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Microalgal Growth in Aquaculture Effluent: Coupling Biomass Valorisation with Nutrients Removal

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Abstract: Natural resources are becoming increasingly scarce, and the need to control their consumption and recycle their use is growing. Water is one of the essential resources for human survival. Therefore, there has been an increasing interest in ways to save, recycle and treat water supplies. Aquaculture is one of the most polluting activities as it produces a significant wastewater volume, which needs proper treatment before being discharged into the environment or recycled. Microalgae are a potential solution for wastewater treatment. Due to their numerous advantages, the use of microalgal biomass is being studied, and, at present, there is already a market and room for profit in the sale of microalgal components in various forms, such as animal and human supplements. From a biorefinery point of view, it is important to take advantage of all the qualities and benefits that microalgae have by combining their great capacity to treat wastewater and exploit the produced biomass, analysing its composition for subsequent valorisation, for example. In this study, *Chlorella vulgaris* was used to treat aquaculture wastewater from a trout farm aquaculture facility, and the treatment efficiency was evaluated. To valorise the resulting biomass, its composition was also assessed. *C. vulgaris* successfully grew in the effluent with growth rates of 0.260 ± 0.014 d^{-1} and with average productivity of 32.9 ± 1.6 mg L^{-1} d^{-1}. The achieved removal efficiencies were 93.5 ± 2.1% for total nitrogen, 98.0 ± 0.1% for nitrate-nitrogen and 92.7 ± 0.1% for phosphate-phosphorus. Concerning biomass composition, the lipids (15.82 ± 0.15%), carbohydrates (48.64 ± 0.83%), and pigment contents (0.99 ± 0.04% for chlorophyll a + b and 0.21 ± 0.04% for carotenoids) were similar to the values of similar studies. However, the protein content obtained (17.93 ± 1.21%) was lower than the ones mentioned in the literature.

Keywords: aquaculture; biomass; circular economy; microalgae; wastewater treatment

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1. Introduction

A highly important issue nowadays concerns the environment and natural resources, namely water resources. Water pollution is mainly caused by the industrial and agricultural sectors and other activities, for instance, aquaculture. These sectors produce wastewaters that contain non-natural synthetic compounds and organic and inorganic substances. If the wastewater is not treated and discharged into the environment, the water quality and the surrounding ecosystems can be jeopardised [1]. Aquaculture farms typically use feed rich in protein and carbohydrates, and, to ensure high growth rates, nitrogen and phosphorus are usually added too. However, oversupply can occur, leading to an accumulation of these nutrients in the medium [2]. The abrupt enrichment of nutrients in water favours the growth of microalgae and aquatic plants that will put at risk the balance of the present ecosystem, overlapping the other organisms [3]. More specifically, algae

growth on the water surface causes a reduction in oxygen levels. This deficit affects aquatic organisms, leading to the destruction of the ecosystem. A sudden increase in algae growth due to the enrichment of the medium with nutrients (nitrogen and phosphorous) is called eutrophication [4]. To prevent eutrophication, the European Union (EU) has taken steps to better control the levels of these nutrients in wastewaters. Accordingly, the limits for urban wastewater discharge are: (i) 10 or 15 $mg_N\ L^{-1}$ for nitrogen, with 70–80% of minimum reduction; and (ii) 1 or 2 $mg_P\ L^{-1}$ for phosphorus, with 80% of minimum reduction [5,6].

To avoid the pollution of watercourses with uncontrolled discharges of effluents, it is necessary to apply effective treatment methods. The techniques commonly used in wastewater treatment plants to remove nitrogen and phosphorus, such as biological nitrification and denitrification and chemical precipitation, are energy-intensive, not eco-friendly and lead to the production of sludge and the release of greenhouse gases [7]. Microalgae are an environmentally friendly and low-cost alternatives that can be used as a solution for urban wastewater treatment and polishing. Microalgae are photosynthetic organisms whose dimensions vary between 2 and 30 µm [8]. These microorganisms are found mostly in aquatic environments and have higher biomass productivity when compared to terrestrial plants [9]. Additionally, microalgal biomass is very rich in lipids (such as eicosapentaenoic and docosahexaenoic acids) and pigments. Some species even produce antioxidant pigments, while others contain vitamins and immunostimulants. Microalgal biomass also contains proteins and carbohydrates. The characteristics of microalgae have several advantages in their use. Since they are photosynthetic organisms, they can capture carbon dioxide (CO_2) from the atmosphere or combustion gases [10]. Microalgal biomass can be further exploited for the production of biofuels and food supplements (for both humans and animals) and extracted pigments can be used in the cosmetic and pharmaceutical industries [11,12]. Since microalgae can grow and survive in adverse aquatic environments and use the nitrogen and phosphorus in wastewater to grow, they are considered an economic wastewater treatment technique [1,13,14]. Viegas et al. [15] used *Chlorella vulgaris*, *Isochrysis galbana*, *Nannocloropsis salina*, *Scenedesmus obliquus*, and *Spirulina major* to treat aquaculture wastewater. In batch mode, all the tested species were able to remove 100% of total nitrogen (TN) and total phosphorus (TP). Additionally, at least 72% of chemical oxygen demand (COD) was remediated by the microalgae. Hawrot-Paw et al. [16] cultivated *Chlorella minutissima* in saline aquaculture wastewater and observed a decrease of 88% in TN and 99% in TP.

In fact, the mass cultivation of microalgae, with application in wastewater treatment, has already captured global interest [17,18]. Moreover, there is great potential in replacing food and fish oils with microalgae in aquaculture. Microalgal biomass has fair amounts of the proteins, lipids, vitamins, and pigments necessary for aquaculture feed. Additionally, using microalgae as a supplement to animal feed enhances growth and, at the same time, decreases feed consumption. Furthermore, this diet boosts the immunological response and resistance to disease by increasing antibacterial and antiviral activity [19]. Sarker et al. [20] used a combination of *Nannochloropsis oculata* and *Schizochytrium* sp. to feed Nile tilapia fish and found that the microalgal-based feed increased the nutritional value and fish growth indicators. Additionally, Sarker et al. [21] studied *Isochrysis* sp. and *Nannochloropsis* sp. as substitutes for fish oil and fish meals in a rainbow trout diet. The authors found that *Isochrysis* sp. enhances the digestibility of proteins and lipids, when compared to traditional fish meal and oil.

However, the feed for aquaculture has to be easy to digest and have a good nutritional diet. Thus, current studies only incorporate microalgal biomass in the cultivation feed and do not use microalgal biomass as a substitute, since the amount that best benefits growth and survival rates has not yet been optimised [22]. It is still necessary to investigate the possible consequences of using the biomass of microalgae grown in wastewater and find effective and achievable solutions in the context of mass production of feed and/or supplements for aquaculture. It is possible to apply the circular economy concept to the use of microalgae in aquaculture. In other words, it is a way of making greener one of the

economic activities that contribute most to water pollution. The aquaculture wastewaters can be treated by microalgae, whose biomass can be later used as an additive or supplement to the aquaculture feed, thus avoiding the addition of extra nutrients and fish oils, which are traditionally used and are one of the main causes of excess nutrients in the aquaculture wastewater. Therefore, this study aimed to assess the growth of *C. vulgaris* in aquaculture wastewater from a trout farm aquaculture facility and valorise the produced biomass through the analysis of its biochemical composition. Nitrogen and phosphorus removal efficiencies were assessed, and the biomass biochemical composition was analysed in terms of lipids, carbohydrates, proteins, and pigments.

2. Materials and Methods

2.1. Microorganisms and Culture Medium

The microalgae *C. vulgaris* CCAP 211/11B was acquired from the Culture Collection of Algae and Protozoa (CCAP, Oban, UK). The stock solutions were made in 100 mL Erlenmeyer flasks using the modified Organization for Economic Co-operation and Development (OECD) test medium described by Salgado et al. [23]. The Erlenmeyer flasks were continuously exposed to light (light:dark ratio of 24:0) with 6.50 µmol m^{-2} s^{-1} of intensity and kept at room temperature. An orbital shaker (Unimax 1010, Heidolph, Germany) provided agitation at 120 rpm.

2.2. Aquaculture Wastewater

The wastewater from a trout farm aquaculture facility was characterised (Table 1) and used as a culture medium for the cultivation of microalgae. This effluent is discharged in the public wastewater collector system and subsequently treated in wastewater treatment plants as urban effluent.

Table 1. Characterisation of the aquaculture effluent used in this study.

Parameters	Values	Unit
pH	7.53 ± 0.01	-
Conductivity	1129 ± 1	µS cm^{-1}
Turbidity	0.655 ± 0.007	NTU
TDC	14.62 ± 0.08	mg C L^{-1}
DOC	5.86 ± 0.43	mg C L^{-1}
DIC	8.76 ± 0.17	mg C L^{-1}
TN	20.23 ± 0.54	mg N L^{-1}
Nitrate-nitrogen (NO_3-N)	10.35 ± 0.22	mg N L^{-1}
Phosphate-phosphorus (PO_4-P)	2.22 ± 0.01	mg P L^{-1}
COD	10.1 ± 0.6	mg O_2 L^{-1}

COD—chemical oxygen demand; DIC—dissolved inorganic carbon; DOC—dissolved organic carbon; TDC—total dissolved carbon; TN—total nitrogen; NTU—nephelometric turbidity unit.

A multi-parameter analyser (Consort's C6010, Brussels, Belgium) was used to measure temperature, pH and conductivity. Turbidity was determined with a turbidimeter (Hanna Instruments HI88703, RI, Smithfield, VA, USA). The effluent composition in terms of different forms of carbon (DIC—dissolved inorganic carbon; DOC—dissolved organic carbon; TDC—total dissolved carbon) and nitrogen (as TN) were measured using a total organic carbon analyser. The Brucine method, used to quantify nitrate-nitrogen (N-NO_3), is based on the reaction between brucine in an acidic medium, resulting in a yellow colouration whose absorbance was measured at 410 nm on a UV/vis spectrophotometer (Genesys 10 UV, Thermo Scientific, Waltham, MA, USA) [24]. Phosphate-phosphorus quantification (P-PO_4) was performed by a reaction with ammonia molybdate, resulting in blue colouration. Then, the absorbance at 820 nm was determined in a UV/vis spectrophotometer (Genesys 10 UV, Thermo Scientific, Waltham, MA, USA) [25]. COD was quantified by the closed reflux method, which is based on the oxidation of organic compounds by potassium dichromate, in an acidic medium, at boiling temperature [26].

2.3. Experimental Setup

Batch experiments were conducted over 11 d in 1 L flasks, with a working volume of 900 mL. Different experimental conditions were tested (Figure 1): (i) a negative control test (C−) consisting only of 1 L of aquaculture wastewater; (ii) an assay with *C. vulgaris* in aquaculture wastewater (AW) in triplicate; and (iii) a positive control test (C+) with *C. vulgaris* in the modified OECD medium, in duplicate.

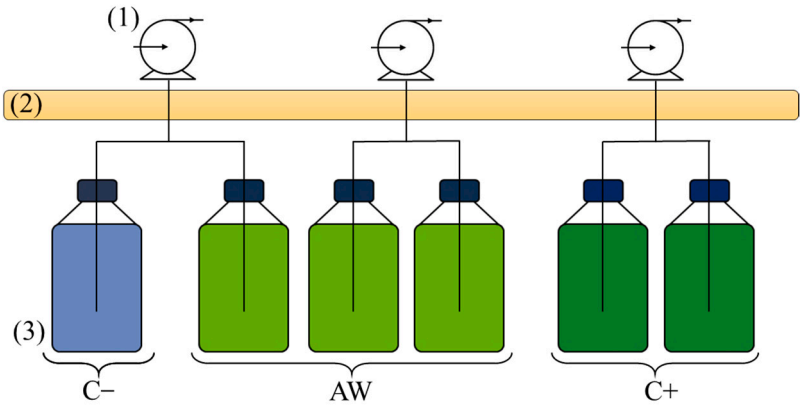

Figure 1. Schematic diagram of the experimental setup: (1) air pumps; (2) light-emitting diode (LED) panel; and (3) culture flasks.

The optical density at a wavelength of 680 nm (OD680) was measured daily with a spectrophotometer (Spectroquant Prove 300, Merck, Darmstadt, Germany) to evaluate the microalgal growth. The initial biomass concentration in the assays in terms of dry weight (dw) was 93.0 ± 2.7 mg_{dw} L^{-1}. The pH and temperature were quantified daily using a pH meter (Consort's C6010, Brussels, Belgium). The defined pH was 7.53 (pH measured in the effluent on day 0), being adjusted daily to this value. The flasks were kept at an ambient temperature of 18.1 ± 1.4 °C. The light was continuously supplied (light:dark ratio of 24:0) by a light-emitting diode (LED) panel with a light intensity of 77.8 ± 4.6 µmol m^{-2} s^{-1}. A radiometer (HD 2102.2, Delta OHM, Caselle, Italy) was used to determine the light intensity. Agitation and CO_2 were ensured by means of air pumps (Sicce Airlight 3300, Pozzoleone, Italy), delivering 1.7 L min^{-1} of atmospheric air flow rate.

The samples were collected throughout the study with different periodicities according to the objective. Daily samples were taken from each flask to determine the kinetic parameters. Meanwhile, to evaluate the intake of nutrients, samples were collected on days 0, 1, 2, 4, 7, 9, and 11, and centrifuged (Himac CT6E Centrifuge, VWR, Amadora, Portugal) for 10 min at 4000 rpm and stored at −20 °C. The quantification of nutrients TN, N-NO_3, P-PO_4 and COD was performed using the previously described methods.

At the end of the experiment, the cultures were centrifuged at 20 °C for 10 min at a speed of 12,000 rpm. The resulting biomass was stored at −80 °C and, finally, lyophilised. Finally, the lyophilised biomass was macerated with a mortar and pestle to obtain a powder and to ensure cell rupture so that the subsequent extraction of proteins, carbohydrates, lipids, and photosynthetic pigments was facilitated. The extraction assays were done in triplicate. The proteins were quantified through the Lowry method [27]. Carbohydrates were quantified using a colourimetric method [28]. The lipids content was obtained by a modified Bligh and Dyer method [29]. Finally, the pigments were analysed in accordance with the method described by Clément-Larosière et al. [30] and Lightenthaler [31] with some modifications.

2.4. Kinetic Growth Parameters

The OD_{680} values collected throughout the experiment allowed the calculation of biomass concentration using a calibration curve previously determined (see Equation (1))

that relates the OD_{680} with the biomass concentration (X, $mg_{dw}\ L^{-1}$), using the method described by Esteves et al. [32].

$$OD_{680} = 0.00543X + 0.06013$$
$$\left(R^2 = 0.995,\ \text{Limit of detection} = 11.12\ mg_{dw}\ L^{-1},\ \text{Limit of quantification} = 37.08\ mg_{dw}\ L^{-1}\right) \quad (1)$$

From the biomass concentration values, the specific growth rate (μ in d^{-1}) was determined for each experiment through Equation (2), where X_1 and X_0 represent the biomass concentration, in $mg_{dw}\ L^{-1}$, at the start (t_0) and at the end (t_1) of the exponential growth phase in d.

$$\frac{dX}{dt} = \mu X \Leftrightarrow \mu = \frac{\ln(X_1/X_0)}{t_1 - t_0} \quad (2)$$

With the biomass concentration values previously determined, it was possible to calculate: (i) the instantaneous productivity (P_x in $mg_{dw}\ L^{-1}\ d^{-1}$) between consecutive samples (X_z and X_{z+1}) through Equation (3); (ii) the average productivity ($P_{x,\,avg}$, see Equation (4)) where X_i and X_f correspond to the biomass concentration ($mg_{dw}\ L^{-1}$) at the start and end of the assay, and t_f represents the final time of the assay (d); and (iii) the maximum productivity ($P_{x,max}$), which consists of the maximum P_x value.

$$P_x = \frac{X_{z+1} - X_z}{t_{z+1} - t_z} \quad (3)$$

$$P_{x,\,avg} = \frac{X_f - X_i}{t_f} \quad (4)$$

2.5. Nutrient Removal

The nutrient concentrations determined by the methods described above allowed us to calculate the removal efficiency (RE, %, Equation (5)) and the removal rate (RR, mg $L^{-1}\ d^{-1}$, Equation (6)), where S_i (mg L^{-1}) represents the concentration of a given nutrient at the initial time (t_i, d) and S_f (mg L^{-1}) the concentration of the nutrient at the final time (t_f, d) of the experiment.

$$RE\ (\%) = \frac{S_i - S_f}{S_i} \times 100 \quad (5)$$

$$RR = \frac{S_i - S_f}{t_f - t_i} \quad (6)$$

To determine the nutrient uptake rates, the experimental data were fitted to the modified Gompertz model [33] (Equation (7)), where S (t) represents the evolution of the nutrient concentration over time, k is the nutrient uptake rate (in d^{-1}) and λ is the lag time (in d). Model parameters were achieved based on minimising the sum of squared errors using Microsoft Excel Solver Add-in. Determined models were evaluated by calculating performance indexes: R^2—coefficient of determination and RMSE—root mean squared error.

$$S\ (t) = S_i + (S_f - S_i) \times \exp(-\exp[k \times (\lambda - t) + 1]) \quad (7)$$

To better understand the relationship between biomass and substrate, the specific biomass yield ($Y_{X/S}$, $g_{dw}\ g_s{}^{-1}$) was determined through Equation (8), where RR is the nutrient removal rate (mg $L^{-1}\ d^{-1}$).

$$Y_{X/S} = \frac{P_{x,\,avg}}{RR} \quad (8)$$

2.6. Statistical Analysis

The differences between each assay were evaluated through the statistical significance of the achieved results (average and standard deviation of each parameter) using the Student's paired *t*-test at a significance level of 0.05.

3. Results and Discussion

3.1. Microalgal Growth

Figure 2 describes the evolution of *C. vulgaris* growth over time. Overall, the results show that *C. vulgaris* grew successfully in aquaculture wastewater. Comparing the growth curves of AW and C+ assays, it is possible to observe that in the beginning they are similar; however, from day 4, the microalgal growth in AW decreases, possibly due to microalgae having already consumed practically all the nutrients in the wastewater. Additionally, as predicted, there was no microalgal growth in C−, proving that there was no presence of microalgae in the effluent.

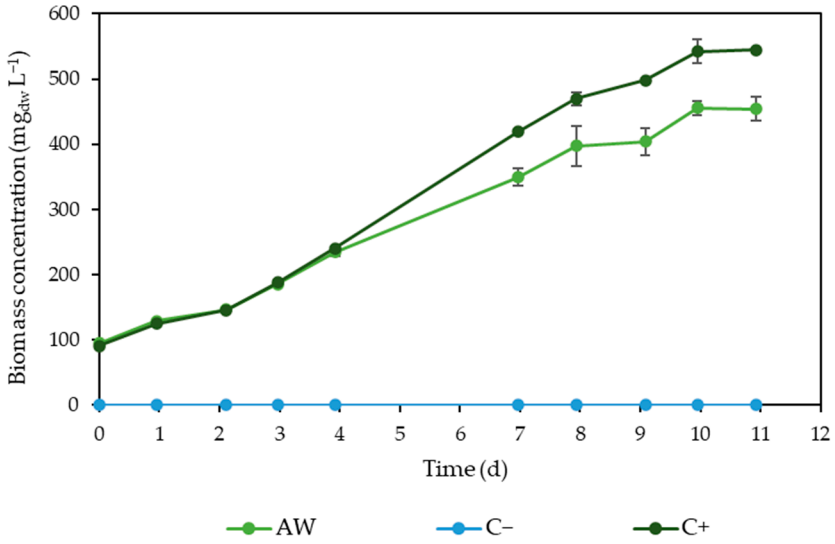

Figure 2. Growth curves of AW, C− and C+ assays.

Table 2 contains the calculated kinetic parameters for each assay. The microalgal specific growth rates in AW (0.260 ± 0.014 d^{-1}) and C+ (0.276 ± 0.004 d^{-1}) assays were not statistically different ($p > 0.05$), which may be an indication that the microalgae in the aquaculture wastewater had the necessary conditions for growth, and that no harmful contaminants were present. Liu et al. [13] used aquaculture wastewater to produce microalgal biomass and obtained a growth rate for the *C. vulgaris* of 0.081 ± 0.008 d^{-1}, a value lower than the one obtained in this study. The C+ assay reached a statistically higher ($p < 0.05$) maximum biomass value (555.8 ± 18.9 mg_{dw} L^{-1}), compared to the AW assay (455.9 ± 10.9 mg_{dw} L^{-1}). The maximum productivity was not statistically different ($p > 0.05$) between the AW (60.0 ± 11.8 mg_{dw} L^{-1} d^{-1}) and C+ (58.8 ± 1.3 mg_{dw} L^{-1} d^{-1}) assays. The average productivity was statistically higher ($p < 0.05$) in C+ (41.6 ± 0.1 mg_{dw} L^{-1} d^{-1}) than in AW assay (32.9 ± 1.6 mg_{dw} L^{-1} d^{-1}). These parameters provide a better understanding of the optimal microalgae conditions, leading to more efficient growth. Therefore, although the microalgae in the AW assay were not cultivated in an optimal medium, they were able to grow as much as the positive control. This reveals that the use of aquaculture wastewater in microalgae cultivation is appropriate and viable.

Table 2. Kinetic growth parameters calculated for the AW and C+ assays.

Experiment	μ (d^{-1})	X_{max} (mg_{dw} L^{-1})	$P_{x,max}$ (mg_{dw} L^{-1} d^{-1})	$P_{x,\,avg}$ (mg_{dw} L^{-1} d^{-1})
AW	0.260 ± 0.014 [a]	455.9 ± 10.9 [a]	60.0 ± 11.8 [a]	32.9 ± 1.6 [a]
C+	0.276 ± 0.004 [a]	555.8 ± 18.9 [b]	58.8 ± 1.3 [a]	41.6 ± 0.1 [b]

$P_{x,max}$—maximum biomass productivity; $P_{x,\,avg}$—average biomass productivity; X_{max}—maximum biomass concentration; μ—specific growth rate. In the same column, values with a common letter ([a] and [b]) are not statistically different ($p > 0.05$).

3.2. Wastewater Treatment

The capacity of *C. vulgaris* to remove nitrogen in the form of TN and NO_3-N and phosphorus in the form of PO_4-P from the aquaculture wastewater was analysed. Figure 3 presents the removal of TN, NO_3-N and PO_4-P by microalgae in each assay and the model fit of the modified Gompertz model to the experimental data. The respective legislation limits (Directive 91/271/EEC concerning discharges from urban wastewater treatment plants to sensitive areas subject to eutrophication) are also represented in the figure. Table 3 presents the initial nutrients concentration, the removal efficiencies and the kinetic parameters determined by adjusting the modified Gompertz model to the experimental data. By analysing Figure 3, it is possible to see that in the AW assay: (i) the TN concentration reached values below the EU legislation after 2 d; (ii) the NO_3-N concentration was already below 10 mg L^{-1} after 1 d; and (iii) the PO_4-P concentration was below the legal limit after 2 d. Therefore, the results show that *C. vulgaris* removed nitrogen and phosphorus efficiently from aquaculture wastewater, reaching values below the above-mentioned legislation limits in a short period of time.

As mentioned before, in the C+ assay, the microalgae were cultivated in a modified OECD medium, containing initial amounts of nutrients different from those present in the AW assay, where aquaculture wastewater was used as the culture medium. Indeed, Table 3 shows that the initial concentration of each nutrient is higher in C+ than in the AW assay. Aquaculture wastewaters usually present high nitrogen and phosphorus concentrations [34]. However, in the present study, the wastewater comes from a small-scale aquaculture plant, which may explain the low concentrations of the N and P obtained.

Even though the initial concentration of TN was statistically higher ($p < 0.05$) in the C+ assay, the removal efficiencies in AW and C+ assays were not statistically different ($p > 0.05$), being 93.5 ± 2.1% and 95.6 ± 1.4%, respectively. Comparing the TN uptake rate obtained from the modified Gompertz model, in AW assays, a value of 0.83 ± 0.26 d^{-1} was achieved, whereas in the C+ assay the nutrient removal rate was 0.44 ± 0.07 d^{-1}. It is also important to mention that the lag time was longer in the C+ assay. Regarding the initial NO_3-N concentration, the value obtained was statistically lower ($p < 0.05$) in the AW assay (10.7 ± 0.8 mg L^{-1}) than in C+ (38.0 ± 0.3 mg L^{-1}). Both assays achieved high values of NO_3-N removal efficiencies, 98.0 ± 0.1% for AW and 99.4 ± 0.1% for C+. The NO_3-N uptake rate given by the modified Gompertz model was higher in the AW assay (0.92 ± 0.21 d^{-1}) than in C+ (0.48 ± 0.18 d^{-1}). The initial PO_4-P concentration was statistically higher ($p < 0.05$) in the C+ assay (10.4 ± 0.1 mg L^{-1}) than in AW (2.2 ± 0.1 mg L^{-1}). The PO_4-P removal efficiency was >90% in both assays, but the one obtained in C+ (98.4 ± 0.1%) was statistically higher ($p < 0.05$) than the removal efficiency in AW (92.7 ± 0.1%). Additionally, the highest PO_4-P uptake rate (1.48 ± 0.16 d^{-1}) and lowest lag time (0.45 ± 0.11 d) were achieved in the AW assay. The uptake rate was higher in the AW assay for all the nutrients analysed, which can be related to the lower initial concentrations of these nutrients in the aquaculture wastewater. As the initial biomass concentration was the same, the microalgae were able to assimilate the nutrients faster in AW, due to the nutrient limitation, resulting in a higher uptake rate. By analysing the performance metrics of the modified Gompertz model (R^2 and RMSE) used in this study, one can see that the obtained R^2 are all near one (≥0.994), and the obtained RMSE values are low (0.013–1.926 mg L^{-1}), demonstrating the adequacy of the model to accurately represent the experimental data.

The NO_3-N and PO_4-P removal efficiencies obtained within this study regarding the aquaculture wastewater treatment, 98.0 ± 0.1% and 92.7 ± 0.1%, respectively, were similar to the literature values. Ansari et al. [35] used *Chlorella sorokiniana*, *S. obliquus* and *Ankistrodesmus falcatus* to treat aquaculture wastewater for 14 d and achieved NO_3-N removal efficiencies from 75.8 to 80.9% and PO_4-P removal efficiencies between 98.5 and 100%. Guldhe et al. [36] also tested *C. sorokiniana* in aquaculture wastewater treatment and obtained a NO_3-N removal efficiency of 84.5% and a PO_4-P removal efficiency of 73.4%. Liu et al. [13] cultivated *Parachlorella kessleri*, *C. vulgaris*, *Scenedesmus quadricauda*, *Chlorococcum*

sp. and *S. obliquus* in real aquaculture wastewater, after 5 d the NO_3-N removal efficiency was 85.7–97.1% and the TP removal efficiency was 90.2–98.9%.

The biomass yield coefficient was also calculated (Table 4). The values obtained for the positive control were statistically lower ($p < 0.05$) than those obtained for AW. This means that the higher the biomass yield values, the higher the biomass productivity is for the same nutrient concentration. Comparing the yields between the different nutrients analysed, the yields regarding phosphorus are higher than the ones regarding nitrogen. Thus, it can be concluded that the nutrient that most impacted biomass production was nitrogen. For the same productivity, microalgae consumed more nitrogen than phosphorus.

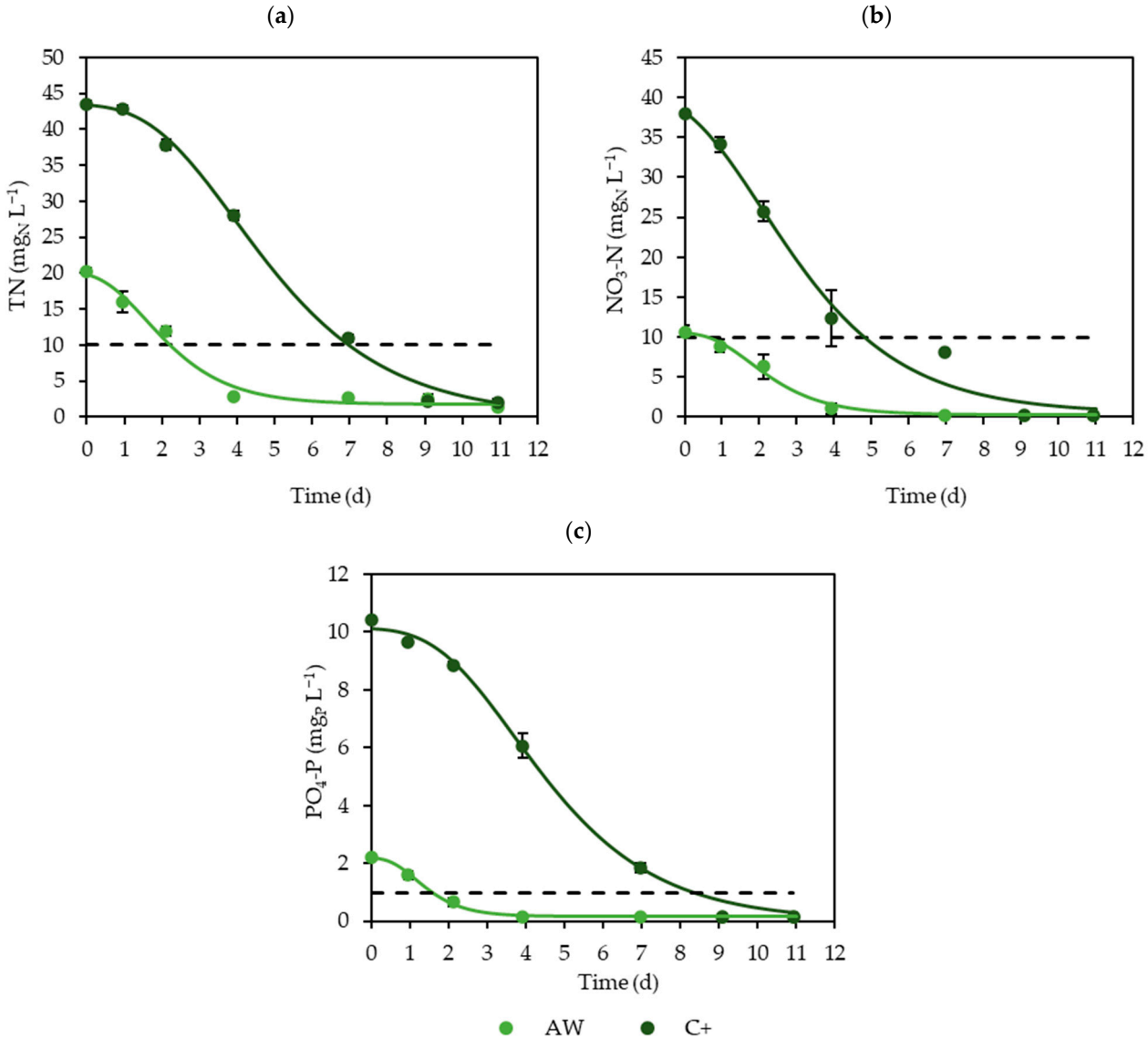

Figure 3. Experimental data and achieved modified Gompertz models (solid lines) for: (**a**) total nitrogen (TN); (**b**) nitrate-nitrogen (NO_3-N); and (**c**) phosphate-phosphorus (PO_4-P) concentrations in AW and C+ assays. The lowest EU legislation limits for treated urban wastewater discharged into aquatic bodies subject to eutrophication (10 mg_N L^{-1} and 1 mg_P L^{-1}) are represented by horizontal dashed lines.

Table 3. Removal efficiencies, modified Gompertz model parameters and fitting performance indexes achieved for each assay.

Nutrient	Assay	S_0 (mg L^{-1})	RE (%)	λ (d)	k (d^{-1})	R^2	RMSE (mg L^{-1})
TN	AW	20.2 ± 0.5 [a]	93.5 ± 2.1 [a]	0.38 ± 0.66	0.83 ± 0.26	0.994	0.797
	C+	43.5 ± 0.5 [b]	95.6 ± 1.4 [a]	1.66 ± 0.42	0.44 ± 0.07	0.998	1.243
NO_3-N	AW	10.7 ± 0.8 [a]	98.0 ± 0.1 [a]	0.67 ± 0.39	0.92 ± 0.21	0.998	0.281
	C+	38.0 ± 0.3 [b]	99.4 ± 0.1 [b]	0.00 ± 1.46	0.48 ± 0.18	0.994	1.926
PO_4-P	AW	2.2 ± 0.1 [a]	92.7 ± 0.1 [a]	0.45 ± 0.11	1.48 ± 0.16	1.000	0.013
	C+	10.4 ± 0.1 [b]	98.4 ± 0.1 [b]	1.63 ± 0.45	0.49 ± 0.09	0.998	0.313

k—uptake rate; R^2—coefficient of determination; RE—removal efficiency; RMSE—root mean squared error; S_0—initial nutrient concentration; λ—lag time. In the same column, values with a common letter ([a] and [b]) are not statistically different ($p > 0.05$).

Table 4. Biomass yield coefficient regarding total nitrogen, nitrate-nitrogen and phosphate-phosphorus.

Assay	$Y_{X/S}$ (g_{dw} $g_s{}^{-1}$)		
	TN	NO_3-N	PO_4-P
AW	19.0 ± 1.4 [a]	21.9 ± 1.1 [a]	62.7 ± 3.2 [a]
C+	10.9 ± 0.3 [b]	10.0 ± 0.1 [b]	38.9 ± 0.1 [b]

$Y_{X/S}$—biomass yield coefficient. In the same column, the values with a common letter ([a] and [b]) are not statistically different ($p > 0.05$).

3.3. Biomass Valorisation

The biochemical composition of the produced biomass was evaluated, and the contents of the analysed compounds are summarised in Table 5.

Table 5. Biochemical composition of the biomass in terms of carbohydrates, proteins, lipids, and photosynthetic pigments.

Parameters	AW	C+
Carbohydrates (% *w*/*w*)	48.64 ± 0.83 [a]	34.10 ± 1.74 [b]
Proteins (% *w*/*w*)	17.93 ± 1.21 [a]	20.98 ± 0.34 [a]
Lipids (% *w*/*w*)	15.82 ± 0.15 [a]	14.98 ± 0.17 [b]
Chlorophyll a (% *w*/*w*)	0.68 ± 0.02 [a]	1.58 ± 0.02 [b]
Chlorophyll b (% *w*/*w*)	0.33 ± 0.01 [a]	0.69 ± 0.01 [b]
Chlorophyll a + b (% *w*/*w*)	0.99 ± 0.04 [a]	2.28 ± 0.03 [b]
Carotenoids (% *w*/*w*)	0.21 ± 0.01 [a]	0.37 ± 0.01 [b]

In the same line, the values with a common letter ([a] and [b]) are not statistically different ($p > 0.05$).

As can be observed from Table 5, the obtained carbohydrate content was statistically higher ($p < 0.05$) in the AW assay (48.64 ± 0.83%), compared to the C+ assay (34.10 ± 1.74%). These values are higher than the typical range of carbohydrate content for *C. vulgaris*, 12 to 17% [37]. The protein content obtained in the C+ assay (20.98 ± 0.34%) and AW (17.93 ± 1.21%) were not statistically different ($p > 0.05$). These values are lower than the ones mentioned in the literature (40 and 60%) [38]. This happened possibly due to a stress response to the nutrient depletion that occurs in the final growth stage (see Figure 3). During stress conditions, the protein content in microalgae decreases [39]. It was also possible to note that the lipid content obtained in AW (15.82 ± 0.15%) was statistically higher ($p < 0.05$) than in C+ (14.98 ± 0.17%) and that both values were similar to the values referenced in the literature (14–22%) [37]. Lastly, in the positive control, there was a higher production of pigments, with chlorophyll a representing the highest content (1.58 ± 0.02%) and carotenoids the lowest (0.37 ± 0.01%). This behaviour was also observed in the AW trials: the pigment produced the most was chlorophyll a (0.68 ± 0.02%), and the least produced was carotenoids (0.21 ± 0.01%). Guldhe et al. [36] grew *C. sorokiniana* in

aquaculture wastewater and achieved 37.1% of carbohydrate content and protein content of 24.9%. Additionally, Liu et al. [13] obtained a carbohydrate content of 29.8 ± 0.1%, protein content of 39.5 ± 0.2% and lipid content of 16.1 ± 0.1% after 5 d of cultivation in real aquaculture wastewater of *C. vulgaris*.

In this study, besides treating aquaculture wastewater, the goal was also to valorise the biomass obtained in the AW assay by analysing the biochemical composition of the produced biomass. One way to valorise microalgal biomass is to integrate it into aquaculture feed. Microalgae have probiotic properties in fish and other properties, such as immunostimulants, antiviral and antibacterial, that impact survival and growth rates, as well as the immune system of fish and other aquatic organisms [22]. However, for the time being, microalgae are more effective as a supplement to existing feed than as a total replacement for feed. Several articles in this area show results that favour supplementary use instead of total use. Madhumathi et al. [40] incorporated 5 to 10% of *Dunaliella salina* in a giant tiger prawn aquaculture and obtained an increase in the survival rate due to an improvement in the immune system and an increase in the antioxidant content. The same occurred with Maliwat et al. [41], who, by incorporating 6 to 8% of *C. vulgaris* in the feed of a giant tiger prawn aquaculture, observed an increase in the growth rate and a better immune response. In addition, the tested prawns gained resistance to *Aeromonas hydrophila* infection. On the other hand, Sarker et al. [42] completely replaced the nutritional diet with microalgae *Schizochytrium* sp. and did not observe significant differences in survival rate. Identically, Haas et al. [43] incorporated the microalgae *Pavlova viridis* and *Nannochloropsis* sp. between 50 and 100% and observed no adverse consequences on growth or nutrient intake, but neither did they reported any advantages. The analysis of the microalgal biomass compounds is important because each one influences the metabolism and organism of fish and other aquatic species differently. Carbohydrates are easily digestible depending on their type and the amount present in the biomass, as well as the species of the aquatic organisms used [22]. A study on Nile Tilapia revealed that microalgae *C. vulgaris* and *Spirulina maxima* present good carbohydrate digestibility, with *C. vulgaris* being the one that is more easily digested [44]. On the other hand, microalgae proteins are a great alternative to the current proteins present in the commercial feed, regarding the amino acid composition. Furthermore, microalgal lipids have a lower degree of contamination than those present in the typical fish oil used in aquaculture [22]. Finally, vibrant colour pigments, such as carotenoids, are widely used because of their ability to enhance the quality and nutritional value of some aquatic organisms. For example, in the case of salmon, these pigments provide it with a more vibrant and striking colour [45]. Despite this, pigment synthesis must fulfil a number of requirements to be used, such as non-toxicity, the enhancement of nutritional components and the existence of digestible cell walls for easy absorption [46].

4. Conclusions

In this study, the removal efficiency of nitrogen and phosphorus by *C. vulgaris* in aquaculture wastewater was evaluated. Additionally, a biochemical composition analysis of the biomass was performed to further valorise the biomass. *C. vulgaris* was able to grow in the aquaculture effluent with a growth rate of 0.260 ± 0.014 d^{-1}, similar to that of the positive control, which was 0.276 ± 0.004 d^{-1}. Additionally, it was found that the wastewater treatment was effective regarding nitrogen and phosphorus contents, as shown by the removal efficiencies of TN, NO_3-N and PO_4-P above 90%. It is important to note that in the AW assay only 3 d and 2 d were required to reduce TN and PO_4-P to values below the EU legal limits, respectively. The biochemical analysis of the biomass revealed contents of lipids, carbohydrates and pigments typical and similar to values reported in other studies. However, the protein content was lower than expected, compared with the literature values.

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