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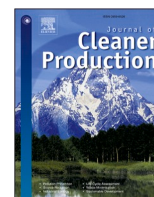

# Cultivation of microalgae at high-density with pretreated liquid digestate as a nitrogen source: Fate of nitrogen and improvements on growth limitations

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ABSTRACT

A liquid digestate rich in ammonium nitrogen (8.3 g $L^{-1}$) was collected from an agricultural biogas plant and supplied to microalgae cultures as their only nitrogen source. *Chlorella vulgaris* was cultivated for up to 21 days, both under controlled conditions in laboratory-scale glass-column photobioreactors as well as outdoors in an open pilot-scale thin-layer photobioreactor. By systematically addressing issues associated with the use of liquid digestate (i.e., turbidity, nutrient imbalance, ammonium toxicity, and acidification), microalgae were robustly cultivated at a high density and cultures achieved a net biomass dry weight of between 10 and 14 g $L^{-1}$, and a productivity of up to 0.93 g $L^{-1}$ $d^{-1}$ (93% of maximum expectation). Cultivation in the thin-layer photobioreactor achieved areal productivities between 7 and 10 g $m^{-2}$ $d^{-1}$. Water acidification due to the uptake of ammonium by microalgae was prevented by a controlled addition of NaOH. A detailed mass balance showed that, despite high removal efficiencies (approximately 3% of the supplied nitrogen remained in the medium), microalgae assimilated only 40–60% of the supplied nitrogen and, consequently, a large amount of nitrogen was lost to the atmosphere.

## 1. Introduction

In recent decades, European renewable energy policies have promoted the valorization of organic matter such as manure, green waste and food waste in biogas plants via anaerobic digestion (Scarlat et al., 2018). Such a use of organic matter may help to establish a sustainable source of bioenergy and, thus, lead to a reduction in greenhouse gas emissions (Bartoli et al., 2019; Paolini et al., 2018). The process, however, produces a large amount of digestate as a byproduct, which must be appropriately disposed of to prevent harm to the environment. When separated, the liquid fraction of the digestate is the largest output and contains a high load of carbon and nutrients such as nitrogen and phosphorus (Zuliani et al., 2016). It is usually disposed of by land-spreading it in the vicinity of the biogas plant as a method of fertilization. This practice is legally regulated in the European Union to reduce the environmental impact of ammonia ($NH_3$) emissions and eutrophication (Nkoa, 2014; Paolini et al., 2018; Scarlat et al., 2018), and, thus, the amount of liquid digestate that can be disposed of is limited. Therefore, finding alternative applications for liquid digestate is crucial if bottlenecks arising from insufficient land availability in the vicinity of biogas plants are to be avoided (Fuchs and Drosg, 2013).

Cultivating microalgae has been suggested as an alternative, which simultaneously treats liquid digestate and produces algal biomass (Monlau et al., 2015). The cultivation of microalgae does not require arable land, as it is carried out in ponds or photobioreactors (PBRs), which facilitates the management of nutrients and wastewater. Additionally, cultivation in close proximity to a biogas plant has the added benefit of access to $CO_2$-rich off-gas and heat produced during electricity generation. By recycling nutrients from liquid digestate and off-gas, the operational costs of microalgae cultivation could be decreased. As a

*Abbreviations:* PLD, pretreated liquid digestate; PBR, photobioreactor; DOC, dissolved organic carbon; PAR, photosynthetically active radiation; SEM, standard error of the mean.

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consequence, microalgae-derived products, such as fuel, feed, nutrition or cosmetics could be promoted (Milledge, 2010; Raheem et al., 2018; Slade and Bauen, 2013). A number of studies have used liquid digestate to cultivate microalgae and various cultivation methods (e.g. cylindrical glass PBR, Erlenmeyer flasks, plastic bags), experimental conditions, digestate types and pretreatment methods have been employed (Kisielewska et al., 2021; Koutra et al., 2021; Ledda et al., 2016). Most of these studies have reported recurrent issues, which are inherent to the cultivation of microalgae with a liquid digestate:

i) Liquid digestates are usually opaque and turbid, which decreases light transmission and contaminates the algal biomass with solid particles (Kwon et al., 2019; Singh et al., 2011).
ii) Liquid digestates often have an unbalanced composition of nutrients, which may limit algal growth (Bjornsson et al., 2013; Park et al., 2010).
iii) Ammonium ($NH_4^+$), usually the main source of nitrogen, can inhibit growth when supplied to microalgae due to its equilibrium with $NH_3$. A decrease in growth was reported for *Chlorella* species when free $NH_3$ concentration increased above 20–37 mg $L^{-1}$ (Jiang et al., 2021; Tan et al., 2016).
iv) The uptake of $NH_4^+$ by microalgae decreases the pH value due to an equimolar release of $H^+$ (Eustance et al., 2013; Fuggi et al., 1981), which can inhibit growth (Rachlin and Grosso, 1991).

Nevertheless, microalgae have successfully been cultivated with liquid digestate when strategies were implemented to mitigate these issues (pretreatment of the digestate, tolerant algal strains, etc.), and algal biomass dry weights from 2.0 to 4.8 g $L^{-1}$ were achieved (Cheng et al., 2015; Zhou et al., 2012, 2019). However, to our knowledge, the aforementioned issues have not been addressed systematically together, and while previous studies have reported promising results, the cultivation of microalgae has never been carried out with liquid digestate in PBRs specifically designed for high density (biomass dry weight ≥10 g $L^{-1}$) (Doušková et al., 2010; Egloff et al., 2018). The cultivation of microalgae at high density has advantages compared to low-density cultivation, such as cheaper dewatering costs, better control of growth conditions, and lower risk of biological contamination (Doucha and Lívanský, 2014). Therefore, PBRs that achieve a high biomass density are promising candidates for use in a biogas plant.

The aim of this study was to achieve stable high-density microalgae cultures with liquid digestate in glass-column (indoor) and thin-layer (outdoor) PBRs by using a systematic approach to address the issues mentioned above:

i) The liquid digestate was pretreated by ultrafiltration to remove particles.
ii) This pretreated liquid digestate (PLD) was only used as a nitrogen source and supplemented with missing nutrients.
iii) The addition of PLD was staggered throughout the day to keep the $NH_4^+$ concentration in the culture low.
iv) pH was controlled by adding NaOH continuously or in batch to compensate for $H^+$ release from the $NH_4^+$ uptake.

Further, growth performance and mass balance of nitrogen were calculated to assess if the cultivation of microalgae results in lower nitrogen loss than landspreading of liquid digestate, where losses are estimated between 20 and 60% due to leaching, runoff, gas emission or volatilization (Duan et al., 2016; Huang et al., 2017).

## 2. Material and methods

The material and methods section consists of four parts. Section 2.1 covers the aspects related to the cultivation of microalgae with mineral medium. Section 2.2 describes the methods used to determine the characteristics of the PLD and the amount of nitrogen supplied to microalgae during the experiments. Sections 2.3 and 2.4 describe the setup and experimental plan used to cultivate microalgae, with PLD as a nitrogen source, at laboratory and pilot scales, respectively.

### 2.1. Microalgae strain and mineral medium

*Chlorella vulgaris* (strain SAG 211-11b) was acquired from the culture collection of algae at the Göttingen University (SAG) in Germany. The strain was cultivated in liquid mineral medium, which was prepared in ultrapure water with the following concentrations (mg $L^{-1}$): 1100 CO$(NH_2)_2$, 237 $KH_2PO_4$, 204 $MgSO_4 \cdot 7H_2O$, 40 EDTA-FeNa, 173.8 $CaCl_2 \cdot 6H_2O$, 0.83 $H_3BO_3$, 0.95 $CuSO_4 \cdot 5H2O$, 3.3 $MnCl_2 \cdot 4H2O$, 0.6

**Table 1**
Characteristics of PLD. Analyses were carried out in triplicate and average values are shown with the standard error of the mean (SEM).

| | | | |
|---|---|---|---|
| pH | 7.85 ± 0.02 | Conductivity (mS $cm^{-1}$) | 94.15 ± 1.05 |
| Salinity (‰) | 67.1 ± 0.9 | Dry weight (g $L^{-1}$) | 72.95 ± 0.55 |
| **mg $L^{-1}$** | | **mg $L^{-1}$** | |
| DOC | 5650 ± 50 | Ca | 392.6 ± 3.5 |
| Phenols | 305.0 ± 8.7 | Si | 82.00 ± 1.26 |
| $NH_4^+$-N | 8285 ± 30 | Fe | 5.20 ± 0.04 |
| $NO_3^-$-N | 78.48 ± 1.86 | Mn | 5.69 ± 0.08 |
| $NO_2^-$-N | 2.98 ± 0.40 | B | 1.96 ± 0.06 |
| $PO_4^{3-}$-P | 7.40 ± 1.97 | Sr | 1.50 ± 0.00 |
| K | 14,485 ± 27 | Se | < 1.4* |
| Na | 5727 ± 17 | Al | < 0.7* |
| Mg | 705.5 ± 2.8 | | |
| **μg $L^{-1}$** | | **μg $L^{-1}$** | |
| Zn | 333.6 ± 13.1 | Pb | 14.47 ± 0.51 |
| V | 308.8 ± 14.9 | Sn | 4.11 ± 0.34 |
| Ni | 297.5 ± 13.9 | Cd | < 24.8* |
| Li | 162.0 ± 5.1 | Cu | < 23.2* |
| Co | 111.2 ± 1.1 | Be | < 16.9* |
| Ba | 107.4 ± 3.3 | Hg | < 13.5* |
| As | 98.81 ± 10.97 | Sb | < 4.3* |
| Cr | 82.44 ± 3.39 | Ag | < 1.9* |
| Mo | 27.56 ± 1.76 | Tl | < 1.2* |

* Below the limit of quantification.

$CoSO_4 \cdot 7H2O$, 2.7 $ZnSO_4 \cdot 7H2O$, 0.17 (NH4)6Mo7O24·4H2O, 0.014 $NH_4VO_3$ (Doušková et al., 2010). A stock solution for each nutrient was also prepared for direct addition to the microalgae cultures. The mineral medium recipe is based on the elemental composition of the microalgae and can sustain an algal biomass production of 6 g $L^{-1}$. This has been empirically confirmed by years of experimentation with *C. vulgaris*.

### *2.2. Characteristics of the pretreated liquid digestate*

PLD was obtained from the *Swiss Farmer Power Inwil* biogas plant located in Inwil, Switzerland. The biogas plant mainly processes waste from agriculture, gastronomy, and industry (e.g., manure, green waste, spoiled food, coffee grounds). The pretreatment of the digestate used in this study was carried out industrially with ultrafiltration and reverse osmosis to remove solid particles and to concentrate nutrients, respectively. While this removed solid particles, the dry weight remained high due to the concentration of salts and dissolved organic matter. At the biogas plant, PLD is stored in an underground tank, from where it was sampled and stored at 4 °C. Due to the prolonged storage under non-sterile conditions at the plant, PLD was not considered sterile thereafter.

The characteristics of PLD, such as physical properties and concentrations of nutrients and elements, are shown in Table 1. Electrodes were used to determine pH value (InLab semi-micro, Mettler Toledo, Switzerland), salinity and conductivity (InLab 738-ISM, Mettler Toledo). Dry weight was determined with a moisture analyzer (HC 103, Mettler Toledo). Photometric tests were used to determine the concentration of dissolved organic carbon (DOC) (LCK 385, Hach, Switzerland), $NH_4^+$-N (LCK 304), $NO_3^-$-N (LCK 339), $NO_2^-$-N (LCK 342), $PO_4^{3-}$-P (LCK 349), and total phenol content (LCK 345). An elemental analysis was carried out as follows: 2 mL from the PLD were mixed in the following order with 2 mL HCl (30%), 4 mL $HNO_3$ (65%) and 2 mL $H_2O_2$. A blank sample solution with the same acid content was prepared in the same way as the PLD. The obtained solutions were diluted in three steps with ultrapure water and an acid-diluted solution (1% $HNO_3$), respectively, resulting in a total dilution factor between 2400 and 9000. Calibration solutions were prepared by using multi-element commercial standards (Bernd Kraft, Germany) in the range of 5–500 μg $L^{-1}$ for K and P and 1–100 μg $L^{-1}$ for a further 28 elements. Samples were measured by Inductively Coupled Plasma Optical Mass Spectrometry (ICP-MS, Agilent, 7000× , USA). Quantification was done by external calibration of each element.

Nutrient concentrations in the PLD were compared with the mineral medium recipe, and both were adjusted to a nitrogen concentration of 85.5 mg $L^{-1}$, which sustains the production of 1 g $L^{-1}$ of algal biomass (Table 2). Higher concentrations in the PLD or similar concentrations in both media were found for five nutrients (N, Mg, V, Ca and B), while concentrations were found to be lower in the PLD for seven others ($PO_4^{3-}$, Fe, Mn, Zn, Cu, Co, Mo). Consequently, the PLD was only used as a nitrogen source and other nutrients were supplemented. Cultivations were carried out with a daily nitrogen supply ($NH_4^+$-N or urea-N) of 85.5 mg per liter of culture volume, allowing for a biomass productivity of 1 g $L^{-1}$ $d^{-1}$.

**Table 2**
Nutrient concentrations of PLD and mineral medium. Concentrations are standardized to 85.5 mg $L^{-1}$ of $NH_4^+$-N and urea-N for PLD and mineral medium, respectively.

| | PLD | Mineral medium |
|---|---|---|
| **mg $L^{-1}$** | | |
| $NH_4^+$-N | 85.51 | 0 |
| Urea-N | 0 | 85.51 |
| $PO_4^{3-}$-P | 0.08 | 8.99 |
| Ca | 4.05 | 5.30 |
| Mg | 7.28 | 3.35 |
| Fe | 0.05 | 1.01 |
| DOC | 58.31 | 0 |
| **μg $L^{-1}$** | | |
| Mn | 58.77 | 152.68 |
| Zn | 3.44 | 102.31 |
| Cu | < 0.2[a] | 40.30 |
| B | 20.29 | 24.19 |
| Co | 1.15 | 20.97 |
| Mo | 0.28 | 15.40 |
| V | 3.19 | 1.02 |

[a] Below the limit of quantification.

### *2.3. Design of glass-column photobioreactors and experimental plan*

The setup of glass-column PBRs is described in a previous study (Pulgarin et al., 2020). Briefly, it consists of a set of glass-columns immersed into an aquarium, which is kept at a steady temperature and illuminated with a vertical panel of fluorescent tubes. Mass flow controllers (Vögtlin, Switzerland) were used to bubble a mixture of air and $CO_2$ into the PBRs to supply carbon and help the microalgae remain in suspension. A peristaltic pump (REGLO digital MS-4/12, Ismatec, Germany) supplied PLD, PLD mixed with NaOH (equimolar to $NH_4^+$, PLD-NaOH), or urea to the microalgae cultures, respectively. Care was taken to supply identical amounts of nitrogen to all cultures. Ultrapure water was used for all cultivations.

Cultivations were carried out as follows: 100 mL of microalgae culture (dry weight: 4.1–4.9 g $L^{-1}$, pH 5.7–6.6) were added to each PBR and cultivated at 25 ± 0.5 °C and constant aeration (0.25 $L_N$ $min^{-1}$ 2% (v/v) $CO_2$). A daytime of 12 h per day was set with an incident photosynthetically active radiation (PAR) of 800 μmol $m^{-2}$ $s^{-1}$ on the vertical surface of the aquarium. Nutrients were supplied to the cultures following a fed-batch strategy. One twelfth of a nitrogen solution prepared with the PLD or urea was supplied to the microalgae cultures every hour during the daytime for a cumulative nitrogen amount of 8.55 mg (i.e., 85.5 mg per liter of culture) per day, which sustained a productivity of 1 g $L^{-1}$ $d^{-1}$. Other nutrients were supplied directly to the cultures every two to four days in amounts sufficient to prevent nutrient depletion. Evaporation loss was approximately 9 mL $d^{-1}$ and was compensated for by the supplied nitrogen solution.

Three different nitrogen solutions (9 mL each) were prepared daily as follows: 1) urea (8.55 mg urea-N) dissolved in ultrapure water, 2) PLD-NaOH, i.e. PLD (8.55 mg $NH_4^+$-N) diluted in ultrapure water and equimolarly mixed with 1 M NaOH (0.61 mmol), 3) PLD (8.55 mg $NH_4^+$-N) diluted in ultrapure water. Cultivations were carried out for up to 21 days in duplicate. pH of the cultures was controlled with an addition of 300 μL 1 M NaOH every day as long as the pH value remained below 6.5. This was not required for the microalgae cultures that were supplied with the PLD-NaOH solution.

Cultures were sampled periodically. To determine dry weight, a sample was washed two times with ultrapure water (centrifugation at 4000 rcf for 3 min, Z323K, Hermle, Germany) and re-suspended in its initial volume prior to analysis. To determine cell density, a sample of 10 μL was observed under a light microscope (400× magnification, Axiolab, Zeiss, Switzerland) and cells were counted in a counting chamber (Neubauer improved, Marienfeld, Germany). Photometric tests were used to determine the concentration of $NH_4^+$-N and total nitrogen (TN) (LCK 138) in the supernatant. The CHN content of dried algal biomass samples (100 μg) was determined by thermal conductivity and infrared spectroscopy (TruSpec Micro CHN, Leco Instruments Ltd., UK).

### *2.4. Design of the thin-layer photobioreactor and experimental plan*

Two cultivations of microalgae at pilot-scale were carried out in an open thin-layer PBR located in an unheated foil greenhouse. First in August/September 2017 and again in June/July 2020. The PBR consisted of an inclined (1.7%) culture surface (18 $m^2$) made of glass sheets in a steel frame (Supplementary Fig. S1). The microalgae were pumped from a tank up to the top of the surface, from where they flowed back to the tank as an 8 mm thick suspension layer. Sensors were used to monitor the conditions of cultivation such as PAR (SKL2620, Skye

Instruments Ltd., UK), partial pressure of $CO_2$ (InPro 5000i, Mettler Toledo), pH value and temperature (InPro 3253i, Mettler Toledo). The thin-layer PBR has been described in detail elsewhere (Egloff et al., 2018; Tejido-Nuñez et al., 2020). This thin-layer PBR differs in several aspects from the glass-column PBRs used in the first part of the study: it has an open design, its temperature fluctuates daily, and the illumination is not artificial. Key features that are comparable are evaporation (which allows for the continued supply of PLD to the cultures) and the high biomass density that can be reached (Doucha and Lívanský, 2006).

The microalgae were cultivated in 200 L of freshwater for 16 to 19 days and supplied with PLD as a nitrogen source. The addition of nutrients followed a fed-batch strategy, like the one used for glass-column PBRs. PLD was continuously supplied for 10 h each day during the daytime via a peristaltic pump to ensure that enough $NH_4^+$-N (17.1 g $d^{-1}$, 85.5 mg per liter of culture) was provided to sustain a productivity of 1 g $L^{-1}$ $d^{-1}$. All other nutrients were added directly to the culture every three to six days to prevent nutrient depletion. Pure $CO_2$ was injected in the microalgae culture during the daytime to maintain a partial pressure of 10 mbar. The $CO_2$ injection was switched off during the night. The pH value of the culture was kept above 7 with the addition of NaOH pellets. Samples were collected periodically to determine the dry weight of the microalgae culture, CHN content of the algal biomass (in duplicate), and $NH_4^+$-N concentration in the supernatant (as well as TN for the cultivation in 2020) following the methods described in sections 2.2 and 2.3. The dominance of *C. vulgaris* in the microalgae culture was confirmed by visual observation under the light microscope.

## 3. Results and discussion

The results and discussion section is divided into three parts. While sections 3.1 and 3.2 discuss the results of the cultivation of microalgae with PLD as a nitrogen source in glass-column and thin-layer PBRs, respectively, section 3.3 discusses the results of the nitrogen mass balance for the different cultivation runs carried out in this study.

### 3.1. Cultivation of microalgae in glass-column photobioreactors

The cultivation of microalgae in glass-column PBRs at high-density with PLD-NaOH as a nitrogen source was successful and growth rates obtained were above those of cultures that were supplied with urea or PLD (Fig. 1 and Supplementary Fig. S2). After 21 days of cultivation, cultures supplied with PLD-NaOH grew an additional 13.7 g $L^{-1}$ (0.65 g $L^{-1}$ $d^{-1}$) and reached a dry weight of 18.6 g $L^{-1}$ ($3.72 \cdot 10^9$ cells $mL^{-1}$). Cultures supplied with urea grew an additional 3.4 g $L^{-1}$ (0.16 g $L^{-1}$ $d^{-1}$) and reached a dry weight of 8.4 g $L^{-1}$ ($1.33 \cdot 10^9$ cells $mL^{-1}$). Thus, growth and productivity were two and four times higher, respectively, for microalgae supplied with PLD-NaOH (Fig. 1A). Microalgae supplied with PLD without the equimolar addition of NaOH did not grow and the cultivation was aborted after six days, as the pH value remained below the tolerance level for *C. vulgaris* (pH value of 6) (Rachlin and Grosso, 1991), despite a daily addition of 300 μL of 1 M NaOH (Fig. 1B). The use of PLD-NaOH stabilized the pH value around 7, which was similar to the cultivation of microalgae with urea.

The results show that microalgae can be robustly cultivated at high-density under laboratory conditions with PLD as a nitrogen source using the approach taken in this study. Indeed, the biomass dry weight (18.6 g $L^{-1}$) was much higher than numbers reported in other studies, which were usually below 4.8 g $L^{-1}$ (Cheng et al., 2015; Marazzi et al., 2017; Veronesiv et al., 2017). This shows that the cultivation potential of microalgae with liquid digestate is not limited to low-density PBRs. The consistent growth suggests that microalgae did not suffer from starvation nor $NH_4^+$-toxicity. The acidification of the growth medium was prevented, thanks to the equimolar addition of NaOH, and the algal biomass produced did not contain solid particles. Surprisingly, the growth of cultures supplied with PLD-NaOH not only matched, but surpassed growth in cultures supplied with urea. As cultures were subjected to a day/night cycle, a light limitation is possible. Indeed, light intensity was usually set to a continuous 800 to 1000 μmol $m^{-2}$ $s^{-1}$ for glass-column PBRs, which enabled a biomass dry weight of up to 16 g $L^{-1}$ (Doušková et al., 2010). This suggests that microalgae cultivated with PLD-NaOH were able to grow mixotrophically by using organic compounds contained in the liquid digestate (Hu et al., 2012; Liang et al., 2009; Markou and Georgakakis, 2011). As the trophic behavior of microalgae was not the focus of this study, this assumption remains to be verified.

In the following section, the cultivation of microalgae supplied with PLD as a nitrogen source was upscaled from 0.1 L to 200 L in a thin-layer PBR. The aim was to assess the feasibility of an upscaling at pilot-scale and under outdoor growth conditions.

### 3.2. Upscaling of the microalgae cultivation in a thin-layer photobioreactor

Microalgae cultures were supplied with PLD as a nitrogen source and successfully cultivated in a thin-layer PBR (Fig. 2). The cultivation was carried out during 16 days in 2017 (Fig. 2A) and 19 days in 2020 (Fig. 2B). Microalgae grew an additional 10.1 and 12.6 g $L^{-1}$, and reached final biomass dry weights of 15.6 g $L^{-1}$ and 13.0 g $L^{-1}$, respectively. The biomass productivity for the full cultivation period was similar between the two runs with a value of 0.63 g $L^{-1}$ $d^{-1}$ in 2017 and 0.66 g $L^{-1}$ $d^{-1}$ in 2020. These values were also similar to the productivity achieved with PLD-NaOH in glass-column PBRs (0.65 g $L^{-1}$ $d^{-1}$). However, closer inspection of the biomass productivities revealed differences over time, with a decline in productivity towards the end of the

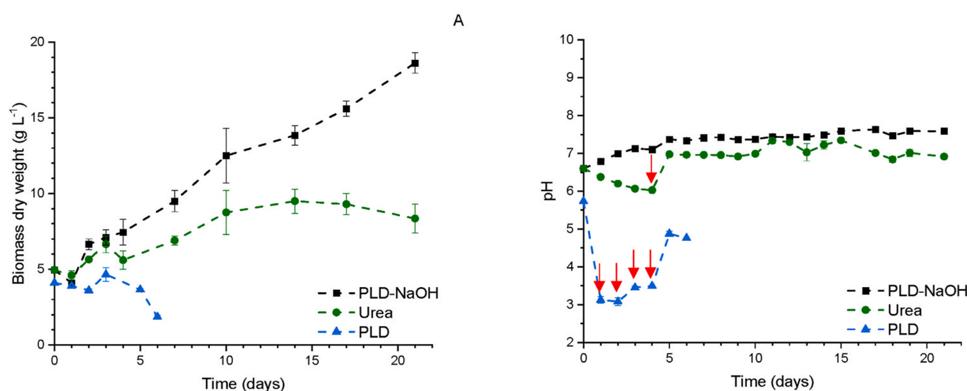


**Fig. 1.** Microalgae biomass dry weight (A) and pH value (B) of cultivations in glass-column PBRs; data points are means from duplicates and error bars are SEM. Microalgae cultures were supplied with three different sources of nitrogen: PLD-NaOH, PLD and urea. Arrows indicate the addition of 300 μL 1 M NaOH to the cultures to adjust the pH value.

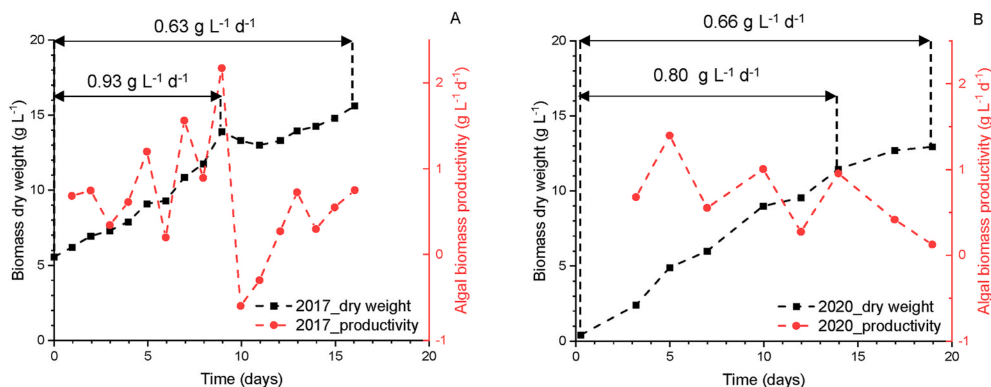


**Fig. 2.** Microalgae biomass dry weight and productivity (between sampling points) during the cultivation at high-density in a thin-layer PBR that received PLD as a nitrogen source. Cultivation was carried out in August/September 2017 (A) and June/July 2020 (B). Arrows show average productivity for specific time intervals.

cultivation period.

Microalgae had a robust growth during the first nine days of the cultivation carried out in 2017 with an average biomass productivity of 0.93 g $L^{-1}$ $d^{-1}$. However, growth abruptly stopped, and loss of biomass occurred over two days, before growth started again at a lower rate. The loss of productivity was associated with intermittent bad weather, which occurred during the cultivation period (Supplementary Fig. S3). Indeed, average light intensity and temperature during the day decreased from 448 μmol $m^{-2}$ $s^{-1}$ and 26.6 °C over the first nine days of cultivation, to 121 μmol $m^{-2}$ $s^{-1}$ and 18.3 °C during the three days of bad weather. In 2020, the average productivity was 0.80 g $L^{-1}$ $d^{-1}$ for the first 14 days. This was associated with good weather conditions that occurred during the entire cultivation period with average light intensity and temperature during the day of 461 μmol $m^{-2}$ $s^{-1}$ and 25.5 °C, respectively (Supplementary Fig. S4). However, productivity decreased during the last five days of the cultivation, which suggests that increased opacity and, thus, reduced light availability, caused by the continued addition of PLD (Supplementary Fig. S5) and the densification of the culture also contributed to the decrease. These results show that microalgae can be robustly cultivated both at high-density and pilot-scale under outdoor weather conditions with PLD as a nitrogen source. To our knowledge, this is the first successful attempt of high-density cultivation. Previous studies at this scale conducted cultivation at a much lower density (i.e., dry weight below 1 g $L^{-1}$) (Fernandes et al., 2020; Hajar et al., 2017; Simonazzi et al., 2019). The productivity of the cultures (0.8 and 0.93 g $L^{-1}$ $d^{-1}$), approached a maximum expectation of 1 g $L^{-1}$ $d^{-1}$, as defined by the daily supply of PLD and nutrients. Possibly, cultivation could have been optimized by harvesting microalgae before they experienced bad weather or approached their stationary phase. The areal productivity can be derived from the volumetric productivity by assuming 200 L of circulating culture and 18 $m^2$ of illuminated surface and, thus, reaching a maximum of 10.3 g $m^{-2}$ $d^{-1}$ (0.93 g $L^{-1}$ $d^{-1}$). It should be noted that the placement of the photobioreactor in a greenhouse results in a loss of sunlight of up to 50% (Egloff et al., 2018) and, thus, productivities are to some degree system-specific.

Controlling the pH value by adding NaOH was a key factor for successful growth (Fig. 3). Indeed, the importance of controlling the pH was demonstrated again during the second run, where insufficient NaOH addition caused the pH value to decrease to 2.5 in the first days of cultivation (Fig. 3B). The water acidification was due to the release of $H^+$ during the uptake of $NH_4^+$ by microalgae, and therefore, its intensity was related to the concentration of $NH_4^+$ supplied to the microalgae, which is higher for a high-density culture than for a low-density one (Scherholz and Curtis, 2013). Despite the addition of NaOH, pH fluctuated daily, yet this was due to the $CO_2$ injection that was switched off during the night. *C. vulgaris* is tolerant to the basification of the growth medium (Ihnken et al., 2014), which also has the benefit of decreasing the risk of biological contamination (Wang et al., 2013). While the PLD

**Table 3**
Nitrogen contained in the algal biomass and supernatant for microalgae cultivated with PLD-NaOH or urea in glass-column PBRs. Concentrations were expressed per liter of microalgae culture and average values are shown with the SEM.

| Time (days) | 0 | 21 | |
|---|---|---|---|
| Source of nitrogen | | PLD-NaOH | Urea |
| Nitrogen in the biomass (mg $L^{-1}$) | 256 ± 2 | 941 ± 34 | 626 ± 71 |
| Nitrogen in the supernatant (mg $L^{-1}$) | 6.5 ± 0 | 73 ± 2 | 24 ± 4 |
| Nitrogen supplied to microalgae (mg $L^{-1}$) | 0 | 1795 | 1795 |

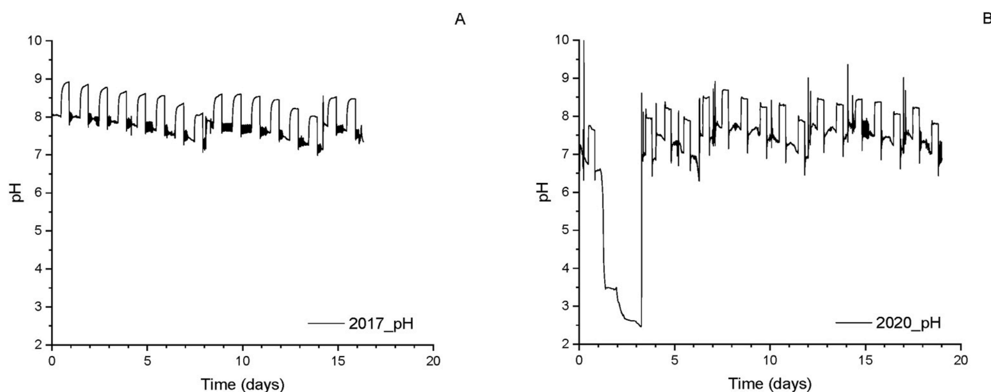


**Fig. 3.** pH values during the high-density cultivation of microalgae in a thin-layer PBR supplied with PLD as a nitrogen source. Cultivations were carried out in August/September 2017 (A) and June/July 2020 (B).

was not sterilized before its addition to the cultures, light-microscopic observations indicated that *C. vulgaris* was the dominant species and no other organisms occurred at noticeable numbers.

### 3.3. Nitrogen assimilation by microalgae cultivated at high-density

A nitrogen mass balance was carried out to determine whether microalgae efficiently assimilated nitrogen in their biomass or if accumulation occurred in the water. The PLD contained a high concentration of $NH_4^+$-N (>8 g $L^{-1}$) and, therefore, the nitrogen mass balance was the focus of this study. Results show that microalgae assimilated only 20.6% and 38.1% of the nitrogen supplied via addition of urea or PLD-NaOH, respectively, during the 21 days of cultivation in glass-column PBRs (Table 3). However, nitrogen did not accumulate in the water and the supernatant contained only 1% and 3.7% of the nitrogen supplied via addition of urea and PLD-NaOH, respectively. Concentration of $NH_4^+$ remained low in the supernatant during the cultivation period with values between 0.25 and 3.0 mg $L^{-1}$ $NH_4^+$-N when microalgae were supplied with PLD-NaOH.

These results show that 78.4% and 58.2% of the nitrogen were removed from the culture system when microalgae were supplied with urea and PLD-NaOH, respectively. When cultures were supplied with PLD-NaOH, volatilization could occur due to the equilibrium of $NH_4^+$ with gaseous $NH_3$. Previous studies, which reported nitrogen assimilation between 20 and 35% and nitrogen loss up to 80%, suggested that $NH_3$ volatilization may be an important contributor (Ledda et al., 2015; Markou et al., 2014). In this study, this is unlikely the sole cause, as nitrogen loss was not observed during a 2-day incubation of diluted PLD without microalgae (concentration of 90 mg $L^{-1}$ $NH_4^+$-N and pH value adjusted to 7). Further, the phenomenon occurred with both $NH_4^+$ and urea. Cultures supplied with urea (which is not volatile) showed higher nitrogen loss and lower growth than microalgae supplied with PLD-NaOH. This suggests that the loss of nitrogen was at least aggravated by biological processes, which occurred in the presence of microalgae. The loss was possibly worsened by the constant feeding rate, which did not consider a possible reduction in demand over time, as well as suboptimal growth conditions, as there was a notable difference between cultures supplied with PLD-NaOH and urea.

The nitrogen mass balance was also calculated for the cultivation in the thin-layer PBR (Fig. 4). Microalgae assimilated 40.8% and 48.3% of the nitrogen supplied via the addition of PLD during the 16-day cultivation period in 2017 (Fig. 4A) and the 19-day period in 2020 (Fig. 4B), respectively. Like glass-column PBRs, nitrogen did not accumulate in the water during the cultivation in the thin-layer PBR. For example, the nitrogen in the supernatant accounted for only 2.4% of the nitrogen supplied to the microalgae during the cultivation carried out in 2020, with a $NH_4^+$ concentration remaining between 0.1 and 2.2 mg $L^{-1}$ (Supplementary Fig. S6).

These results show that a large fraction of nitrogen was lost to the atmosphere during the cultivation of microalgae in glass-column and thin-layer PBRs. Suboptimal growth conditions such as light-limitation or bad weather most likely contributed to the loss of nitrogen. However, the productivity was high during the first nine days of cultivation in 2017 and the first 14 days in 2020, and the assimilation of nitrogen for these specific time periods was 63.5% and 52.1%, respectively. While these percentages are higher than the ones calculated for the full cultivation period, they still show that microalgae assimilated only about half of the nitrogen supplied to the culture even at high productivity. The recipe of the mineral medium, to which the supply of PLD was scaled, may have contributed to the loss of nitrogen. Indeed, the nitrogen content of *C. vulgaris* may vary from about 4.5% to 8.5% but the average during the cultivation in the thin-layer PBRs was 5.5 ± 0.1%. In comparison, the mineral medium recipe set the nitrogen content to 8.5%, which may result in excessive fertilization. Therefore, tuning the nitrogen supply may contribute to decreased loss.

Loss due to the volatilization of $NH_3$ was not assessed in the thin-layer PBR, and therefore, it cannot be excluded. However, PLD was supplied gradually during the daytime to prevent a high concentration of $NH_4^+$ that favors conversion to $NH_3$. Further, pH value was kept at 7.5 ± 0.5 during the feeding period, to favor an equilibrium towards $NH_4^+$. Additionally, results obtained in glass-column PBRs as well as other studies showed a relatively good stability of $NH_4^+$ for the pH and temperature range used in this study (Collos and Harrison, 2014; Wen et al., 2019). Therefore, it is assumed that biological processes were an important contributor to the loss of nitrogen. While the composition of these emissions remains unknown, this raises the question of the environmental impact of microalgae cultivation. For example, recent studies showed that *C. vulgaris* may emit nitrous oxide ($N_2O$), a greenhouse gas, via the oxidation of intracellular nitrite (Alcántara et al., 2015; Guieysse et al., 2013). Until now, significant $N_2O$ emissions were measured only when microalgae were supplied with nitrate or nitrite (Plouviez et al., 2017, 2019). However, the large nitrogen loss reported in the present study during the cultivation of microalgae with $NH_4^+$-rich PLD indicates how vital it is to better understand the processes that cause these emissions and the different gas species involved.

## 4. Conclusions

Microalgae were successfully cultivated at high-density at laboratory and pilot scales with pretreated liquid digestate as a nitrogen source. Microalgae achieved biomass productivities up to 0.93 g $L^{-1}$ $d^{-1}$ under optimal weather conditions, which was close to expected values. The robust growth was made feasible by anticipating and systematically resolving the bottlenecks associated with the use of liquid digestate.

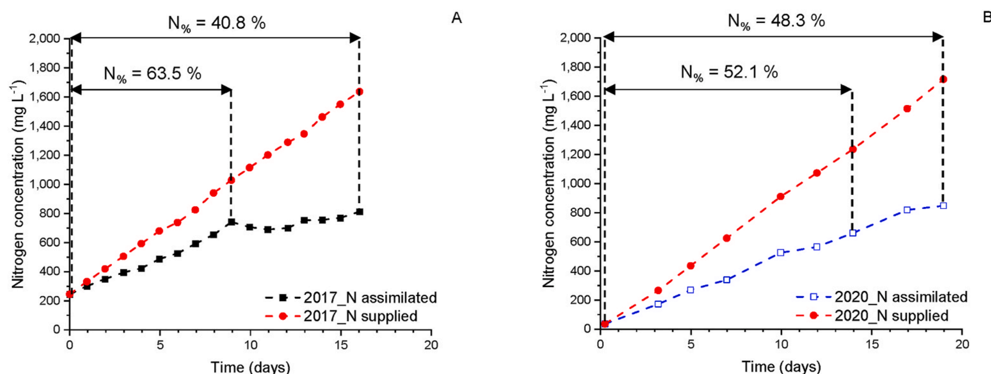


**Fig. 4.** Nitrogen mass balance during the cultivation of microalgae in the thin-layer PBR in 2017 (A) and 2020 (B), with PLD as a nitrogen source. The concentration of nitrogen assimilated by the microalgae was compared to the concentration of nitrogen supplied to the culture. Concentrations of nitrogen are expressed per liter of microalgae culture. $N_{\%}$ shows the percentage of supplied nitrogen, which was assimilated by the algal biomass for specific time intervals represented by arrows.

High-density cultivation also mitigated the risk of contamination and cultures were not jeopardized using a non-sterile liquid digestate. The nitrogen mass balance showed that at least half of the nitrogen supplied to the culture was neither assimilated in the algal biomass nor accumulated in the supernatant. It is noteworthy that this observation was true both at laboratory and pilot-scale despite distinct differences in the cultivation systems. Therefore, the cultivation of microalgae has a nitrogen loss similar to that resulting from the landspreading of liquid digestate. Formation of volatile nitrogen compounds by microalgae is considered to be a probable cause and must be investigated to better assess the environmental impact of microalgae cultures. Further optimization of the cultivation, e.g., via improved pH control and PLD dosage, will also allow for a reduction in the loss of nitrogen. Closed reactor systems may also be designed to recover nitrogen from gases.

## CRediT authorship contribution statement

**Adrian Pulgarin:** Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, Writing – review & editing, Visualization. **Alexander Garcia Kapeller:** Methodology, Investigation, Formal analysis, Writing – review & editing. **Mohamed Tarik:** Formal analysis, Resources. **Sophia Egloff:** Investigation, Resources. **Marina Mariotto:** Investigation, Resources. **Christian Ludwig:** Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Dominik Refardt:** Conceptualization, Validation, Resources, Writing – review & editing, Project administration, Funding acquisition.

## Declaration of competing interest

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

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jclepro.2021.129238.

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