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Microalgae Cultivation on Nutrient Rich Digestate: The Importance of Strain and Digestate Tailoring under PH Control

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Abstract: The bioremediation of digestate using microalgae presents a solution to the current eutrophication issue in Northwest Europe, where the use of digestate as soil fertiliser is limited, thus resulting in an excess of digestate. Ammonium is the main nutrient of interest in digestate for microalgal cultivation, and improving its availability and consequent uptake is crucial for optimal bioremediation. This work aimed to determine the influence of pH on ammonium availability in cultures of two green microalgae, additionally screened for their growth performances on three digestates produced from different feedstocks, demonstrating the importance of tailoring a microalgal strain and digestate for bioremediation purposes. Results showed that an acidic pH of 6–6.5 resulted in a better ammonium availability in the digestate media, translated into better growth yields for both *S. obliquus* (GR: 0.099 ± 0.001 day^{-1}; DW: 0.23 ± 0.02 g L^{-1}) and *C. vulgaris* (GR: 0.09 ± 0.001 day^{-1}; DW: 0.49 ± 0.012 g L^{-1}). This result was especially true when considering larger-scale applications where ammonium loss via evaporation should be avoided. The results also demonstrated that digestates from different feedstocks resulted in different growth yields and biomass composition, especially fatty acids, for which, a digestate produced from pig manure resulted in acid contents of 6.94 ± 0.033% DW and 4.91 ± 0.3% DW in *S. obliquus* and *C. vulgaris*, respectively. Finally, this work demonstrated that the acclimation of microalgae to novel nutrient sources should be carefully considered, as it could convey significant advantages in terms of biomass composition, especially fatty acids and carbohydrate, for which, this study also demonstrated the importance of harvesting time.

Keywords: digestate; microalgae; pH control; ammonium; bioremediation

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1. Introduction

Nutrient-rich digestate is a by-product resulting from the anaerobic digestion (AD) of food and farm waste, and is currently used as liquid fertiliser across Northwest Europe [1]; however, in the last decade, nutrient run-off into ground water and natural environments has been an increasing issue [2], leading to the implementation of the Nitrate Vulnerable Zones (NVZs) policy, designated under the European Nitrate Directive 91/676/CEE, that limits the annual load of nitrogen applied onto arable land. Consequently, the majority of AD plants are under pressure to find alternative solutions for their excess digestate, which is currently stored or buried. Microalgae have been widely studied to remediate wastewater from water treatment processes, aquaculture facilities, and other industries [3–5], and there has been a growing interest in utilising their bioremediation properties to tackle the growing digestate issue in Northwest Europe [6–8]. Indeed, digestate composition shows a vast potential to support microalgal growth, especially in terms of macronutrients such as phosphorus and nitrogen [9,10], and also other microelements, which, at the right concentrations, can provide essential nutrition to microalgae [11].

While some studies have shown the capacity of microalgae to grow on digestate, only a few have looked at optimising the process, and, more precisely, increasing the digestate uptake by microalgae. Indeed, digestate is characterised by a high turbidity and pH [12–14], as well as a high particle content, which can decrease the light availability and consequently disturb the photosynthesis process and thus microalgal growth [15]. Additionally, the wide range of feedstocks used in the AD industry result in a variety of digestates with very different compositions [16,17]. Therefore, there is a need to tailor specific nutrients to suit microalgal growth requirements. Different strains of microalgae can also have different abilities to uptake digestate, demonstrating the need to screen specific strains for different types of digestates, allowing for the optimisation of the bioremediation process.

The value of digestate for microalgae cultivation lies in its high nitrogen content, and, more specifically, ammonium, which is the preferred nitrogen source for microalgae; indeed, the metabolic cost to process ammonium into organic matter is lower than for other forms of nitrogen [18,19]. However, ammonium is also known for its tendency to evaporate, reducing the availability for microalgae [20]. Indeed, the volatilisation of ammonium is a common phenomenon in cultures of microalgae grown on digestate due to the high pH environment caused by both digestate, which has a naturally elevated pH, and microalgal growth, which increases the pH in cultures during respiration. This combined effect results in a shift in ammonium from a liquid to gaseous form [21]. Consequently, the pH should also be considered when using microalgae to remediate digestate, as this factor could play an essential role in improving the ammonium uptake by microalgae, and therefore digestate remediation.

Beyond remediation purposes, growing microalgae on digestate also has significant potential in providing high-value products for an array of industries, such as the animal feed or biofertiliser and biostimulant sectors [22–25]. Indeed, within the context of a circular economy, it is crucial to generate value from the biomass grown using waste nutrients from digestate, contributing to reducing the production cost of microalgal systems. However, strain screening for specific digestates while looking closely at the biomass composition has been investigated only superficially, despite the tremendous potential to optimise microalgal composition by tailoring digestates.

In the presented work, two green microalgae (*Chlorella vulgaris* and *Scenedesmus obliquus*) were selected for their ability to grow on waste products [24,26], and were cultivated on three digestates produced from the anaerobic digestion of three different feedstocks, aiming to identify which strain would perform better on the different digestates tested. Furthermore, to improve the ammonium uptake by the microalgae (and hence remediation potential), the pH was also manipulated to avoid ammonium evaporation during culture, improving its availability for microalgal growth. Combining pH manipulation with strain and digestate screening and tailoring presents a novelty to the field of digestate bioremediation using microalgae, by aiming to improve the remediation potential using a dual approach based on abiotic and biological factors. Additionally, the composition of the generated biomass was analysed to determine if different digestates would have a different influence on the microalgal composition, bringing additional information on the potential of microalgae grown on digestate as a high-value product for further applications.

2. Materials and Methods

2.1. Digestates

Digestates resulting from the anaerobic digestion of different waste streams were used in the experiment. The digestate provided by the Langage AD facility (Plymouth, UK, described as SU hereafter) was produced from kitchen and food waste. Digestate from COOPERL (France) resulted from the processing of pig manure and agricultural waste; finally, digestate supplied by INNOLAB (Belgium) was plant-based with a feedstock dominated by potato peel. Resulting digestates all had a dark-brown colour and a high turbidity (see data from Fernandes et al., 2020). Consequently, all three digestates were treated according to the technique developed in Fernandes et al. [12] to allow for microalgal

cultivation. To summarise, digestates were filtered using membrane filtration at a pore size of 0.22 µm and stored at 4 °C prior to the experiment.

The ammonium (NH_4^+) and phosphorus (PO_4^{3-}) compositions of each digestate were assessed using test kits, determining the level of dilution (using di-ionised water) required to bring the ammonium concentration below 100 mg L^{-1} (corresponding to 2% of the total culture volume of 800 mL) for the growth experiment and providing an insight on the N:P ratio. Results are summarised in Table 1.

Table 1. Ammonium and phosphorus composition of the three digestates used in the experiment.

	NH_4^+	PO_4^{3-}	N:P	Dilution Factor Required
SU	4815 mg L^{-1}	202 mg L^{-1}	23.8	48
COOPERL	3102 mg L^{-1}	440 mg L^{-1}	7.1	31
INNOLAB	5295 mg L^{-1}	395 mg L^{-1}	13.4	53

2.2. Experimental Design

The microalgae *Chlorella vulgaris* (*C. vulgaris*) and *Scenedesmus obliquus* (*S. obliquus*) were used in the present study for their known ability to grow on waste [26]. Both microalgae are freshwater species belonging to the Chlorophyta division. *C. vulgaris* and *S. obliquus* were grown in triplicates separately on the three digestates and on an F/2 control medium to assess repeatability of the work. The composition of the F/2 control was based on the CCAP formulation; however, the main source of nitrogen in the medium, which is sodium nitrate ($NaNO_3$), was replaced by ammonium chloride (NH_4Cl, Sigma-Aldrich, Dorset, UK) in this experiment. Ammonium chloride was selected, allowing for a comparison of the nitrogen consumption between the F/2 control and the digestate conditions, where ammonium was the main form of nitrogen available. Ammonium chloride was added to the F/2 medium to obtain a concentration of 100 mg L^{-1}. Furthermore, magnesium in the form of $MgSO_4 \cdot 7H_2O$ (Sigma-Aldrich, Dorset, UK) was added to the medium at a concentration of 0.2 g L^{-1} to reflect microalgal needs in micronutrients.

2.2.1. Culture Conditions and Inoculation

Cultures were grown in 1 L Erlenmeyer flasks at a total working volume of 800 mL, which allowed for a sufficient volume of culture for sampling throughout the experiment. Cultures were maintained at a temperature of 25 °C under a light intensity of 70 µmol m^{-2} s^{-1} and a photoperiod of 12 L:12 D. Flasks were supplied with filtered (0.22 µm) air for constant aeration, and culture agitation was provided by a shaking platform at a speed of 90 rpm, twelve hours a day. Inoculum volume was calculated to obtain an initial optical density at 750 nm of 0.5.

Nutrient addition in the form of F/2 medium and the three digestates occurred at day 0, and no further nutrients were added for the rest of the experiment, which was continued for 10 days.

2.2.2. pH Manipulation

The pH was measured daily and adjusted using solutions of 1 M HCl or 1 N NaOH (Sigma-Aldrich, Dorset, UK) to maintain a pH between 6 and 6.5. The use of an acidic pH aimed to reduce losses of ammonium due to evaporation from the culture media and increase its availability for microalgae. The pH manipulation condition (pHM^+) was compared against no manipulation of the pH (pHM^-).

2.3. Growth Measurements

One millilitre of culture was sampled daily by pipetting to assess growth rates through measurements of the absorbance at 750 nm. This specific wavelength was used as it avoids light absorption by pigments and can be treated as a light scattering measurement [27]. The growth rate was calculated using the following equation:

$$\mu = \ln(OD_2/OD_1)/(t_2 - t_1)$$

where μ is the specific growth rate (day^{-1}) and OD_1 and OD_2 are the optical density measured at 750 nm at time 1 (t_1) and time 2 (t_2).

Flowcytometry was also performed daily on the same sample to assess the cell counts in the cultures.

Dry weight (in g L^{-1}) was measured at day 0 and day 10 (last day of the experiment) by filtering 20 mL of a sample using pre-dried and pre-weighed filters (Whatman 47 mm GF/C glass microfiber filters, pore size: 1.2 µm). Samples were oven-dried for 24 h at 80 °C and dry weight was calculated as the weight difference between the dried-filtered sample and the pre-weighed-filter in relation to the volume of sample filtered as follows:

$$dw\ (g\ L^{-1}) = ((fs - f)/Vs) * 1000$$

where dw is the dry weight in g L^{-1}, fs is the weight of the filtered and dried sample (g), f is the weight of the pre-dried filter (g), and Vs is the volume of sample filtered (mL).

2.4. Nutrient Analysis

Every other day, namely at day 0, 2, 4, 6, 8, and 10, 7 mL of cultures was sampled to perform ammonium and phosphorus analysis. The NH_4^+ concentration was measured using an ammonium reagent kit (Spectroquant®) based on the colorimetric quantification of NH_4^+ (method analogous to EPA 350.1, APHA 4500-NH3 F, ISO 7150-1, and DIN 38406-5). The absorbance of treated samples was measured at 690 nm according to supplier instructions and measured against a calibration curve to determine NH_4^+ concentration. PO_4^{3-} was measured using a reagent test kit (Spectroquant®), also based on colorimetric reactions (method analogous to EPA 365.2 + 3, APHA 4500-P E, and DIN EN ISO 6878). Absorbance was recorded at 410 nm and P concentration was assessed for ammonium.

2.5. Determination of Total Proteins and Carbohydrates

Pellets of biomass for biochemical analysis were sampled every other day. Fifteen millilitres of culture was collected and centrifuged for 30 min at 4500 rpm. The supernatant was discarded, and the pellets were washed with 2 mL of DI water. Samples were further centrifuged at 10,000 rpm for 25 min. Supernatants were discarded and pellets were stored at −80 °C for further analysis.

For the determination of the biochemical composition, a multi-assay procedure was modified for the quantification of total protein and carbohydrate [28]. Previously collected pellets were freeze-dried and weighed (1–1.5 mg) in 2 mL Safe-Lock microcentrifuge tubes. The dry pellets were resuspended in 24.3 µL of phosphate buffer (pH 7.4) and 1.8 mL of 25% (*v*/*v*) methanol in 1 N of NaOH, along with an equal volume of glass beads (425–600 µm i.e., acid washed). Cells were treated using a cell disruptor (Scientific Industries Inc., Bohemia, NY, USA) for 3 cycles (ten-minute bead beating and two-minute stand). For carbohydrate analysis, two aliquots of 0.2 mL extract were transferred to 2 mL PTFE capped glass vials: for the control, by adding 1.2 mL 75% H_2SO_4; for the experimental samples, by adding 0.4 mL 75% H_2SO_4 and 0.8 mL anthrone reagent. Samples were incubated at 100 °C for 15 min followed by measurement in polystyrene cuvettes (absorbance at 578 nm). The remaining extract after cell disruption was stored at −80 °C in 4 mL PTFE capped glass vials and later saponified by incubating at 100 °C for 30 min. Saponified extracts of 25 µL were first placed directly into 96-well assay plates with the following additions: controls, 0.2 mL BCA reagent alone (Thermo Scientific, Waltham, MA, USA); experimental, 0.2 mL BCA/Cu mix (Thermo Scientific) and incubated at 37 °C for 30 min, measuring (absorbance at 562 nm) [29]. The carbohydrate and protein assay standard curves were generated separately and are presented in the Supplementary Material (Figure S1).

2.6. Determination of Fatty Acid Methyl Esters (FAMEs)

All chemicals and analytical reagents were of high-performance liquid chromatography grade unless stated otherwise (Sigma-Aldrich, Dorset, UK). On the day of analysis, freeze-fried algal biomass was weighed (~1 to 2 mg) followed by direct transesterification as described elsewhere [30–32]. A total of 290 µL of toluene, 300 µL of 0.5 M sodium methoxide, and 10 µL of hexane containing C13:0 as an internal standard were added to the weighed samples, followed by incubation at 80 °C for 20 min. After cooling at room temperature, 300 µL of 10% boron tri-fluoride in methanol was added and the mixture was incubated at 80 °C for 20 min. After cooling to room temperature, 300 µL of water and 600 µL of hexane were added. The mixture was vortexed for 1 min and centrifuged at 18,000× *g* at 4 °C for 10 min. The organic phase was recovered, measured, and evaporated to dryness under inert nitrogen gas using a six-port mini-vap evaporator (Sigma-Aldrich, Dorset, UK).

The dried fatty acid methyl esters (FAMEs) were reconstituted in 300 µL hexane prior to identification and quantification on a GC-ToF-MS (Waters Corporation, Milford, MA, USA) using a TR-FAME capillary column (25 m × 0.32 mm × 0.25 µm). The sample volume of 1 µL was injected in split injection mode at 250 °C. The split ratio was 1:20, split flow 30 mL min^{-1}, and helium carrier gas flow 1.5 mL min^{-1}. GC oven temperature was held at an initial temperature of 150 °C for 1 min, then increased by 10 °C min^{-1} up to 250 °C and held at 250 °C for 1 min; total run time was 17 min. The GC-ToF-MS was operated in EI mode at 70 eV, scanning m/z 50–700 with an initial 2 min solvent delay. EI source temperature was held at 200 °C and GC re-entrant temperature at 250 °C. Fatty acids were identified by comparing the obtained retention times with that of known standards (37 component FAME mix, Supelco™, Bellefonte, PA, USA). A representative GC-ToF-MS chromatogram of 37 FAME Mix (C8-C24) standard is available in the Supplementary Material. Data acquisition and post-acquisition processing for peak identification were performed using MassLynx (version 4.1) software (Waters Corporation, Milford, MA, USA). A six-point calibration curve was generated using one internal (C13:0) and two external standards (C17:0 and C19:0). Quantitation of FAMEs in the biomass extract was then determined by comparing experimentally derived component peak areas with the calibration curve generated by the reference internal and external standards (Supplementary Material, Figure S2). In total, n = 3 replicates (biological) were run, among which, FAMEs identified in only 2 or more replicates were considered as true hits. The data were later normalised to the dry weight of the biomass and FAMEs were reported on a percentage dry weight basis.

2.7. Statistical Analysis

Statistical analysis was carried out on the studied factors using the R project software. Crossed factors ANOVAS were carried out on normally distributed data and normality was determined using Shapiro tests. When statistical significance was found, post hoc Tukey tests were implemented. Results were deemed significant when the p-value (p) was below 0.05.

3. Results

3.1. Growth Performances: Absorbance at 750 nm and Dry Weight

Chlorella vulgaris grew better on all three digestates in comparison to the F/2 control medium, both under pHM^+ and pHM^- conditions ($p < 0.001$, Figure 1). Growth under pHM^- was consistent over the 10 days of experiment, and the highest growth rate was recorded for the SU digestate (0.09 day^{-1}). In pHM^+, the growth of *C. vulgaris* was less linear, and cell clumping was observed in the cultures. The highest absorbance at 750 nm was recorded for the Innolab digestate, reaching 1.26 ± 0.15 after 6 days of the experiment; however, this was followed by a decrease until the end of the experiment (Figure 1). The Cooperl digestate showed the second best performance, with an absorbance of 1.08 ± 0.05, followed by the SU digestate (0.93 ± 0.05) at the end of the experiment. The same growth

trends were observed for the flowcytometry data, which recorded the cell count in the cultures (Supplementary Material, Figure S3).

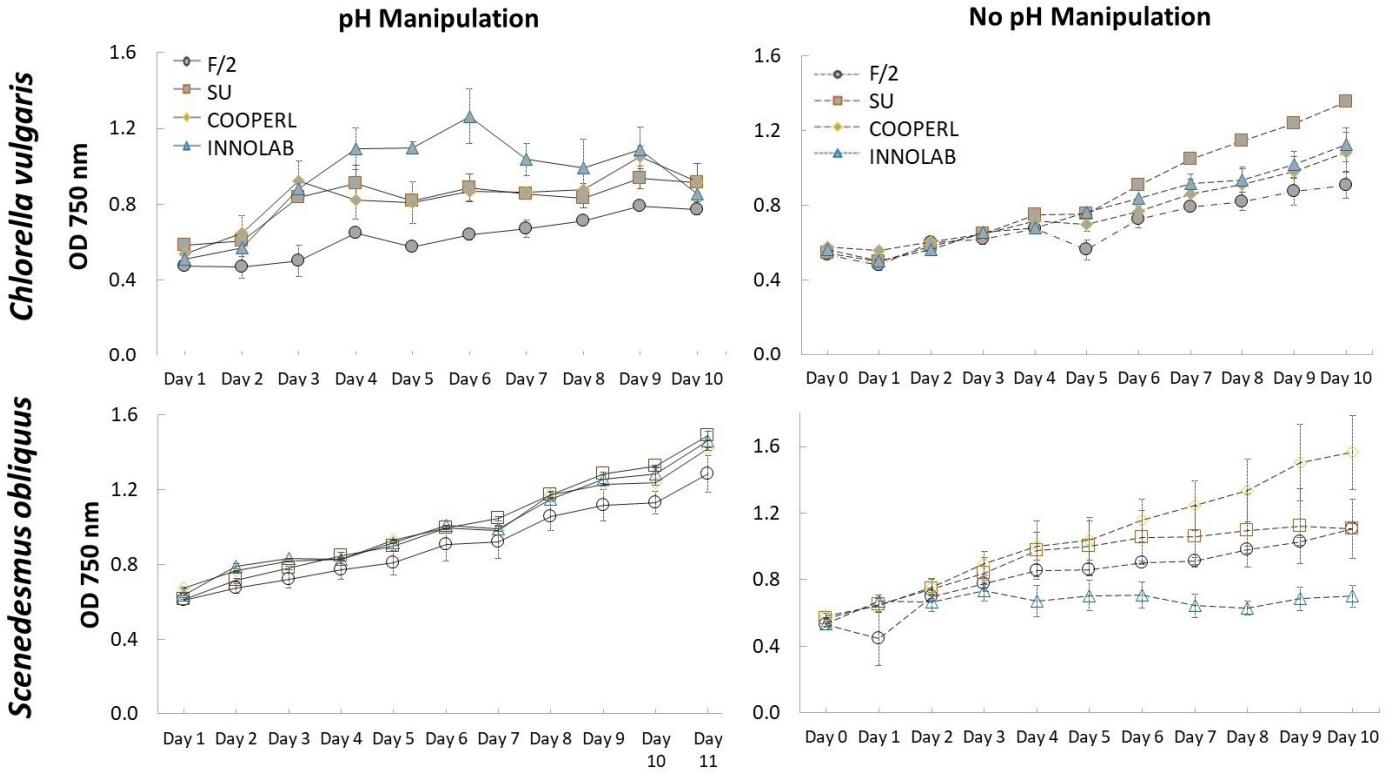

Figure 1. Absorbance at 750 nm for *C. vulgaris* and *S. obliquus* grown on three different digestates under pH manipulation and no pH manipulation for 10 days of the experiment. Error bars represent the standard deviation on three replicates.

Under pHM^-, *S. obliquus* showed the best performances in the Cooperl digestate ($p < 0.001$; GR: 0.099 ± 0.002 day^{-1}), followed by the SU digestate (GR: 0.074 ± 0.001 day^{-1}), the F/2 control (GR: 0.067 ± 0.001 day^{-1}), and the Innolab digestate (GR: 0.027 ± 0.001 day^{-1}). Under pHM^+, growth was more consistent between conditions, and the performances were very similar between digestates (GR: 0.081 ± 0.001 day^{-1}; 0.067 ± 0.001 day^{-1} and 0.078 ± 0.001 day^{-1}, for SU, Cooperl, and Innolab digestates, respectively). Growth was slightly lower for the F/2 control (GR: 0.059 ± 0.002 day^{-1}), but no significant differences were found between all of the tested conditions ($p > 0.05$, Figure 1).

Additionally, there were no significant differences between the performances of the two strains when the pH was not manipulated ($p < 0.05$); however, *S. obliquus* showed significantly better growth under an acidic pH ($p < 0.001$), overall reaching a higher absorbance at 750 nm at the end of the 10 days of the experiment (Figure 1).

The dry weight analysis showed discrepancies at day 0 between the different conditions tested, despite inoculum concentrations being the same for both cultures of *C. vulgaris* and *S. obliquus* (Figure 2). Indeed, despite the use of membrane filtration to treat the three different digestates, a significant number of particles can still be found in the filtration permeates, therefore contributing to the dry weights recorded at the start of the experiment.

The dry weight significantly increased during the time of the experiment ($p < 0.01$), and differences observed between conditions after 10 days were mainly related to microalgal growth. Results showed that the dry weight in cultures of *C. vulgaris* was higher in the Innolab digestate and under pHM^- (0.28 ± 0.025 g L^{-1}), whereas, in pHM^+, dry weight was the highest in the Cooperl digestate (0.49 ± 0.012 g L^{-1}). Overall, the DW was significantly higher in cultures of *C. vulgaris* in pHM^+ for all digestates tested and the F/2 control ($p < 0.001$), even doubling in some of the studied conditions (Figure 2).

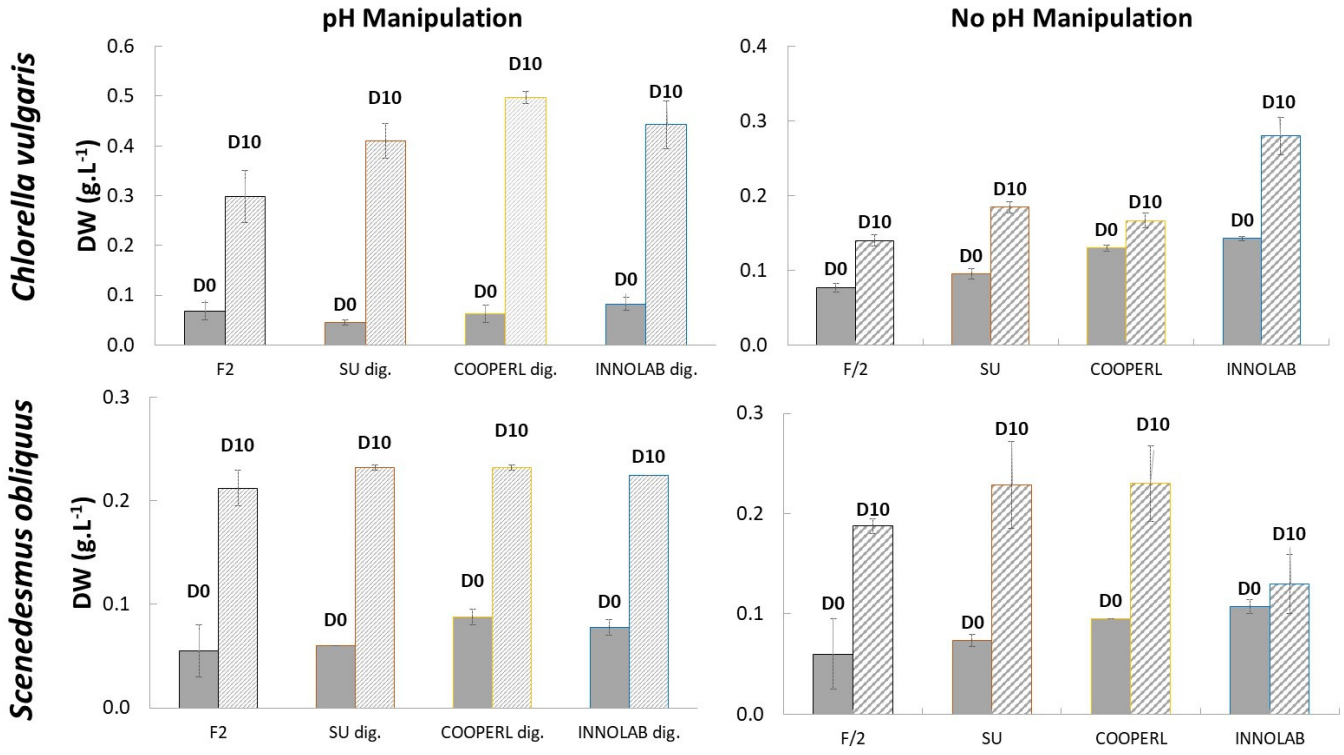

Figure 2. Dry weight of *C. vulgaris* and *S. obliquus* grown on three different digestates under pH manipulation and no pH manipulation on day 0 and 10 of the experiment. Error bars represent the standard deviation on three replicates.

In cultures of *S. obliquus*, the pH manipulation resulted in a higher dry weight in the Innolab digestate and the F/2 control ($p < 0.001$); however, no significant changes were observed in the Cooperl and SU digestates, with a DW of around 0.23 ± 0.002 g L^{-1} in $pHM^{-/+}$ and in both digestates ($p > 0.05$). Finally, *C. vulgaris* yielded a higher dry weight at the end of the experiment under pHM^{+}, in comparison to cultures of *S. obliquus* under the same conditions of pH (Figure 2).

3.2. Nutrient Consumption: Ammonium and Phosphorus

The concentration of NH_4^+ decreased rapidly after the start of the experiment in both cultures of *C. vulgaris* and *S. obliquus*, and for all three digestates tested when the pH was not manipulated. The decrease continued steadily and the final NH_4^+ concentration in the different media was significantly lower than at the beginning of the experiment ($p < 0.001$, Figure 3). No decrease in the NH_4^+ concentration was observed in the F/2 control, which maintained a consistent content of ammonium throughout the experiment and for both strains. The observed decrease in ammonium, however, was not in accordance with the dry weight data measured previously (Figure 2), and it could be assumed that the recorded decrease was mainly linked to the evaporation of the ammonium, most likely induced by the naturally high pH of digestate. Indeed, NH_4^+ did not decrease in the F/2 control where the pH remained neutral. However, the microalgae did not consume this source of nitrogen, despite its availability.

Under pH manipulation, the concentration of NH_4^+ remained the same in cultures of *C. vulgaris* for all three digestates and the control, with a concentration averaging 83.7 ± 1.83 mg L^{-1} ($p > 0.05$). Therefore NH_4^+ was not consumed by *C. vulgaris* during the 10 days of the experiment. The same result was observed in cultures of *S. obliquus*, up to day 8 of the experiment. However, NH_4^+ decreased significantly between day 8 and day 10 ($p < 0.001$) and reached values below 20 ± 3.93 mg L^{-1} in the three tested digestates, showing a rapid consumption of ammonium by the microalgae (Figure 3). In this instance, it was demonstrated that the ammonium evaporation did not occur under an acidic pH;

however, NH_4^+ was still not consumed by the microalgae, or only until a later stage of the experiment. Therefore, it could be hypothesised that both *C. vulgaris* and *S. obliquus* were acclimating to the novel source of nitrogen (ammonium from digestate or ammonium chloride in the F/2 control).

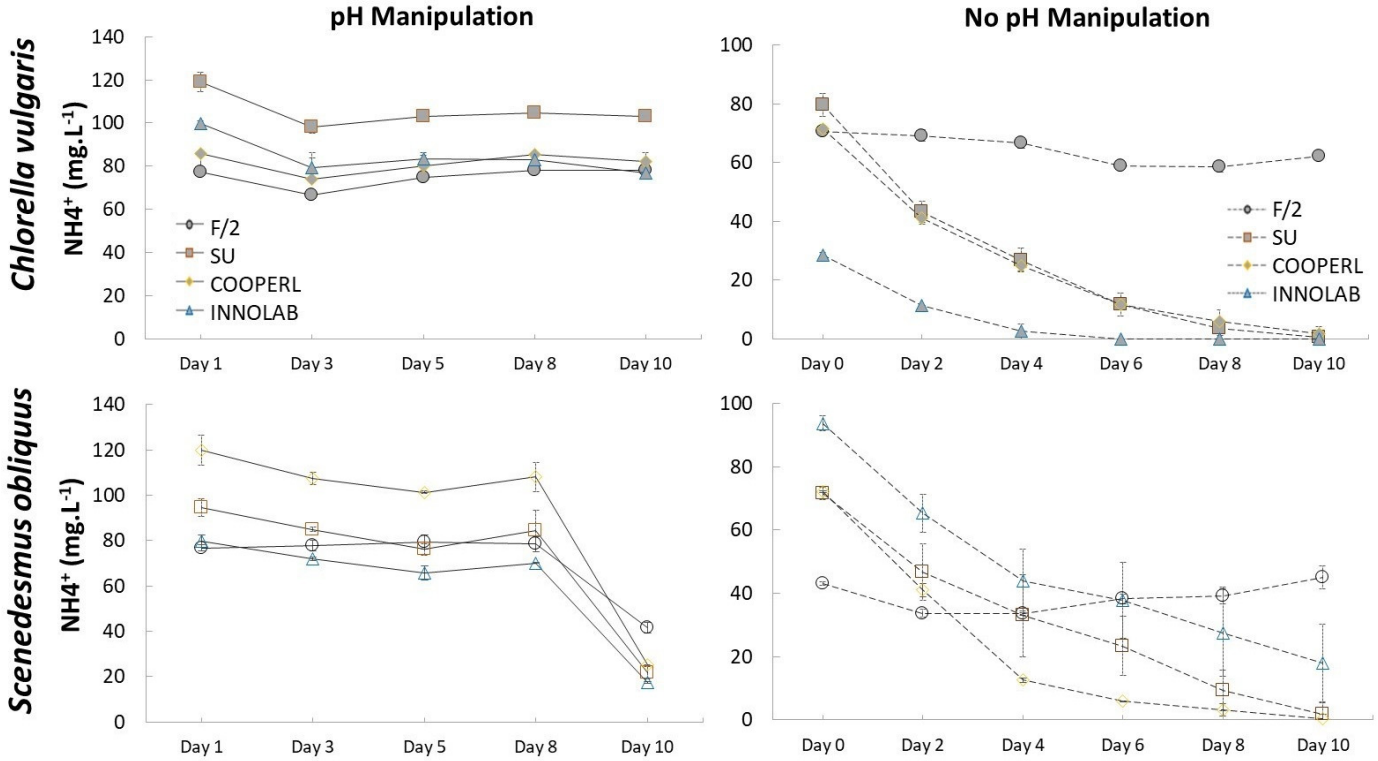

Figure 3. Concentration of ammonium NH_4^+ in cultures of *C. vulgaris* and *S. obliquus* grown on three different digestates under pH manipulation and no pH manipulation for 10 days of experiment. Error bars represent the standard deviation on three replicates.

Phosphorus decreased over time in cultures of *C. vulgaris* when the pH was not manipulated, showing a consumption of the nutrient, especially in the Cooperl and Innolab digestates. A decrease in the phosphorus concentration was also observed in cultures of *S. obliquus* in pHM^- for all of the conditions tested, except for cultures grown in the Innolab digestate, where phosphorus increased after 4 days of cultivation, reaching 8.86 ± 0.154 mg L^{-1} (Figure 4). The growth data presented previously showed that *S. obliquus* performed less well when grown on the Innolab digestate (Figures 1 and 2). Microalgal cells are known to release phosphorus when decaying; therefore, the observed increase in phosphorus could be linked to cells of *S. obliquus* degrading in the Innolab digestate and releasing stored phosphorus.

Under an acidic pH, phosphorus increased during the time of the experiment in cultures of *C. vulgaris*, but decreased overall in cultures of *S. obliquus* (Figure 4). While regulating the pH using HCl, lower pHs were recorded in cultures of *C. vulgaris*, and a pH lower than 6 was measured several times across the time of experiment. Under an acidic pH, particles dissolve more easily, and, in this study, it was possible that particles remaining in the different digestate would have released phosphorus when dissolving in the acidic environment, explaining the observed results. Additionally, growth data showed that *S. obliquus* performed better in pHM^+, which could also explain the decrease in phosphorus for this strain, using the nutrient for growth purposes.

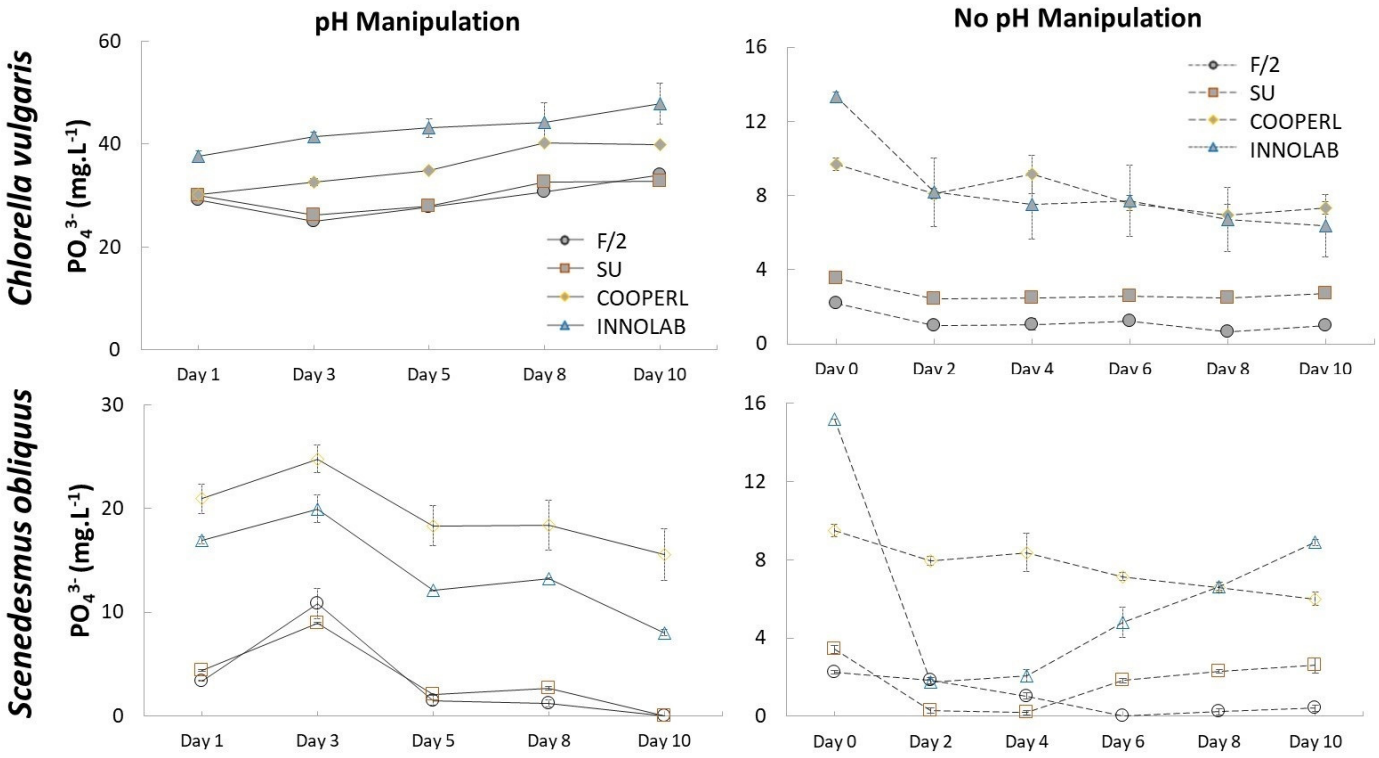

Figure 4. Concentration of phosphorus PO_4^{3-} in cultures of *C. vulgaris* and *S. obliquus* grown on three different digestates under pH manipulation and no pH manipulation for 10 days of experiment. Error bars represent the standard deviation on three replicates.

3.3. Composition Analysis: Fatty Acids, Proteins, and Carbohydrates

The analysis of the biomass composition was performed on the cultures where pH was manipulated. Indeed, cultures under pHM^+ yielded more biomass (Figure 2), and efforts were therefore concentrated towards analysing these specific samples.

In cultures of *C. vulgaris*, the total fatty acids increased significantly over the time of the experiment for all conditions tested ($p < 0.001$), with a most significant increase between day 6 and day 10 for the F/2 control and both SU and Innolab digestates, whereas the FAs concentration was the highest after 6 days in the Cooperl digestate, reaching 4.91 ± 0.3% DW (Figure 5). A similar trend was observed in the total MUFAs and PUFAs concentrations, especially for the SU digestate, with a five-fold increase between day 0 and day 10 (Supplementary Material, Figure S4). Additionally, most of the fatty acids measured as part of this work were found in higher concentrations in cultures grown using the different digestate than in the F/2 control ($p < 0.001$).

In cultures of *S. obliquus*, the highest concentrations of fatty acids were measured on day 6 of the experiment for all tested conditions. Additionally, the highest fatty acids content was recorded in cultures grown on the Cooperl digestate (6.94 ± 0.33% DW); however, there were no significant differences between the F/2 control and the digestates conditions on day 6 ($p > 0.05$, Figure 5). Finally, the statistical analysis showed that the total fatty acid content was not significantly different between the two tested strains at the beginning of the experiment, but that, over the course of the study, concentrations of fatty acids were significantly higher in cultures of *S. obliquus* ($p < 0.001$).

The protein content generally decreased over time or maintained a constant concentration for all digestates studied and the control for both cultures of *C. vulgaris* and *S. obliquus*. Apart from the odd increase on day 6 of the experiment, the protein content after 10 days of culture was significantly lower ($p < 0.001$) or similar ($p > 0.05$) to the protein concentration recorded at the beginning of the experiment. For example, in the Cooperl digestate, the protein content decreased from 46.8 ± 0.001 to 27.8 ± 0.001% DW in cultures of *C. vulgaris*, and

from 43.5 ± 0.001 to 29.6 ± 0.001% DW in cultures of *S. obliquus* (Figure 5). The observed results were in accordance with the low nutrient consumption described previously and shown in Figure 3. Additionally, there were no significant differences between the protein content of the two strains ($p > 0.05$).

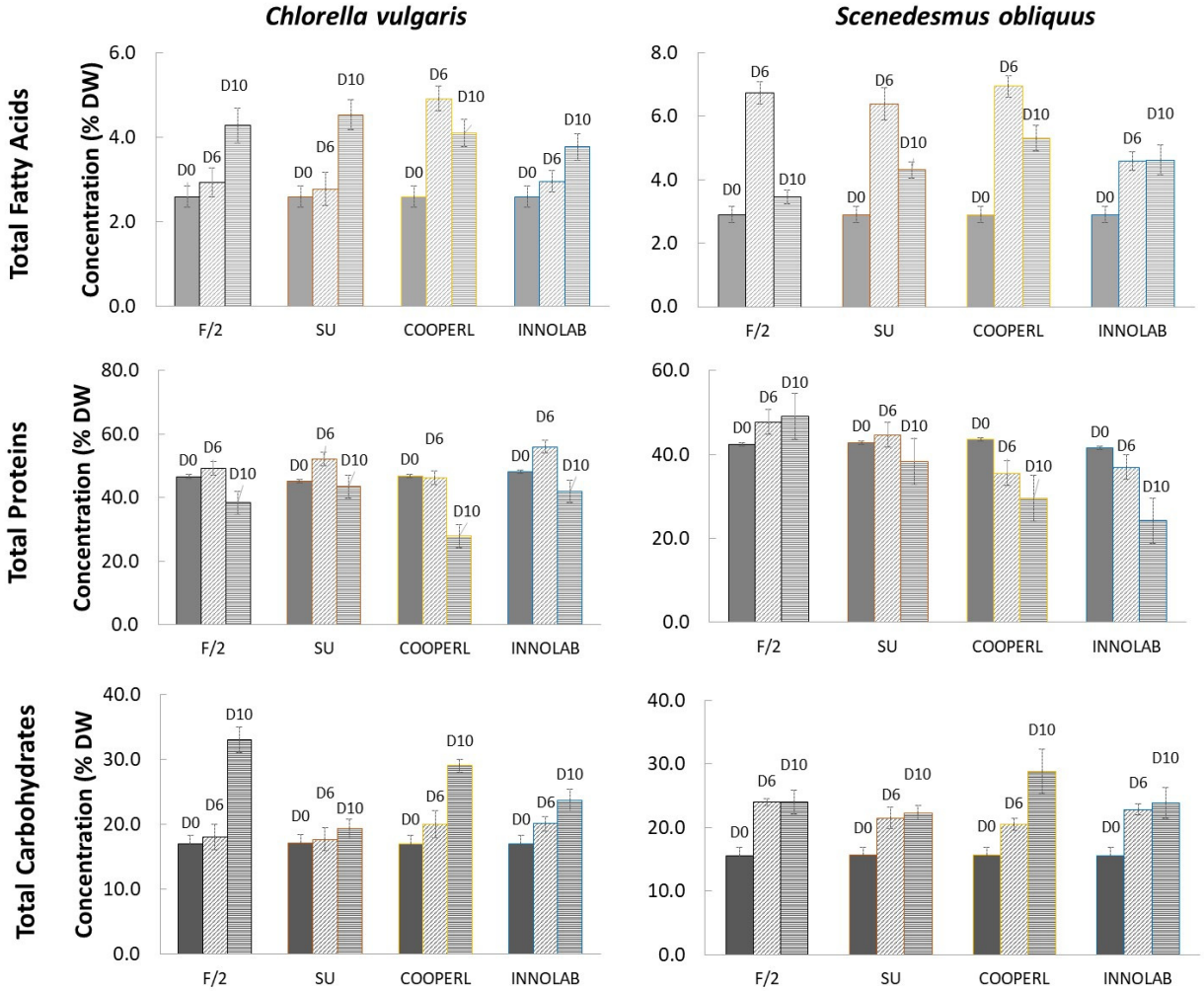

Figure 5. Total fatty acids, proteins, and carbohydrates content in cultures of *C. vulgaris* and *S. obliquus* grown on three different digestates under pH manipulation on day 0, 6, and 10 of the experiment. Error bars represent the standard deviation on three replicates.

Carbohydrates increased during the experiment in both cultures and for all conditions tested. In cultures of *C. vulgaris*, the most significant increases occurred between day 6 and day 10 of the experiment in the F/2 control and cultures grown on the Cooperl and Innolab digestates ($p < 0.001$), with the highest carbohydrate concentration of 33 ± 2% DW measured in the F/2 control (Figure 5). In cultures of *S. obliquus*, an increase in carbohydrate content was most significant between day 0 and day 6 ($p < 0.001$) for the F/2 control and the SU and Innolab digestates. In cultures grown on the Cooperl digestate, carbohydrates increased the most between days 6 and 10 and reached a highest recorded carbohydrate concentration of 28.8 ± 1.0% DW. Statistically, carbohydrates were significantly higher in cultures of *C. vulgaris* in the F/2 control ($p < 0.001$, Figure 5); however, no significant differences were found between the two strains in the digestates conditions ($p > 0.05$).

4. Discussion

In this study, two microalgal strains were grown on three digestates resulting from the anaerobic digestion of different feedstocks. Digestates were treated to suit microalgal needs, and a concentration of 2% of the three digestates was used to grow *C. vulgaris* and

S. obliquus for 10 days (digestate optimisation based on Fernandes et al. [12]). Due to the volatile nature of ammonium, especially at a high pH, which is a characteristic of digestate, the pH was manipulated to reach acidic values of 6–6.5 and a comparison was made against cultures where the pH was not modified.

Results showed that pH manipulation to limit NH_4^+ evaporation from the different tested media generated better performances from the microalgae *S. obliquus*, with an overall better growth rate and dry weights recorded across all digestates. The same pH manipulation did not necessarily yield better growth in cultures of *C. vulgaris*, which demonstrated signs of stress, mainly via the clumping of cells. However, the dry weight was higher when the pH was maintained to be acidic in comparison to no pH manipulation. Additionally, nutrient data revealed that evaporation likely took place when the pH was not manipulated; indeed, the observed decrease in ammonium during the experiment did not coincide with a corresponding gain in the biomass dry weight. A delayed consumption of ammonium was observed for *S. obliquus* under an acidic pH, and the gain in dry weight was more in accordance with recorded ammonium data. The slow consumption of ammonium by both *S. obliquus* and *C. vulgaris* could be the result of an acclimation to the ammonium present in digestate; the same acclimation occurred in the modified F/2 control where the nitrogen source was supplied by ammonium chloride, demonstrating that ammonium was the main factor resulting in the observed acclimation and that digestate did not present other toxicity to both microalgal strains. Acclimation to a newly introduced source of nitrogen has been described in the literature; for example, Chuka-ogwude et al. [33] showed that 6 weeks of acclimation time to a high ammonium concentration was needed for both *C. vulgaris* and *S. obliquus*. In the present study, *C. vulgaris* did not appear to consume ammonium from digestate after 10 days of the experiment; however, *S. obliquus* consumed ammonium almost instantly after 8 days of acclimation, showing better acclimation capabilities in the presented work. Phosphorus was mainly consumed under no pH manipulation, except in decaying cultures, where increasing concentrations of phosphorus were recorded and likely linked to a release from dying cells [34,35]. In cultures of *C. vulgaris*, an acidic pH induced a phosphorus release via the dissolution of digestate particles; thus, an increase in the compound over the course of the experiment was observed [36–38]. However, in cultures of *S. obliquus*, a decrease in phosphorus was observed after several days, showing consumption by the microalgae. In environments with high concentrations of phosphorus, the phenomenon of P luxury uptake can occur [39,40], and phosphorus is accumulated under the form of polyphosphate in microalgal cells [41,42]. Polyphosphates facilitate the incorporation of metal ions and, consequently, can contribute to the remediation of heavy metals in waste streams [43]; additionally, the excess phosphorus uptake by microalgae promotes phosphorus removal from waste [44]. In the presented work, an acidic pH seemed to promote luxury uptake in *S. obliquus*, demonstrating the relevance of pH control in waste bioremediation systems.

In this study, an acidic pH provided better growth performances, especially in cultures of *S. obliquus*; this was in accordance with findings from Guedes et al. [45], who demonstrated that the highest growth rate and biomass productivity in *S.obliquus* were associated with a pH of 6. The better growth performances in acidic pH could be linked to a better nutrient availability as discussed above. Indeed, while an acclimation phase occurred, ammonium was available for the microalgae to uptake beyond this acclimation, rather than evaporating into the environment. This conveyed a significant advantage in terms of reducing ammonium leakage into the atmosphere, especially when considering a larger scale for industrial applications of waste remediation by microalgae. Furthermore, a higher ammonium availability allowed for greater biomass accumulation, which was reflected in the dry weight recorded at the end of the experiment. Bouras et al. [46] found that a neutral pH of 7 provided the best performances in terms of growth and DHA accumulation in *Schizochytrium limacinum*; however, they also tested a pH of 6, which yielded the second-best performances in cultures. On the contrary, higher pHs of 8 and 9 resulted in less growth, as well as a rapid ammonium decrease from the medium, linked to evaporation,

and the authors directly linked the lesser growth to the lack of ammonium availability in the medium following evaporation.

The presented work demonstrated that an acidic pH had clear advantages in terms of an improved nutrient availability and uptake, resulting in a more consistent and higher biomass production from microalgae grown on digestate, and this result could be further improved by looking at acclimation strategies to novel sources of nitrogen. Due to the naturally high pH of digestate, efforts should be made towards controlling this factor to ensure that ammonium recovery is mainly performed via microalgal growth, rather than evaporation. This is particularly true when considering the larger-scale system, for which, abiotic parameters are more difficult to control and evaporation can easily occur.

Another aspect of this study was to assess if there was any specificity between microalgal strains and digestates, in an effort to further optimise remediation by tailoring the combination of digestate and strain. Regardless of pH manipulation, both microalgal strains yielded less growth on the modified F/2 control in comparison to the three tested digestates. This could be explained by ammonium chloride being less available to microalgae in comparison to ammonium from digestate. Additionally, digestate can be enriched with dissolved CO_2 generated during the methane fermentation process; this CO_2 can be utilised for photosynthesis [47]. Under no pH manipulation, *C. vulgaris* grew better on the SU digestate, whereas growth was higher in the Innolab digestate when an acidic pH was maintained. In cultures of *S. obliquus*, better performances were recorded for SU and Cooperl digestates in both pHM^+ and pHM^-, which was reflected in the absorbance and dry weight data. Therefore, it can be assumed that certain types of digestates can be favourable to some microalgal strains; however, this can be modified by environmental factors, and, in the case of the presented study, by pH manipulation, which was particularly true for *C. vulgaris*. It could hence be assumed from previous results that an acidic pH had the potential to modify digestates properties, notably by increasing the nutrient availability, and hence shifting strain performances from one digestate to another. Similarly, digestates yielding less growth could have inhibitory factors specific to the tested strains [48]. The literature focuses on ammonium toxicity [49–51], but the digestate composition can be extremely complex and other inhibitory elements can be at play. Another well-known property of digestate is its dark colour, limiting light availability for photosynthesis, and this has been mainly counteracted by dilution (including in this study) [14,25,52–55]. The transmittance of the different digestates used in this work could explain why the two microalgal strains yielded different growth rates on the three tested digestates. Indeed, while the three digestates were diluted to obtain a similar ammonium concentration, transmittances were different as the digestates did not have the same particle composition and were produced from different feedstocks. Wang et al. [56] demonstrated that microalgal growth was negatively correlated to the digestate transmittance; therefore, light availability could be one of the factors behind the different growth rates observed in the present experiment. The presented work demonstrated that microalgal strains can perform differently on different types of digestates, with a clear influence of environmental factors such as pH or light transmittance. Thus, when considering microalgae for digestate remediation, it is crucial to implement an initial strain screening to optimise the digestate uptake and, consequently, bioremediation.

Composition analysis of *C. vulgaris* and *S. obliquus* showed a higher fatty acid content when the strains were grown on the different digestates in comparison to the F/2 control. Similar findings can be seen in the literature [57], especially when diluted digestate is used (which was the case in this work). Indeed, the lower nitrogen content of diluted digestate allows for an increase in lipid accumulation, and this was found for many microalgal species, including *Neochloris oleoabundan* [58], *Synechocystis* sp. and *Nannochloropsis salina* [59], and *Chlorella* sp., *Scendesmus* sp. and *Nannochlorpsis gaditana* [14,52]. Additionally, fatty acid accumulation was different between the two microalgal strains studied; indeed, higher fatty acids concentrations were found after 10 days of the experiment for *C. vulgaris*, whereas the same result was observed after 6 days of experiment in cultures of *S. obliquus*. Fatty acid accumulation in microalgal cells is mainly based on nutrient deprivation [46,60]. In

this study, the nitrogen results showed very little consumption of the ammonium from the digestates until the end of the experiment due to an acclimation period. This could explain why *C. vulgaris* had the highest fatty acids content at the end of the experiment, as nutrients from the upscaling phase were depleted and the microalgae had not acclimatised to the ammonium from digestate yet. *S. obliquus* had the highest concentration of fatty acids at day 6, and ammonium results showed that the microalgae consumed ammonium almost instantly between day 8 and day 10 of the experiment. Therefore, fatty acids were accumulated until day 6, when nutrients were depleted and decreased once *S. obliquus* acclimatised to the novel source of ammonium, shifting from nutrient depletion to nutrient consumption. However, the very limited consumption of nitrogen linked to the observed acclimation of both strains led to a decrease in proteins over the time of experiment. Indeed, protein accumulation is directly correlated to nitrogen uptake [61,62], which was very minimal in the present study. Carbohydrates, on the other hand, increased significantly during the experiment, reaching maximum concentrations after 10 days for all digestates and the F/2 control. Similarly to the lipid content, nitrogen starvation can increase the accumulation of carbohydrates [61,63–65]. Indeed, under nitrogen depletion, microalgae can transform proteins into carbohydrates by shifting the use of photosynthetic carbon from the metabolic pathway of protein synthesis to carbohydrates synthesis, hence accumulating this compound as energy storage [66,67]. This was also in accordance with the observed decrease in proteins during the present experiment. Additionally, the synchronic increase in lipids and carbohydrates can be explained by their parallel synthesis pathways, where energy is firstly stored as carbohydrates and then converted into lipids [68]. Similar results were found in the literature; for example, Brányiková et al. [69] reported a carbohydrate accumulation of up to 410 g kg^{-1} in cultures of *C. vulgaris*, where nitrogen was depleted, and Ji et al. [70] obtained a carbohydrate content in *Tetraselmis subcordiformis* of 350 g kg^{-1}.

In this study, the acclimation of *C. vulgaris* and *S. obliquus* to ammonium from digestate, which resulted in conditions of nitrogen depletion, also conveyed advantages in terms of macronutrient accumulation, with significant increases in lipids and carbohydrates in the produced biomass. It can be further assumed that, after acclimation and when ammonium consumption resumes, other compounds such as proteins could, in turn, be accumulated. Therefore, the harvesting time of the biomass is crucial when targeting specific compounds from microalgae grown on digestate, and different accumulation stages can occur, demonstrating the versatility of digestate to obtain a variety of high-value products.

5. Conclusions

The presented work demonstrated the importance of pH control, especially in terms of nutrient availability for microalgae, in order to ensure that bioremediation takes place via microalgal growth rather than by ammonium loss to the atmosphere. While looking at upscaling this technology, lower pHs of 6–6.5 should be maintained to minimise these ammonium losses, which are bound to occur in larger-scale systems. This study also emphasised the necessity for strain screening when considering digestate bioremediation; indeed, strains can perform differently on different digestates from different feedstock. Here, a pig-manure-based digestate yielded better dry weights in both *S. obliquus* (0.23 ± 0.002 g L^{-1}) and *C. vulgaris* (0.49 ± 0.012 g L^{-1}). Additionally, environmental factors and specific inhibitors can also influence performances and should be carefully considered. Finally, this study shed some light on acclimation processes in microalgae and on the resulting biomass composition, showing some advantages in terms of lipids and carbohydrate composition, where nitrogen starvation resulted in 6.94% DW of fatty acids in *S. obliquus*. The harvesting time was also found to be an impactful factor when targeting high-value compounds, and hence the importance of this work, which revealed some of the dynamics linked to nitrogen uptake from digestate. The presented work showed promising results and current findings could be utilised to further the field of digestate bioremediation using microalgae, especially for larger-scale applications and for high-value ingredient production within a circular economy.

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