initial N-NH_4^+ concentrations. The process performances were evaluated in terms of N-NH_4^+ stripping efficiency and rates. Subsequently, MP production from CWP using N recovered through the optimised direct air stripping process was investigated and compared with MP production using direct addition of digestate and mineral N (NH_4Cl) as N sources. The MP production process was evaluated with respect to key process performance indicators such as biomass concentration, protein content, sCOD removal and residual N-NH_4^+. Finally, considering the potential presence of high heavy metals content in the digestate, which could hinder biomass growth as well as compromise product quality (Dragicevic et al., 2017), the work evaluated how the latter may be transferred and/or accumulated in the final product when different N recovery and feeding strategies are applied.

2. Materials and methods

2.1 Source, characterization and pretreatment of anaerobic digestate

The anaerobic digestate was sourced from anaerobic digesters located in the Campania region (Italy) treating buffalo manure and agricultural residues. Digestates were stored at 4 °C prior to physic-chemical characterization (Table S1). A high-N digestate was used for batch air stripping, while a low-N digestate was used for continuous air stripping. The low-N digestate, amended with NH_4Cl to achieve different N concentrations, was used for other continuous air stripping tests and as direct N source for the aerobic MP production.

Before being used, the digestate was filtered through a 1 mm stainless steel filter mesh to separate the liquid from the solids, then centrifuged at 6000 rpm for 10 minutes to remove residual solids, improving liquid-to-gas mass transfer in air stripping and nutrient assimilation in MP production.

When used as N source directly supplemented in the synthetic C-rich CWP, the liquid fraction was pasteurized (70 °C for 1 hour) and diluted to achieve 1.3 $g \cdot L^{-1}$ $N\text{-}NH_4^+$, ensuring a C/N ratio of 15. After pasteurization and dilution, the dissolved organic carbon (DOC) of the digestate, used as indicator of the dissolved organic matter, was equal to 1.40 $g \cdot L^{-1}$. This allowed to consider the DOC fed through the digestate as negligible, as the DOC provided through the lactose of the CWP used was 14.40 $g \cdot L^{-1}$ (Table S2).

Furthermore, to investigate the effect on biomass of the heavy metals potentially present in the digestate, the anaerobic digestate was chemically amended with metals (Table S3) to reach typical concentration values of digestate from animal manure (Dragicevic et al., 2017; Jin and Chang, 2011; Li et al., 2018).

2.2 Composition of the synthetic cheese whey permeate

A synthetic lactose-based solution, adapted from Dubois Frigon (2020) and reported in Table S2, was used to simulate a CW-based organic substrate for MP production. The nitrogen concentration provided either through mineral nitrogen (NH_4Cl) or through direct digestate supply was calculated to achieve a C/N ratio equal to 15.

2.3 Direct air stripping tests

Direct air stripping tests were performed under both batch and continuous modes in 250 mL Schott bottles with 150 mL of liquid digestate in a water bath at 25, 35, 45 and 55 °C. Air was

125 provided as stripping agent by a stainless steel air sparger (bubble size 0.5 – 2.0 mm) connected
126 to a 520S peristaltic pump (Watson Marlow, United Kingdom). The sparger was placed 5 cm
127 below the surface to enhance pH elevation and ammonia desorption (Zhao et al., 2015). A:D
128 ratios of 1:1, 2:1 and 4:1, were guaranteed by providing airflows of 150, 300 and 600 $mL \cdot min^{-1}$
129 , respectively. To avoid foaming, a volume of 1 mL of antifoaming agent (Arauner defoamer,
130 Germany) was added to 150 mL of digestate.

131 *2.3.1 Batch tests*

132 Batch tests lasting 24 hours, performed with high-N digestate (Table S1), screened temperature
133 and A:D combinations. Gas and liquid samples were taken periodically to monitor the time-
134 dependent evolution of gas composition, pH and $N\text{-}NH_4^+$ concentration.

135 *2.3.2 Continuous tests*

136 Continuous tests, performed with low-N digestate (Table S1), were conducted with an A:D ratio
137 of 4:1 at temperatures of 25, 35 and 45 °C and with an A:D ratio of 2:1 at temperature of 55 °C.
138 Continuous tests were carried out with a hydraulic retention time (HRT) equal to 8 hours and
139 lasted 48 hours. Furthermore, additional continuous tests were performed using digestate
140 amended with NH_4Cl (ITW Reagents, Italy) to evaluate the effects of different initial $N\text{-}NH_4^+$
141 concentrations of 1.5, 2.5, 3.5 and 4.5 $g \cdot L^{-1}$. Liquid samples were collected periodically to
142 monitor pH and $N\text{-}NH_4^+$ concentration.
143

144 *2.4 Aerobic MP production*

145 *2.4.1 Source and activation of the microbial inoculum*

146 A commercial lyophilized mixed culture of yeasts and bacteria (Genesis Laboratories, Bulgaria)
147 for kefir production served as microbial inoculum. Prior to its use for the aerobic MP production
148 process, the inoculum was activated under batch conditions. Activation was performed in a 500

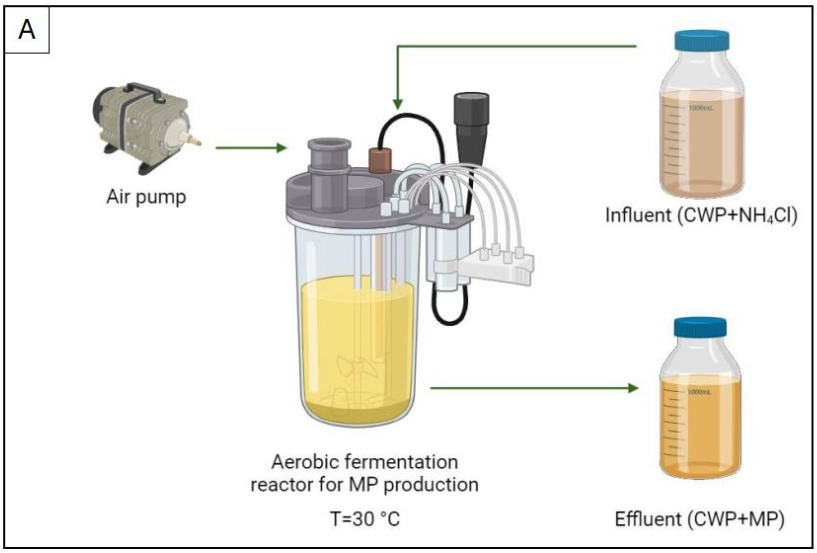
149 mL Schott bottle with 200 mL synthetic CWP, incubated at 30 °C and stirred at 500 rpm for 48
150 hours. Aeration was supplied through a porous stone connected to a 520S peristaltic pump
151 (Watson Marlow, United Kingdom).

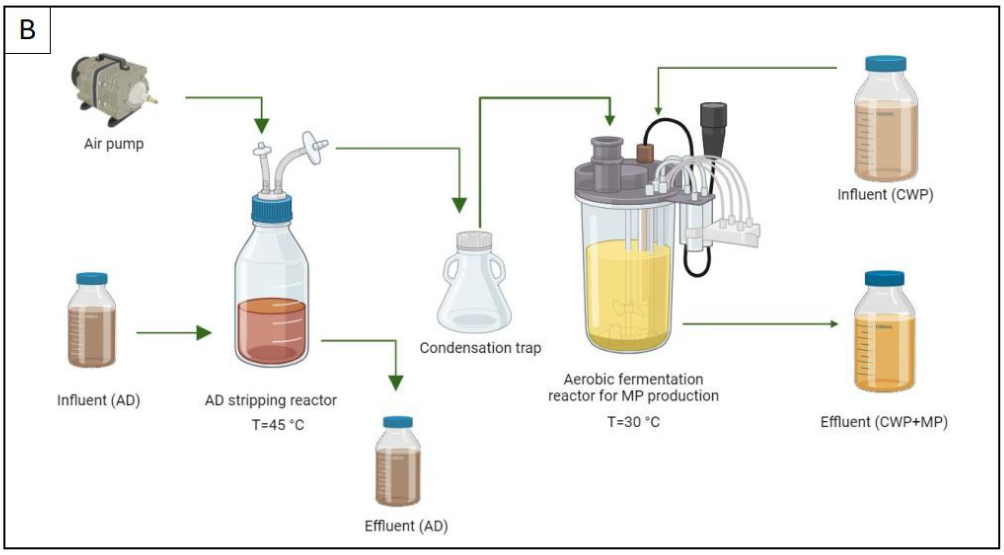
152 *2.4.2 Experimental setup of the aerobic MP production process under different N*
153 *recovery/feeding strategies*

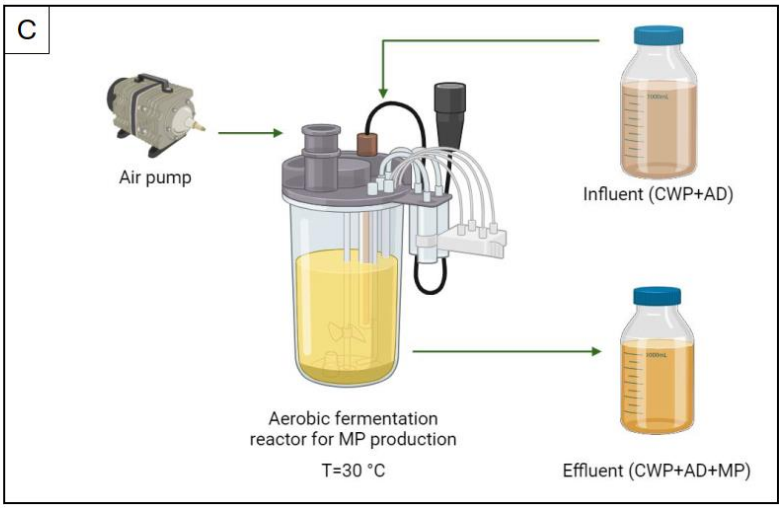
154 Microbial biomass growth was tested under different N feeding strategies (Figure 1). While the
155 C source was always provided by lactose contained in synthetic CWP, N was provided either
156 through NH_4Cl addition in the synthetic CWP medium fed in the bioreactor (Figure 1 panel A),
157 or by direct air stripping of the digestate connected to the aerobic reactor (Figure 1 panel B), or
158 by directly mixing the digestate with the synthetic CWP free of NH_4Cl (Figure 1 panel C). The
159 first N feeding strategy, involving the addition of chemical N, was used as control condition
160 since it reproduces the typical MP production scenario from CWP (Scotto di Uccio et al., 2023).
161 The latter two conditions relating to waste N recovery and valorisation were also tested using a
162 heavy metal-contaminated digestate as N source.

163 The continuous MP production setup consisted of a Biostat B Plus (Sartorius, Germany) with a
164 2 L vessel operated as continuous stirred-tank reactor (CSTR) with a 1-day HRT. The working
165 volume of 1 L was inoculated at 10% of the volume with the activated inoculum. Aerobic
166 conditions (>2 $mgO_2 \cdot L^{-1}$) were guaranteed by sparging air with a flowrate of 600 $mL \cdot min^{-1}$ and
167 by stirring at 600-800 rpm. The process was maintained at 30 °C, with pH controlled at 4.5 ±
168 0.1 using 1 M NaOH or HCl.

169 Based on initial results, air stripping was performed in 500 mL Schott bottles filled with 250
170 mL of liquid digestate at 45 °C with an A:D ratio of 4:1. The air stripping process was
171 performed in continuous mode under the same experimental set up described in Section 2.3
172 under a 8 h HRT.


A
Air pump
Influent (CWP+NH4Cl)
Aerobic fermentation
reactor for MP production
T=30 °C
Effluent (CWP+MP)


B
Air pump
Influent (CWP)
Influent (AD)
AD stripping reactor
T=45 °C
Condensation trap
Aerobic fermentation
reactor for MP production
T=30 °C
Effluent (CWP+MP)
Effluent (AD)


C
Air pump
Influent (CWP+AD)
Aerobic fermentation
reactor for MP production
T=30 °C
Effluent (CWP+AD+MP)

173

Figure 1. Overview of the experimental conditions and setup. Panel A: MP production using synthetic CWP medium including NH_4Cl as N source. Panel B: MP production using synthetic CWP medium (free of NH_4Cl) and ammonia from the digestate stripping process as N source. Panel C: MP production using synthetic CWP medium (free of NH_4Cl) mixed with digestate as N source. AD=anaerobic digestate.

2.5 Analytical procedures

Total (TS) and volatile solids (VS) concentrations were analyzed according to the Standard Methods (APHA, 2007). Total suspended solids (TSS) concentrations, analyzed according to the Standard Methods (APHA, 2007), were used as indicator of the biomass growth. When digestate was directly supplied to the aerobic reactor as N-source, the TSS related to the biomass concentration were calculated excluding the TSS contribution from the digestate. The pH was measured by means of a HI98100 pH-meter (Hanna Instruments, Italy). $N\text{-}NH_4^+$ concentration in the digestate was detected spectrophotometrically, through the indophenol blue method (Aminot et al., 1997), on the liquid fraction obtained by centrifuging the digestate at 1000 rpm for 10 min and filtering it through 0.45 μm polypropylene membranes (VWR, Italy). The same concentration in the CWP medium was analysed through a steam distillation unit (Behr Labor-Technik, Germany) according to Standard Methods (APHA, 2007). Soluble (sCOD) and total chemical oxygen demand (tCOD) were determined through the closed reflux colorimetric method (APHA, 2007), while DOC was determined through a TOC-L analyser (Shimadzu, Japan). Protein content in the biomass was evaluated spectrophotometrically by the Folin method (Lowry et al., 1951). At the end of each experiment, the biomass was harvested through centrifugation at 4000 rpm for 10 min, frozen at −20 °C and then dried at 70 °C for 24 h. Then, the extraction of heavy metals was performed on grinded biomass thorough a microwave assisted acid digestion with a mixture 9:1 of HNO_3 and H_2O_2 according to the EPA 3051A (EPA, 2007) method. The extracted heavy metals were analysed through an atomic absorption spectrophotometer (GBC Avanta, Germany).

199

200 *2.6 Calculations*

201 The N-NH_4^+ removal efficiencies along the tests were calculated according to Equation 1:

$$\text{N-NH}_4^+ \text{ removal efficiency} = \frac{\text{N}-\text{NH}_4^{+\,in} - \text{N}-\text{NH}_4^{+\,\text{i}}}{\text{N}-\text{NH}_4^{+\,in}} \cdot 100\ [\%] \quad (1)$$

202 where $\text{N}-\text{NH}_4^{+\,\text{in}}$ indicates the initial ammonium nitrogen concentration and $\text{N}-\text{NH}_4^{+\,\text{i}}$
203 indicates the ammonium nitrogen concentration at a given time or at the end of the test. Since
204 water evaporation occurred during the batch tests, the N-NH_4^+ concentration values were
205 calculated by taking into account the change in volume and were always referred to the initial
206 digestate volume.

207

208 The N-NH_4^+ stripping rate (SR), which represents the ammonium nitrogen removed per unit
209 of time per unit of volume ($g \cdot L^{-1} \cdot h^{-1}$), was calculated, for batch tests, based on Equation 2:

$$\text{SR} = \frac{\text{N}-\text{NH}_{4\,i-1}^{+} - \text{N}-\text{NH}_{4\,i}^{+}}{\Delta i} \left[\frac{\text{gN}-\text{NH4}+}{\text{L}\cdot\text{h}}\right] \quad (2)$$

210 where the subscripts *i* and *i-1* indicate the values of ammonium nitrogen concentration at the
211 chosen sampling time and at the previous one, respectively, and Δi indicates the interval time
212 between the two moments considered.
213 The difference between N-NH_4^+ at 0 and 24th hour was used to calculate the daily SR value in
214 batch mode.
215 With reference to continuous test, the SR was calculated based on Equation 3:

$$\text{SR} = \frac{\text{N}-\text{NH}_{4\,i-1}^{+} + \left(\text{N}-\text{NH}_{4\,IN}^{+} \cdot \Delta i\right) - \text{N}-\text{NH}_{4\,i}^{+} - \left(\frac{\text{N}-\text{NH}_{4\,i-1}^{+} + \text{N}-\text{NH}_{4\,i}^{+}}{2} \cdot \Delta i\right)}{\Delta i} \left[\frac{\text{gN}-\text{NH4}+}{\text{L}\cdot\text{h}}\right] \quad (3)$$

216 Where $\text{N}-\text{NH}_{4\,IN}^{+}$ indicates the N-NH_4^+ concentration in the influent digestate.

The average hourly SR, calculated over the 48-hours test and multiplied by 24 hours, was used to calculate the daily SR in continuous mode.

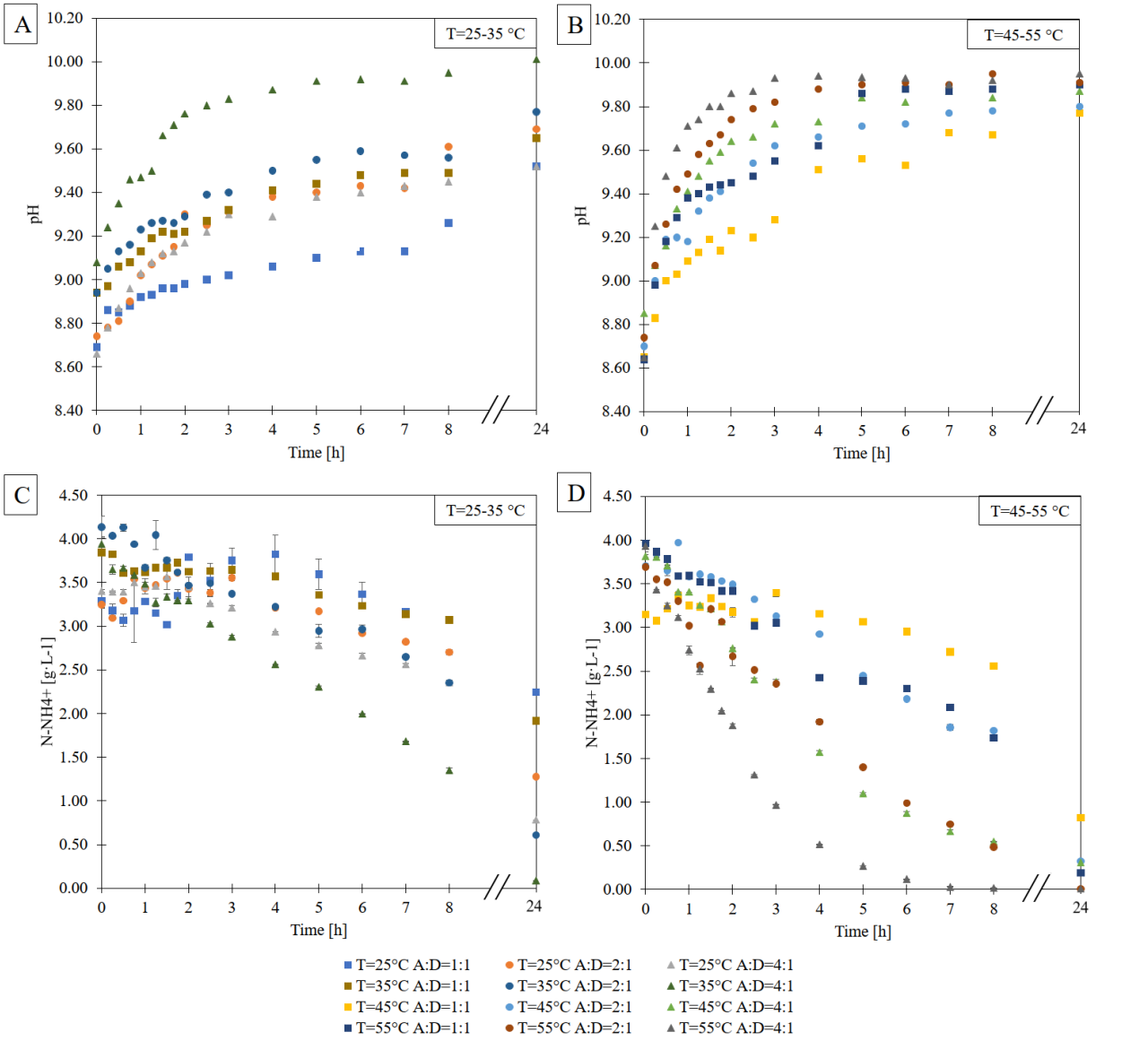
3. Results and discussion

3.1 *Effect of temperature and air to digestate ratio on direct air stripping under batch and continuous conditions*

Figure 2 shows the evolution of pH and N-NH_4^+ concentration along the 24 hours of batch experiments under different temperatures and A:D ratios. As expected, the pH increase was positively correlated with the increase in temperature. As clearly visible in Figure 2, panels A and B, the increase in pH mainly occurred in the first few hours of the stripping process, regardless of the operating condition adopted. However, such an increase in pH did not immediately correspond to a decrease in ammonium nitrogen (Figure 2 panels C and D). Indeed, the temporal evolution of N-NH_4^+ shows an initial lag phase associated with CO_2 volatilization, being the latter more volatile than ammonia due to its lower solubility (1.7 g CO_2 L^{-1} compared with 535 g NH_3 L^{-1} in pure water at 20 °C) (Alitalo et al., 2012; Laureni et al., 2013). CO_2 volatilization is needed to increase the pH up to a value sufficient to favor the dissociation of NH_4^+ into NH_3 and prompt ammonia volatilization.

At A:D ratios of 1:1 and 2:1 and with a temperature of 25 °C, the process showed low ammonia stripping performances since, after 24 hours of treatment, a N-NH_4^+ removal efficiency of only 32 and 61%, respectively, was obtained (Figure 2 panel E). When increasing the temperature to 45 °C, as shown in Figure 2, the A:D ratio increase from 2:1 to 4:1 did not have relevant effects on the N-NH_4^+ removal efficiency, which achieved values of 91 and 92%, respectively. Similar values higher than 90% were obtained by Laureni et al. (2013) by performing an air stripping process on liquid digestate at 50 °C for 225 minutes with an A:D ratio equal to 10:1, thus much higher than the values used in this study. By further increasing the temperature at 55

°C, a complete N stripping (100% efficiency) was obtained with both A:D ratios of 2:1 and 4:1. Along with the increasing temperature, not only the percentage of free ammonia increases, but also the molecular diffusion coefficient of ammonia in both liquid and gas films is enhanced, while the viscosity, the surface tension of the liquid phase and the liquid-gas distribution ratio of ammonia are reduced (Zhao et al., 2015).


A
T=25-35 °C
pH
Time [h]
B
T=45-55 °C
pH
Time [h]
C
T=25-35 °C
N-NH4+ [g·L-1]
Time [h]
D
T=45-55 °C
N-NH4+ [g·L-1]
Time [h]
T=25°C A:D=1:1
T=25°C A:D=2:1
T=25°C A:D=4:1
T=35°C A:D=1:1
T=35°C A:D=2:1
T=35°C A:D=4:1
T=45°C A:D=1:1
T=45°C A:D=2:1
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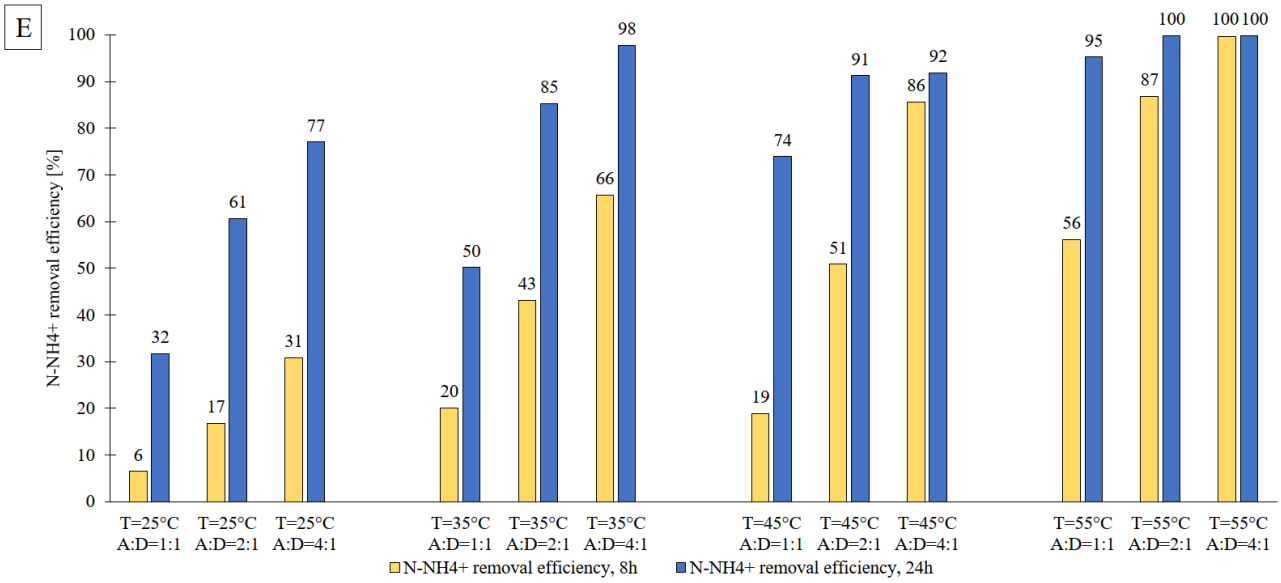
T=55°C A:D=4:1


E
N-NH4+ removal efficiency [%]
6
32
17
61
31
77
20
50
43
85
66
98
19
74
51
91
86
92
56
95
87
100
100
100
T=25°C A:D=1:1
T=25°C A:D=2:1
T=25°C A:D=4:1
T=35°C A:D=1:1
T=35°C A:D=2:1
T=35°C A:D=4:1
T=45°C A:D=1:1
T=45°C A:D=2:1
T=45°C A:D=4:1
T=55°C A:D=1:1
T=55°C A:D=2:1
T=55°C A:D=4:1
N-NH4+ removal efficiency, 8h
N-NH4+ removal efficiency, 24h

247

Figure 2. Evolution of pH (Panels A, B), residual N-NH_4^+ concentrations in the liquid fraction of digestate (Panels C, D) and N-NH_4^+ removal efficiencies (Panel E) along the 24 h batch mode direct air stripping process of ammonia under different temperature and air-to-digestate (A:D) conditions.

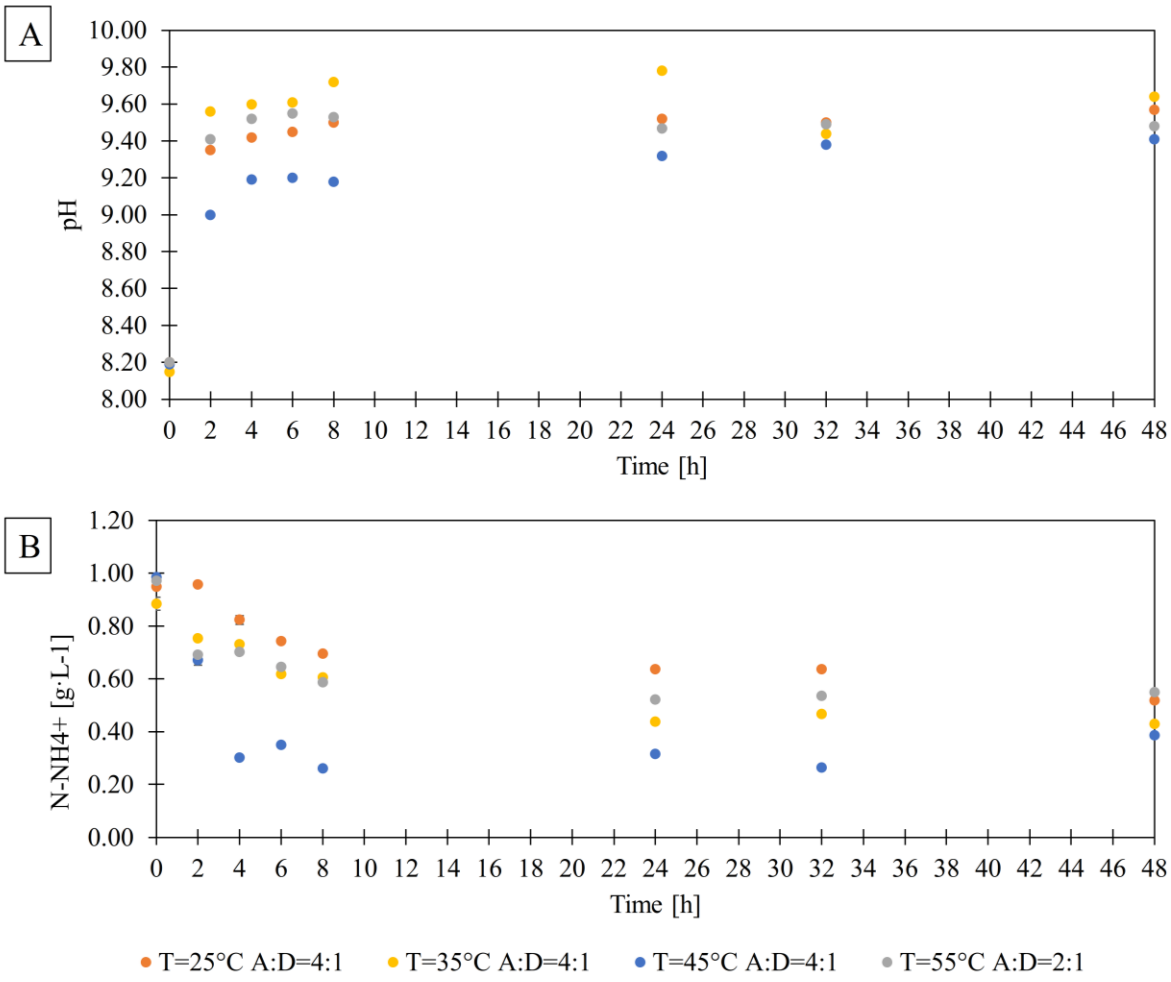
The SR values obtained during the ammonia stripping process in batch (reported in supplementary information in Table S4) were strongly dependent on both the temperature and air-to-digestate (A:D) ratio. The results are in good agreement with those referred to ammonia removal from the digestate at higher A:D ratios (2:1 or 4:1), with increased temperatures enhancing ammonia stripping and achieving the maximum SR values earlier. At 35 °C with A:D ratios of 2:1 and 4:1, or at 55 °C with similar ratios, SR values of up to 1.19 g N-$NH_4^+ \cdot L^{-1} \cdot h^{-1}$ were reached within the first hour. In general, the values reported in Table S4 highlight that, given a specific temperature, the maximum stripping rate is reached earlier or achieves higher values when a higher A:D ratio is applied. Daily SR values peaked at nearly 4 g N-$NH_4^+ \cdot L^{-1} \cdot d^{-1}$ at 55°C with an A:D ratio of 4:1 being only slightly lower (3.85 g N-$NH_4^+ \cdot L^{-1} \cdot d^{-1}$) under milder process conditions, such as 35°C and A:D of 4:1.

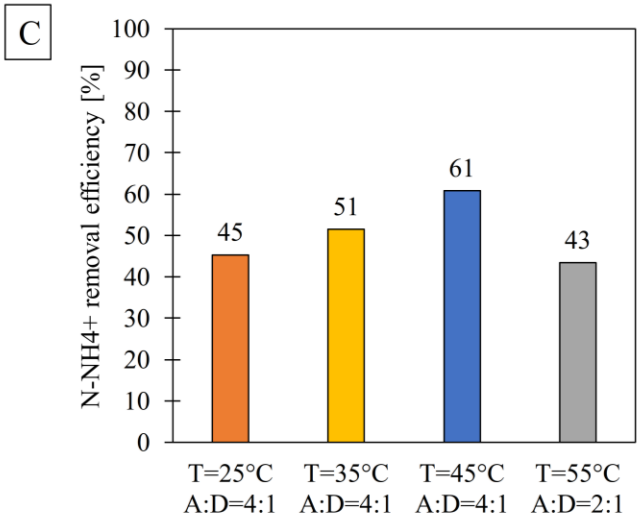
Figure 3 shows the evolution of pH and N-NH_4^+ concentration along the 48 hours of direct ammonia stripping in continuous experiments at different temperatures and A:D ratios. The results highlight how the most significant increase in pH (panel A) and the lowest residual N-NH_4^+ concentration (panel B) were achieved already within the first 8 hours of operation. Subsequently, stationary conditions appeared to be established in terms of both pH value and residual N-NH_4^+ concentration. Such stationary conditions were reached earlier, after 4 hours of operation, when both a temperature of 45 °C and an A:D ratio of 4:1 were applied.

At the same A:D ratio of 4:1, the N-NH_4^+ removal efficiency increased along with temperature, reaching values of 45, 51 and 61% when moving from 25 to 35 and 45 °C, respectively (Figure

3 panel C). Increasing the temperature by an additional 10 °C, thereby reaching 55 °C, while halving the A:D ratio from 4:1 to 2:1, did not improve the stripping process. As shown in panel B of Figure 3, after eight hours of stripping process at 45 °C and with an A:D ratio of 4:1, the residual $N\text{-}NH_4^+$ concentration was about 0.26 $g \cdot L^{-1}$ while, at 55 °C and with an A:D ratio of 2:1, the latter was about 0.55 $g \cdot L^{-1}$, i.e. approximately twice as much. In terms of stripping efficiency, the process operated with a temperature of 55 °C and an A:D ratio equal to 2:1 performed worse than the process carried out at a temperature of 25 °C and an A:D ratio equal to 4:1, reaching a value of only 43% $N\text{-}NH_4^+$ removal efficiency. The latter results show that, also under continuous stripping conditions, the effect caused by the increased driving force of the stripping flow is predominant over the effect of temperature. This agrees with Laureni et al. (2013), who obtained ammonia removal efficiencies higher than 90% by performing an air stripping process at 50 °C for 225 minutes with an A:D ratio equal to 10:1.

By comparing the results reported in panel E of Figure 2 with those presented in panel C of Figure 3, it is clearly visible that the values of ammonia removal efficiency, at the same temperature and applied A:D ratio, are higher in the batch operational mode. In particular, operating in batch, an ammonia removal efficiency of up to 87% (with a temperature of 55 °C and an A:D ratio of 2:1) was achieved, while operating continuously under the same conditions the highest ammonia removal efficiency was 43%. Such a difference can be explained by the higher initial $N\text{-}NH_4^+$ concentration in the digestate used in batch tests compared to the digestate used in the continuous tests, which affects the rate of diffusion of dissolved liquid ammonia into stripped ammonia gas.


A
pH
Time [h]
B
N-NH4+ [g·L-1]
Time [h]
T=25°C A:D=4:1
T=35°C A:D=4:1
T=45°C A:D=4:1
T=55°C A:D=2:1


C
N-NH4+ removal efficiency [%]
45
51
61
43
T=25°C A:D=4:1
T=35°C A:D=4:1
T=45°C A:D=4:1
T=55°C A:D=2:1

294

Figure 3. Evolution of pH (Panel A), residual N-NH_4^+ concentrations in the liquid fraction of digestate (Panel B) and N-NH_4^+ removal efficiencies (Panel C) along the 48-h continuous direct air stripping process of ammonia under different temperature and air-to-digestate conditions.

The SR values obtained during the continuous stripping process under the tested experimental conditions (see supplementary information Table S5) increased with the temperature at constant A:D ratio. At 25 °C and 35 °C with an A:D of 4:1, maximum hourly SR were 0.07 and 0.08 g N-$NH_4^+ \cdot L^{-1} \cdot h^{-1}$, respectively. At 45 °C, the SR reached 0.17 g N-$NH_4^+ \cdot L^{-1} \cdot h^{-1}$ within 2 hours and peaked at 0.24 g N-$NH_4^+ \cdot L^{-1} \cdot h^{-1}$ after 4 hours. At 55 °C and an A:D of 2:1, the SR peaked at 0.15 g N-$NH_4^+ \cdot L^{-1} \cdot h^{-1}$ before stabilizing at lower values. Thus, given a specific A:D ratio, the maximum stripping rate is reached earlier or achieves higher values when a higher temperature is applied. The daily SR varied with temperature and A:D ratio. At 25 °C and 35 °C with an A:D of 4:1, SR were 1.12 and 1.36 g N-$NH_4^+ \cdot L^{-1} \cdot d^{-1}$, respectively. Increasing the temperature to 45 °C at the same A:D ratio significantly raised SR to 2.69 g N-$NH_4^+ \cdot L^{-1} \cdot d^{-1}$. However, raising the temperature to 55°C while reducing the A:D ratio to 2:1 led to a decrease in SR to 1.44 g N-$NH_4^+ \cdot L^{-1} \cdot d^{-1}$. Also for the SR, lower values were observed for the stripping process operated in continuous vs. batch mode. The limitation was likely due to the lower initial concentration of dissolved ammonium nitrogen present in the continuous tests, which limited the driving force of the overall stripping process.

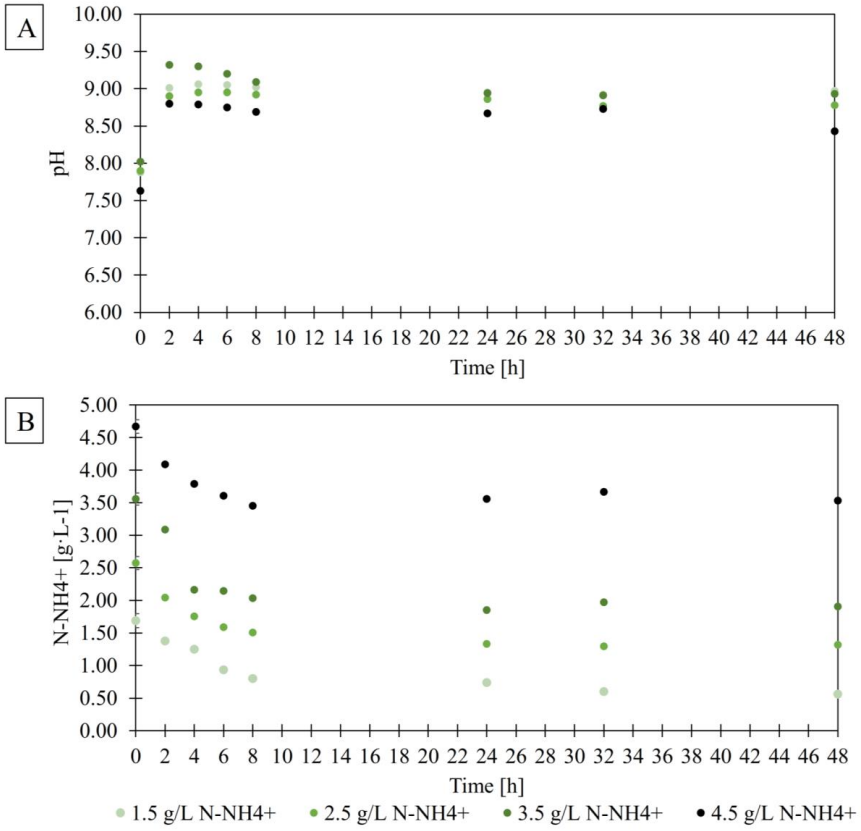
3.2 Effect of initial nitrogen content on continuous direct air stripping of ammonia

As previously discussed, under continuous conditions, the highest N-NH_4^+ removal efficiency was obtained at a temperature of 45 °C and an A:D ratio of 4:1. Therefore, additional continuous stripping tests were performed under these operating conditions using digestate amended with

320 NH_4Cl to evaluate the effects of different initial ammonium nitrogen contents of 1.5, 2.5, 3.5

321 and 4.5 $g \cdot L^{-1}$.

322



C

Time of operation [h]	Initial $N\text{-}NH_4^+$ concentration $[g \cdot L^{-1}]$			
	1.5	2.5	3.5	4.5
2	18	21	13	12
4	26	32	39	19
6	45	38	40	23
8	53	41	43	26
24	56	48	48	24
32	65	50	45	21
48	67	49	46	24

323

324 Figure 4. Evolution of pH (Panel A), residual $N\text{-}NH_4^+$ concentrations in the liquid fraction of digestate (Panel B)

325 and $N\text{-}NH_4^+$ removal efficiency [%] (Panel C) along the 48-h continuous direct air stripping process of ammonia

326 from digestate characterized by initial $N\text{-}NH_4^+$ concentrations of 1.5, 2.5, 3.5 and 4.5 $g \cdot L^{-1}$. In panel C, different

colors have been used to indicate how values appear over time: variable (red), almost stable (orange) and stable (green) values.

As already observed with the lower N concentrations, pH and residual ammonium nitrogen concentration values tend to stabilize after four to eight hours of operation (Figure 4 panels A, B). Figure 4 panel C shows the average efficiency of continuous stripping, calculated with reference to the initial $N\text{-}NH_4^+$ concentration to evaluate its effect on the process. The latter results show that, under each tested condition, the $N\text{-}NH_4^+$ removal efficiencies tend to stabilize and be constant over time. Such a stabilization in terms of $N\text{-}NH_4^+$ removal efficiency occurs faster at higher initial $N\text{-}NH_4^+$ loading. Nevertheless, the value of the final stripping efficiency decreases from 67 to 49, 46 and 24% as the initial ammonium nitrogen concentration increases from 1.5 to 2.5, 3.5 and 4.5 $g \cdot L^{-1}$, respectively. However, despite such differences in stripping efficiency, the total mass of nitrogen stripped is approximately the same under all tested conditions. In particular, a very similar decrease of ammonium-nitrogen concentration of 1.12 and 1.14 $g \cdot L^{-1}$ was obtained at an initial nitrogen concentration of both 1.5 and 4.5 $g \cdot L^{-1}$. Therefore, the initial concentration of ammonium nitrogen in the digestate does not seem to affect the total amount of N removed under continuous process conditions.

The SR values obtained during the continuous stripping process of ammonia from the digestate characterized by initial $N\text{-}NH_4^+$ concentrations of 1.5, 2.5, 3.5 and 4.5 $g \cdot L^{-1}$ under the different experimental conditions (see supplementary information Table S6) stabilized between 0.14 and 0.21 g $N\text{-}NH_4^+ \cdot L^{-1} \cdot h^{-1}$ regardless of the initial $N\text{-}NH_4^+$ concentration. In all cases, the stabilization of the SR value occurred after the first 8 hours, which corresponds to one HRT. Under continuous conditions, daily SR values increased with the initial $N\text{-}NH_4^+$ concentration of the digestate. At 45°C and an A:D ratio of 4:1, SR values were 3.74, 4.68, 4.96, and 4.80 g $N\text{-}NH_4^+ \cdot L^{-1} \cdot d^{-1}$ for initial concentrations of 1.5, 2.5, 3.5, and 4.5 $g \cdot L^{-1}$, respectively, while the

one obtained with the digestate characterized by an initial $N\text{-}NH_4^+$ concentration of 0.89 $g \cdot L^{-1}$ was 2.69 g $N\text{-}NH_4^+ \cdot L^{-1} \cdot d^{-1}$. These results confirm that higher initial concentrations of dissolved ammonium nitrogen in the digestate increase the driving force of the stripping process.

3.3 From waste-N to protein-N: evaluating the potential of direct air nitrogen stripping for microbial protein production from digestate and C-rich side streams

As previously discussed in Section 3.1, the highest $N\text{-}NH_4^+$ removal efficiency in continuous mode was obtained at a temperature of 45 °C and an A:D ratio of 4:1. The latter allows to recover up to 4.96 g $N\text{-}NH_4^+ \cdot L^{-1} \cdot d^{-1}$ from the digestate, which would be sufficient to support a microbial growth during aerobic fermentation of 50 g TSS $L^{-1} \cdot d^{-1}$ (Scotto di Uccio et al., 2023). Based on the obtained results, the direct air stripping of ammonia from digestate was combined with an aerobic fermentation process aimed at MP production. The final MP product obtained was compared with those obtained by operating aerobic fermentation with either an external mineral nitrogen source or through direct digestate supply, with and without heavy metals addition. The latter comparison was aimed at evaluating how N recovery from digestate through stripping process can minimize the transfer and accumulation of heavy metals in the final MP product, thereby enhancing its quality.

3.3.1 Effect of different N sources on the aerobic MP production process and protein content

Figure 5 shows the daily biomass concentrations measured during the tests carried out with the different N sources. In the control, when N was provided by NH_4Cl, the biomass concentration rapidly increased during the first day of operation until stabilising at a value close to 10 g $TSS \cdot L^{-1}$. The latter was similar to the value of 10.55 g $TSS \cdot L^{-1}$ obtained from the cultivation of the same inoculum under the conditions previously investigated by the authors (Scotto di Uccio et al., 2023).

When the N source was the N stripped from digestate and then solubilised in the aerobic reactor, a lower biomass concentration was observed and a TSS concentration of 4.78 g $TSS \cdot L^{-1}$ (Figure 5) was achieved. The highest biomass concentration here obtained from the combined stripping-aerobic process was similar to the average concentration equal to 4.05 g $TSS \cdot L^{-1}$ obtained by Matassa et al. (2022), who used a similar experimental configuration but performed the stripping process at 30 °C and under batch conditions. Furthermore, when N was provided through the direct mixing of digestate in the aerobic reactor, the biomass concentration rapidly increased until reaching a value of approximately 12 g $TSS \cdot L^{-1}$ (Figure 5).

The latter observation suggests that N provided through NH_4Cl and digestate favoured the biomass growth. Indeed, NH_4Cl provided a readily available and efficiently metabolizable form of N, while the digestate may have provided not only N but also a wide range of other nutrients to the microorganisms. The biomass concentrations obtained in the present study when using digestate as N-source directly supplied in the reactor are higher than those obtained in similar studies. Bertasini et al. (2022) reached concentrations up to 1.95 g $TSS \cdot L^{-1}$ by performing aerobic MP production process of *Saccharomyces cerevisiae* using a candies production effluent as C source (70 g $sCOD \cdot L^{-1}$) and the liquid fraction of agricultural digestate as N source (0.44 g Total Kjeldal Nitrogen$\cdot L^{-1}$). Qin et al. (2019) obtained biomass concentrations up to 1.15 g $TSS \cdot L^{-1}$ while treating the liquid fraction of digestate from the yeast production industry with a pure culture of the *Yarrowia Lipolytica* yeast. The biomass concentration increased up 1.53 g $TSS \cdot L^{-1}$ by co-culturing *Yarrowia Lipolytica* with the *Chlorella Vulgaris* microalga. Conversely, when N was in this study provided from the stripping and solubilization processes, the low availability of N in the initial exponential growth phase (the phase in which the highest N consumption occurs, see panel 6 of Figure 6) likely led to a limitation of the biomass growth.

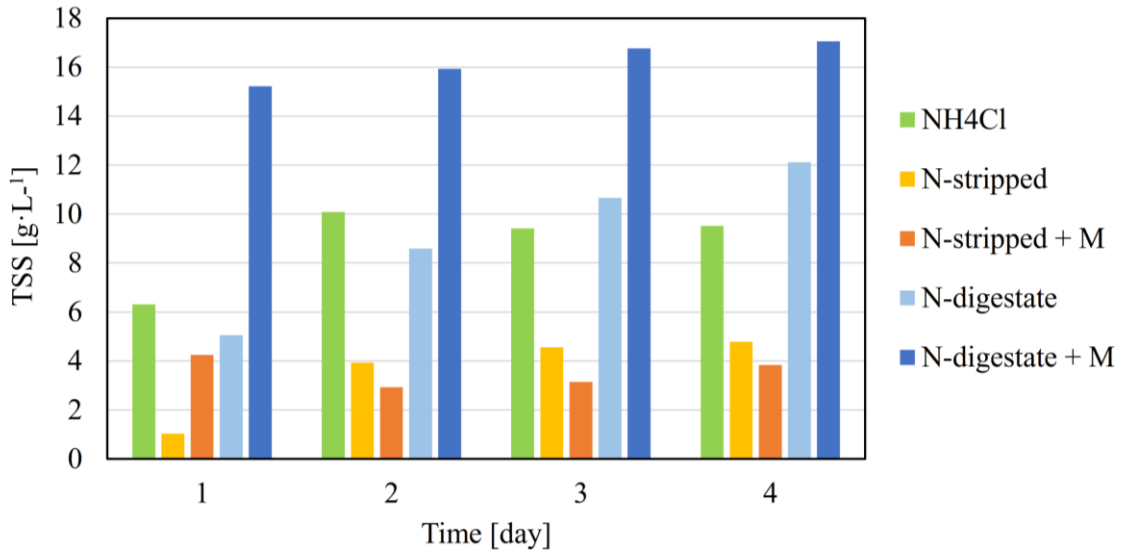


Figure 5. Performance of the aerobic microbial protein production process in terms of daily biomass concentration under the different experimental conditions. NH_4Cl: N supplied as a chemical, N-stripped: N supplied through the stripping process, N-stripped + M: N supplied through the stripping process using digestate contaminated with heavy metals, N-digestate: N supplied through digestate in the aerobic reactor, N-digestate + M: N supplied through digestate contaminated with heavy metals directly fed in the aerobic reactor.

The daily DOC and N-NH_4^+ concentrations detected in the reactor under the different experimental conditions are reported in Figure 6. Except in the case where N was provided through the stripping process from the digestate contaminated with heavy metals, the DOC consumption mainly occurred during the first day of operation, while its concentration remained relatively constant afterwards. The difference in N-NH_4^+ concentration visible in Figure 6 (panel B) at the time zero is related to the different N-supply strategies, as the test using stripped N was characterized by an initial negligible nitrogen concentration. In all cases, in the first day of reactor operation, almost all available N-NH_4^+ was consumed, while in the subsequent days either a decrease in consumption or even an accumulation of N-NH_4^+ was observed. These observations are in line with the biomass growth trends previously discussed (Figure 5).

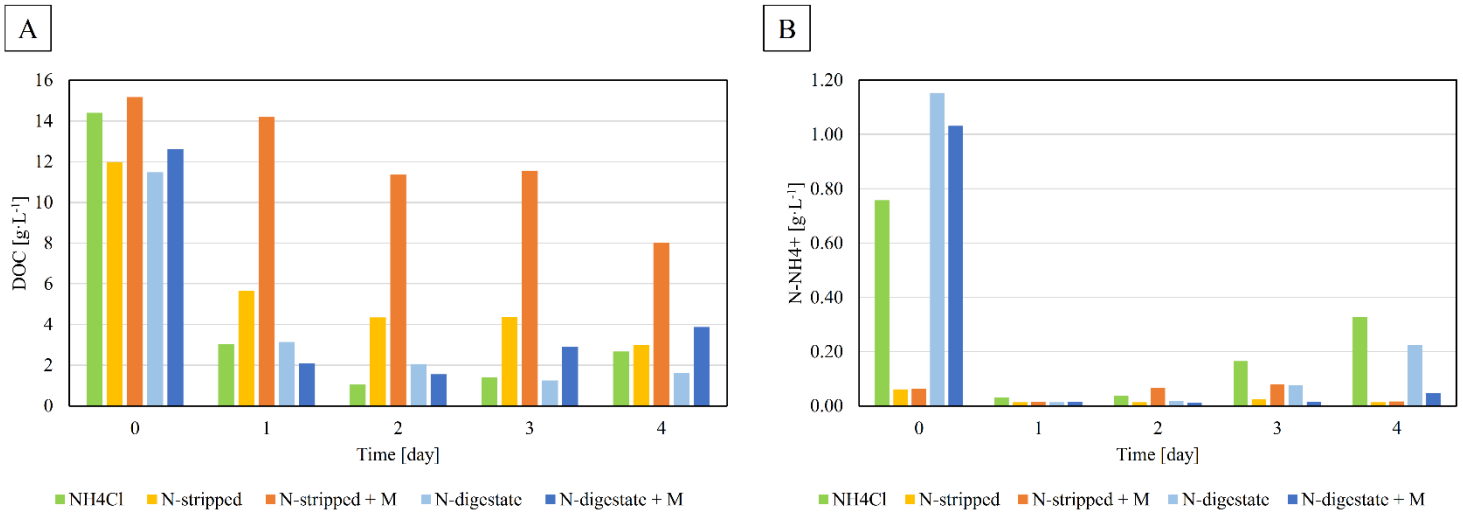


Figure 6. Performance of the aerobic microbial protein production process in terms of daily DOC and N-NH_4^+ concentrations under the different experimental conditions. NH_4Cl: N supplied as a chemical, N-stripped: N supplied through the stripping process, N-stripped + M: N supplied through the stripping process using digestate contaminated with heavy metals, N-digestate: N supplied through digestate in the aerobic reactor, N-digestate + M: N supplied through digestate contaminated with heavy metals directly fed in the aerobic reactor.

Figure 7 shows the protein content of the biomass produced under different N sources. In the control, when N was provided by NH_4Cl, a high protein content of 67% was detected. As NH_4Cl provides an easily assimilable form of nitrogen, it likely allowed an efficient protein synthesis leading to a biomass characterized by a protein content similar to those reported in other studies on MP production through aerobic production process from CWP (Matassa et al., 2022; Scotto di Uccio et al., 2023). When the N was stripped from digestate and then solubilised in the aerobic reactor, the protein content decreased to 17%, suggesting that the stripping process led to the microbial growth under N-limiting conditions. The issue primarily revolves around the low initial availability of N for microorganisms when provided with stripped N. This limitation, which significantly restricted both the final biomass concentration and the protein content of the resulting MP, might be overcome by inoculating the reactor only once a sufficient initial N concentration is established, or by directly adding mineral N in the growth substrate to support

the process in the initial phase. The latter was already demonstrated by Matassa et al. (2022), who produced biomass with a protein content as high as 75% by performing a process similar to that adopted in the present study combining direct nitrogen stripping and aerobic fermentation of cheese whey amended with NH_4Cl. Directly feeding the digestate into the reactor led to a biomass characterized by a 44% protein content. In this case, the raw digestate likely provided a more complete nutrient supply, including also organic nitrogen under the form of amino acids and peptides, better supporting the microbial growth and protein production compared to stripped N.

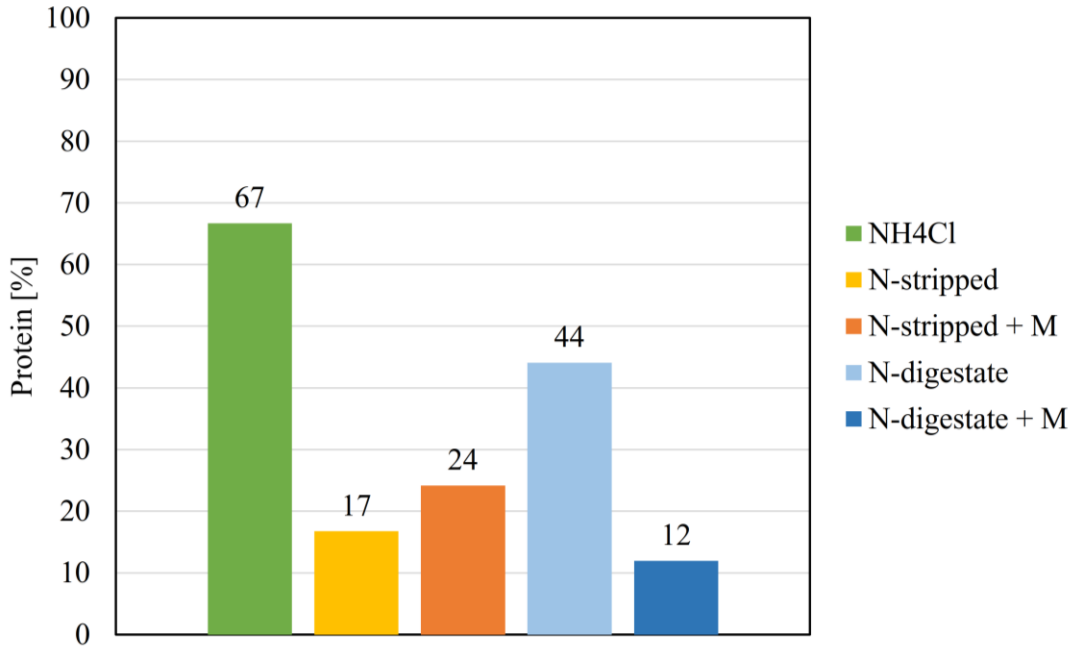


Figure 7. Protein content of the biomass produced under the different experimental conditions. NH_4Cl: N supplied as a chemical, N-stripped: N supplied through the stripping process, N-stripped + M: N supplied through the stripping process using digestate contaminated with heavy metals, N-digestate: N supplied through digestate in the aerobic reactor, N-digestate + M: N supplied through digestate contaminated with heavy metals directly fed in the aerobic reactor.

448 *3.3.2 Effect of heavy metals in the digestate on the aerobic MP production process*

449 As clearly visible in Figure 5, the presence of heavy metals contamination in the digestate used
450 in the present study seems not to have affected the biomass growth when N was supplied
451 through the stripping process. The TSS value varied from a minimum of 2.92 to a maximum of
452 4.24 g $TSS \cdot L^{-1}$, thus similar to those reported in the absence of added metals in the digestate.
453 Conversely, the presence of heavy metals in the digestate, used as direct N source in the aerobic
454 MP production reactor, resulted in an increase of the biomass concentration up to a value of
455 17.06 g $TSS \cdot L^{-1}$. The increase in biomass growth in kefir-based fermentation process when the
456 digestate is contaminated with heavy metals may be linked to specific microbial responses
457 (Mrvčić et al., 2013). Kefir microorganisms, including those belonging to the *Lactobacillus* and
458 *Saccharomyces* genera, showed mechanisms helping them to resist and tolerate metal stress,
459 such as producing metal-chelating substances or altering their metabolism to cope with
460 oxidative stress induced by metals.

461 However, when the digestate was contaminated with heavy metals, the protein content
462 decreased from 44 (in the absence of heavy metals) to 12%. The latter suggests that, while
463 heavy metals can induce some beneficial stress responses for biomass growth, as already
464 discussed in Section 3.3.1, an excessive presence of metals might inhibit protein synthesis,
465 leading to a lower overall protein content. In particular, it has been shown that the high presence
466 of metals such as Cu assimilated by biomass (as occurred in that obtained in this condition, see
467 Section 3.3.3) leads to stress response that promotes the degradation of proteins and the repair
468 of DNA to mitigate the toxic effects (Mrvčić et al., 2013).

469

470 *3.3.3 Effect of heavy metals in the digestate on the quality of microbial protein*

471 Table 1 reports the heavy metals content measured in the biomass produced by using as N
472 source the digestate contaminated with heavy metals, and the respective regulatory limits

established at the European level. When the heavy metals-contaminated digestate was directly supplemented to the aerobic reactor, the produced biomass presented a higher heavy metal concentration than the biomass produced by feeding N through the stripping process.

The extent of the increase in heavy metal content in biomass varied significantly depending on the metal. While for Cd no accumulation was detected under both N-feeding strategies (i.e., N-stripping and N-digestate supply), for Ni, Pb and Zn a low to moderate metal accumulation was measured, achieving a 1.4, 2.0 and 4.6 higher concentration, respectively, in the biomass produced through N-digestate supply as compared to that produced through N-stripping. A very high increase of almost 66-fold was finally observed for Cu, the concentration of which varied from 5 to 329 $mg_{METAL} \cdot kg_{DRY\ BIOMASS}^{-1}$ in the biomass produced through N-stripping and N-digestate feeding, respectively). These results suggest that when metal-contaminated digestate is used as N source directly in the aerobic process, heavy metals are readily available and assimilable by biomass. On the contrary, removing and recovering the nitrogen through stripping allows to physically disconnect the contaminated waste matrix from the aerobic reactor, thereby avoiding the transfer and accumulation of contaminants such as heavy metals in the final MP product.

The MP produced in the bioreactor coupled with the stripping process from contaminated digestate meets all the standards to be valorised as organic fertiliser, while the MP produced using the N from the contaminated digestate slightly exceeded the standards for fertilisers use only for Cu (329 against 300 $mg_{METAL} \cdot kg_{DRY\ BIOMASS}^{-1}$ allowed). Furthermore, the biomass produced through both N-stripping and N-digestate addition exceeded the Pb limit of 10 $mg_{METAL} \cdot kg_{DRY\ BIOMASS}^{-1}$ in the case of use as feed, achieving values of 36 and 71 $mg_{METAL} \cdot kg_{DRY\ BIOMASS}^{-1}$, respectively. In the same way, being the latter Pb contents also higher than the limit of 3 $mg_{METAL} \cdot kg_{DRY\ BIOMASS}^{-1}$ allowed for food products, the biomass would not be suitable for human nutrition. Ni concentration values were equal to 11 and 15 $mg_{METAL} \cdot kg_{DRY}$

498 $_{BIOMASS}^{-1}$ in the present study under both experimental configurations, being comparable with
499 the value of 8 $mg_{METAL} \cdot kg_{DRY\ BIOMASS}^{-1}$ obtained by Van Peteghem et al. (2023), who cultivated
500 a mixed culture of *Metschnikowia pulcherrima* and *Corynebacterium glutamicum* using
501 recovered N from pig manure and municipal organic waste.

502 Table 1. Heavy metals content in the biomass produced via aerobic process by providing N by means of
503 contaminated digestate through stripping or by direct feeding. Maximum heavy metals content allowed for
504 different purposes according to the European regulatory limits.

Metal	Metal content in MP from N-stripped	Metal content in MP from N-digestate	Maximum permitted content in food[a]	Maximum permitted content in organic fertilisers[b]	Maximum permitted content in feed[c]
	$mg_{METAL} \cdot kg_{DRY\ BIOMASS}^{-1}$	$mg_{METAL} \cdot kg_{DRY\ BIOMASS}^{-1}$	$mg_{METAL} \cdot kg_{DRY\ BIOMASS}^{-1}$	$mg_{METAL} \cdot kg_{DRY\ BIOMASS}^{-1}$	$mg_{METAL} \cdot kg_{DRY\ BIOMASS}^{-1}$
Ni	11	15	-	50	-
Pb	36	71	3	120	10
Cu	5	329	-	300	-
Zn	28	128	-	800	-
Cd	0	0	3	1.5	2

505 [a] (European Parliament and the Council of the EU, 2023)
506 [b] (European Parliament and the Council of the EU, 2019)
507 [c] (European Parliament and the Council of the EU, 2006)
508

509 ## 4. Conclusions

510 The direct air stripping process of ammonia nitrogen from digestate showed promising
511 performances at temperatures equal to or lower than 55 °C and without chemical pH
512 adjustment, reaching efficiencies of up to 100% under batch conditions (55 °C, A:D=4:1) and
513 up to 61% under continuous conditions (45 °C, A:D=4:1). The initial $N\text{-}NH_4^+$ concentration
514 did not affect the amount of nitrogen recovered, as the mass of nitrogen stripped remained
515 relatively constant, although the final stripping efficiency decreased from 67 to 24% along
516 with the increasing initial nitrogen loading. The different N supply modes influenced the MP
517 production process. When $N\text{-}NH_4^+$ was already present in the culture medium, either provided

by NH_4Cl or digestate addition, biomass concentrations up to 10.08 and 17.06 g $TSS \cdot L^{-1}$ were reached. Conversely, when N was provided by stripping and solubilising ammonia from digestate, a lower biomass concentration of 4.78 g $TSS \cdot L^{-1}$ was observed, likely due to the low availability of N in the early exponential growth phase. The presence of heavy metals in the digestate used as N source via stripping did not affect the biomass growth due to the gas phase transition of ammonia that avoided their transfer and accumulation in MP, while it led to a metal increase in biomass when the digestate was fed directly into the aerobic reactor. The latter also affected the quality of the final MP product. As a matter of fact, the biomass obtained from the aerobic MP production process fed with heavy metals-contaminated digestate presented a lower protein content and higher contents of each heavy metal tested with respect to the same biomass grown by using stripped nitrogen, making it unsuitable for human food while not completely suitable for animal feed and fertilizers production.

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Supplementary material

Supplementary data of this work can be found in online version of the paper.

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CRediT author contribution statement

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Declaration of Interest Statement

☒ 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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