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Feasibility of the Hybrid Use of *Chlorella vulgaris* Culture with the Conventional Biological Treatment in Urban Wastewater Treatment Plants

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Abstract: Currently, most wastewater treatment plants do not meet the legal requirements, especially regarding phosphorus and nitrogen contents. In this work, real primary urban wastewater (P-UW) was used as culture medium for the growth of *Chlorella vulgaris*. Experiments were carried out in batch photobioreactors at laboratory scale. To determine the maximum nutrient removal levels and the optimal pH value for *C. vulgaris* growth, the following pH values were studied: 5, 6, 7, 8, 9, 10, and 11. Additionally, two control experiments were conducted using UW and tap water at the same conditions but without microalgae inoculation. The operational conditions were agitation rate = 200 rpm, T = 25 °C, aeration rate = 0.5 L/min, and continuous light with illumination intensity = 359 μE m^{-2} s^{-1}. Significant higher growth was obtained at pH = 7. The direct use of *C. vulgaris* for P-UW treatment demonstrated high removal percentages of organic (COD and BOD_5 removal = 63.4% and 92.3%, respectively) and inorganic compounds (inorganic carbon removal = 99.6%). The final biomass was characterized by an accumulation of high energetic compounds, mainly carbohydrates, which ranged between 63.3% (pH = 5) and 82.8% (pH = 11) and represent a source of biofuels. These new achievements open up the possibility of new horizons in urban wastewater treatment.

Keywords: urban wastewater; *Chlorella vulgaris*; nutrient's removal; biochemical composition; biofuels

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1. Introduction

Global population is estimated to be increased to nine billion people by 2050. The continuous population growth, urbanization, industrial development, and diversification of human activities has resulted in a water crisis that affects, nowadays, 4000 million people around the world, who undergo water scarcity [1,2]. Furthermore, large amounts of wastewaters are generated because of industrial, agricultural, and domestic activities. Residual waters must be treated to avoid numerous environmental problems and ensure public health with safe water supplies [3].

Wastewater physicochemical characteristics are highly heterogeneous depending on its origin (domestic, commercial, and industrial activities). Within the different types, urban wastewaters (UW) are generated by industrialized countries as a combination of liquid and solid residues from domestic and commercial activities, and sometimes from pre-treated industrial activities [4]. Physicochemical characteristics of these effluents are related to the standard of living, behavior, and lifestyle of the inhabitants of the regions where UW are generated. The main physicochemical characteristics of untreated UW are chemical oxygen demand (500–1200 mg/L), total nitrogen (30–100 mg/L), ammonium (20–75 mg/L), total phosphate (6–25 mg/L), and high levels of suspended and volatile solids between 250–600 mg/L and 200–480 mg/L, respectively [5]. In addition, numerous pathogens microorganisms are commonly found in untreated UW, representing a major health risk for natural environments [6].

Conventional UW treatment is performed in wastewater treatment plants (WWTP) through the following stages: preliminary, primary, secondary, and tertiary treatment. Physicochemical and biological operations are combined along the process to improve the water quality by the removal of biological oxygen demand, suspended solids, nutrients (mainly nitrogen and phosphorous), coliforms, and toxic compounds [7]. Due to the increasingly stringent legislation, it is necessary to incorporate more advanced treatments to WWTP that only perform primary and secondary treatments. In this sense, tertiary treatment is applied in some WWTP for wastewater reuse and disposal in sensible places. This stage is intended to completely remove organic ions, nutrients, suspended solids, microorganisms, and remaining pollutants [8]. The European Parliament, and the Council of the European Union in 2020, have published the Regulation (EU) 2020/741 of the European Parliament and of the Council of 25 May 2020 concerning minimum requirements for water reuse, which updates the European Council Directive of 21 May 1991, in which it is established minimum requirements for discharges referring to the values of biological oxygen demand (BOD_5), chemical oxygen demand (COD), total suspended solids (TSS), phosphorus and nitrogen at the outlet of WWTPs that must comply with the following values 25, 125, 35, 1 and 10 mg/L, respectively [9]. This new regulation establishes minimum water quality, monitoring requirements, and risk management provisions, for the safe use of reclaimed water in the context of integrated water management and to ensure that reclaimed water is safe for agricultural irrigation, thus ensuring a high level of protection of the environment and of human and animal health, promote the circular economy, support adaptation to climate change, and contribute to the objectives of Directive 2000/60/EC by addressing water scarcity and the resulting pressure on water resources in a coordinated manner across the Union, thereby also contributing to the effective functioning of the internal market [10].

Microalgae represent the oldest photosynthetic organisms on earth, and they are the primary producers in the aquatic ecosystems together with the protists. Microalgae have ability to transform atmospheric inorganic carbon (CO_2) to organic biomass in presence of natural sunlight. They are unicellular, which makes it easier for large-scale cultivation. Microalgal biomass has many industrial applications such as pharmaceutical, nutraceutical, human and animal feed, aquaculture, biofuels, and health care products [11]. In addition, wastewater bioremediation has been used in marginal land to remove nutrients from any wastewater for biomass production and hence resolve eutrophication problem [12]. In fact, Transparency Market Research Consulting expected for the global algae market growth at a 7.42% for Compound Annual Growth Rate (CAGR) during the forecast tenure 2019–2027 to earn around USD 1.37 billion by the end of 2027 [13].

Abdel-Raouf et al. [7], Arbib et al. [14], Hodaifa et al. [4,15], Malvis et al. [16], Maaitah et al. [17], and other authors have demonstrated the feasibility of industrial wastewater treatment by microalgae as an environmentally friendly alternative with great industrial and economic potential. Microalgae can grow in wastewaters removing organic and inorganic nutrients such as nitrogen, phosphorous, heavy metals, and toxic compounds as phenols. In addition, the microalgal biomass, rich in lipids, carbohydrates, and proteins, constitutes a promising alternative to the production of third generation biofuels such as methane or biodiesel. Biofuels from microalgae are considered as a carbon-neutral energy resource and allow reduction on CO_2 emission levels up to 50% on comparison to fossil fuels. Microalgae are considered as a promising feedstock to produce third-generation biofuels [18]. Other bioactive compounds can be extracted from microalgae, such as pigments, functional polysaccharides, and polyunsaturated fatty acids, which are green and natural alternatives to be used in health care and cosmetics [19].

Chlorella vulgaris is a green microalga, which can accumulate large amounts of energy-rich molecules, specifically carbohydrates and lipids in the form of starch and triacylglycerol (TAG), respectively. The carbohydrate content can be converted to useful bioethanol, biomethane, and biohydrogen. In addition, the accumulated lipids in the biomass mainly

contains palmitic acid (C16:0), stearic acid (C18:0), palmitoleic acid (C16:1), and oleic acid (C18:1), which contribute to the biodiesel production [20].

At present, most urban wastewater treatment plants have difficulties in achieving the parameters required by current legislation for final discharges, especially as regards to the final nitrogen and phosphorus contents; in addition, the generation of large quantities of activated sludge, which these plants are not able to reduce completely. This work aims to use primary urban wastewater (P-UW) as culture media for the green microalgae *Chlorella vulgaris*. This allows the reduction of the activated sludge generated in the aerobic reactor (secondary treatment), the reduction of the final nitrogen and phosphorus contents and the generation of a high added-value algal biomass. The biochemical composition of the microalgae biomass is governed by the pH of the culture medium. Therefore, different pH values (5, 6, 7, 8, 9, 10, and 11) of the culture medium were evaluated. Two control experiments were also performed with UW and tap water at the same conditions (aeration rate = 0.5 mL/min, agitation rate 200 rpm, artificial continuous illumination at intensity 395 μE cm^{-2} s^{-1}, and temperature = 25 °C) but without microalgae inoculation.

2. Experimental

2.1. Microalga Used

A green microalga *Chlorella vulgaris* strain SAG 9.88, available in the laboratory of Chemical Engineering Area of the University of Pablo de Olavide (Spain), was grown in urban wastewater from primary treatment (without dilution) as culture media. Stock cultures were maintained at room temperature and continuous artificial illumination in solid mineral Rodríguez-López Medium [21], which solidified with agar. The artificial illumination provided by 120 cm white-light fluorescent lamps.

2.2. Microalga Culture and Experimental Set-Up

The crude wastewater samples were taken from the primary treatment on an urban wastewater treatment plant located in Seville (Spain), specifically, from the clear fraction of the primary settling tank. Algal experiments were performed without sterilization, at laboratory scale, in stirred batch tank photoreactors with 1 L work volume and 10 cm (diameter) × 16 cm (high) dimensions. All materials and glass bioreactors were used without sterilization. Culture media and air supply were added to the photoreactors without previous filtration or sterilization.

Algal cultures were designed to study the pH of the culture media influence in *C. vulgaris* growth. With this aim, experiments at different pH values (5, 6, 7, 8, 9, 10, and 11) were performed. During the course of the experiments, at least two samples at different time were taken daily, each sample was centrifuged and separated into two fractions: liquid, corresponding to the wastewater, and solid, corresponding to the algal biomass. The liquid fraction has been determined for the parameters of total carbon, total organic carbon, inorganic carbon, and total nitrogen. The solid fraction was first washed twice with ultrapure water. Then, the biomass concentration in g/L and the parameters of total carbon, total organic carbon, inorganic carbon, and total nitrogen were determined.

The biochemical composition of the biomass (total pigments, total proteins, total lipids, and total carbohydrates) has only been determined at the end of the experiments.

In addition, a control experiment was conducted using P-UW but without microalgae inoculation in which only P-UW was treated in the photoreactors in presence of air.

The liquid inoculum for each experiment consisted of a suspension of precultured cells in sterile solid Rodríguez-López mineral medium [21] and incubated for seven days. At the beginning of each experiment, the inoculum was transferred to the photobioreactor in a laminar flow cabin.

All experiments were conducted at 25 °C with mechanical stirring at 200 rpm. pH value was initially adjusted and maintained along the culture at the established value by adding 0.1-M NaOH or 0.1-M HCl solutions. Constant continuous illumination was provided from one

side of the photobioreactor by fluorescent lamps with intensity = 359 μE/(m^2 s). Aeration was supplied by an air compressor at a flow rate of 0.5 L/min.

2.3. Kinetic Growth of the Microalga

For the biomass concentration determination (x, g/L), a volume of 5 mL of suspended microalga was collected from the photobioreactors and centrifugated at 3000× *g* rpm for 10 min. The obtained biomass was washed three times with ultrapure water and measured at 600 nm [22] using a UV-Visible Spectrophotometer, type Evolution 201 (Thermo Scientific). The net biomass generation ($x-x_o$, g/L) was calculated during the cultures as the difference between the biomass concentration at a specific time and the initial value.

The maximum specific growth rate (μ_m) of *C. vulgaris* in each culture was calculated in the exponential growth phase and determined according to Equation (1):

$$\ln (x/x_o) = \mu_m t + a \quad (1)$$

where 'μ_m' is the slope of the line and corresponds to the maximum specific growth rate and 'a' is the intercept.

The volumetric biomass productivity (P_b) was calculated during the deceleration phase of growth and determined by the lineal Equation (2):

$$x = P_b t + b \quad (2)$$

where 'P_b' is the slope of line and corresponds to the value of the volumetric biomass productivity and 'b' is the intercept.

2.4. Biochemical Composition of the Biomass

The harvested biomass was characterized with the aim to determine the biochemical composition on pigments, proteins, carbohydrates, and lipids. The sum of the percentage of these four fractions is approximately 99% of the biomass since the genetic material fraction is normally around 1% [17]. At the end of each experiment, the microalgal biomass was separated by centrifugation at 3000 rpm for 5 min and washed three times with distilled water.

Total pigments were formed by total chlorophylls (chlorophyll a and chlorophyll b) and total carotenoids. These pigments were determined spectrophotometrically, using a UV-Visible Spectrophotometer, type Evolution 201 (Thermo Scientific), throughout the cultures after its extraction according to Jeffrey and Humphery [23] and Strickland and Parsons [24].

Total crude proteins were determined according to Equation (3) [25]. Nitrogen percentage was determined with a Total Carbon and Nitrogen Analyzer provided by Skalar Analytical B.V Company, mod. FormacsHT and FormacsTN (Breda Nederland):

$$\% \text{ Crude proteins} = \% \text{ TN} \times 6.25 \quad (3)$$

The total carbohydrate content (total reducing sugars) was determined by using the DNS (dinitrosalicylic acid) method as described by Miller [26]; 3 mL of DNS reagent is mixed with 2 mL of washed (twice) and sonicated microalgae sample for 30 min. Then, the sample is immersed in hot water at 80–85 °C for 10 min. The cooled sample is measured photometrically at 640 nm using a UV-Visible Spectrophotometer, type Evolution 201 (Thermo Scientific). In addition, a calibration line using glucose as reference reagent is needed.

At the end of each experiment, the algal biomass was separated and washed to determine the total lipids and fatty acid profiles. A minimum of 5 mg and 100 mg of dried algal biomass at 105 °C were used, respectively. Fatty acid content in the lipid fraction has been identified and measured by gas chromatography [27]. For total lipids, the dried biomass was extracted in a micro-soxhlet extractor with n-hexane as solvent (50 mL). The

extraction was performed for 24 h, after which the n-hexane was removed and samples were dried and weighed.

2.5. Analytical Methods

Real crude P-UW was characterized by measuring the following parameters: pH value, electric conductivity, turbidity, moisture and volatile materials, total solids, organic matter, ashes, dissolved oxygen, biological oxygen demand (BOD_5), chemical oxygen demand (COD), total carbon (TC), total organic carbon (TOC), inorganic carbon (IC), total nitrogen (TN), and nitrate+nitrite content.

The value of pH was measured using a CRISON pH meter, mod. LPG 22 (Barcelona, Spain).

Electric conductivity was determined directly by a CRISON conductivity meter, GLP31 model.

Turbidity was measured by a Turbidimeter Hanna, mod. HI93703 (Barcelona, Spain).

Total solids and moisture, together with volatile materials were determined according to the weight loss of the sample after being placed in an oven, type Memmert mod. UF110 Memmert GmbH+Co KG (Schwabach, Germany) at 105 ± 1 °C.

Ashes were determined by using an oven, type CARBOLITE mod. ELF14 of Gero Ovens (Chelmsford, United Kingdom) at 575 ± 5 °C.

The percentage of organic matter was calculated as the percentage of total solid minus the percentage of ashes.

Dissolved oxygen and BOD_5 were determined by using an Oximeter, Crison type Oxi 45+ with dissolved oxygen electrode type D.O. 5120, and supplied by Crison Instruments S.A., Alella (Spain). The BOD_5 value was calculated by measuring the initial and final oxygen content in the sample kept at 20 °C for five days [28–30].

Chemical oxygen demand (COD) was measured by photometric determination, by using a UV-Visible Spectrophotometer, type Evolution 201 (Thermo Scientific) at 620 nm of the concentration of chromium (III) after 2 h of oxidation with potassium dichromate/sulphuric acid/silver sulphate at 148 °C [28–30].

Total carbon (TC) represents all the carbon contained in a sample, which includes organic and inorganic carbon (TC = TOC + IC); total organic carbon (TOC) is the organic carbon that is converted into carbon dioxide after oxidation (TOC = TC−IC); inorganic carbon (IC) is the inorganic carbon in a sample that, after acidification, turns into carbon dioxide. IC includes all carbonates, bicarbonate, and dissolved carbon dioxide (IC = TC−TOC), and total nitrogen (TN) is all nitrogen in the sample, which includes organic and inorganic nitrogen. Total carbon and nitrogen and NO_3+NO_2 were determined using an analyzer provided by Skalar Analytical B.V Company, mod. FormacsHT and FormacsTN (Breda Nederland).

2.6. Statistical Analysis Applied

To confirm the reproducibility of the experimental data reported, the cultures were made at least in duplicate, and the analytical methods were applied at least in triplicate. In the duplicated experiments, biomass growth was monitored, and the final wastewater quality was determined. Graphics and statistical methods used (such as Standard deviation, Coefficient of variation (CV = standard deviation ∗ 100/mean); Reduced chi-square statistic; Residual sum of squares or Sum of squared errors of prediction (SSE); Coefficient of determination (R-square); Adjusted R-square; ANOVA analysis with the sum of square, F-value and *p*-value at the 0.05 level, etc.) were available in OriginPro 8.0 and Excel programs. Model calculation and statistical methods used were made on OriginPro 8.0 program.

3. Results and Discussion

3.1. Characterization of Primary Urban Wastewater and C. vulgaris Growth

The urban wastewater used in this work was obtained in a WWTP after a pre-treatment based on the separation of large particles as wet wipes, wood, leaves, stones, etc., and

after natural sedimentation (without any coagulants or flocculants) in a settling tank where primary sludge and clear (P-UW) fractions were obtained. Table 1 shows the characterization of the P-UW. This wastewater was characterized by a neutral pH value (6.9) and high values for turbidity (69 FTU), COD (284 mg O_2/L), BOD_5 (81.5 mg O_2/L), and TN (101 mg/L) in comparison to those determined for tap water (1.2 FTU, 0.0 mg O_2/L, not detect, and 0.51 mg/L, respectively). In addition, the dissolved oxygen (3.1 mg O_2/L) in the P-UW is lower than that determined for tap water, 8.2 mg O_2/L, which is due to the presence of the organic matter in the P-UW. Similar observations were made in relation to the total solids, organic matter, and ash when the P-UW was compared with the reference (tap water).

Table 1. Characterization of primary urban wastewater (P-UW) before and after treatment by *Chlorella vulgaris* at different pH values and control cultures.

Parameter	Tap Water	P-UW	UW Treated by *Chlorella vulgaris* at Different pH Values						Control Experiment
			5	6	7	9	10	11	UW-Aeration [1]
pH	6.85	6.86	4.63	6.03	7.42	9.3	9.99	10.5	9.3
Conductivity, µS/cm	0.00256	1505	1600	1780	1956	2000	1998	1990	2010
Turbidity, FTU	1.19	69	7.7	5.59	5.48	5.57	6.07	5.93	9.71
COD, mg O_2/L	0.00	283.8	192	297	204	104	125	3.57	– [2]
BOD_5, mgO_2/L	ND [3]	81.5	5.34	6.84	6	6.42	6.94	0.67	0.01
Disolved O_2, mg O_2/L	8.2	3.07	8.01	8.11	7.32	8.28	8.11	7.65	7.8
Total solid, %	0.020	0.101	–	–	–	–	–	–	–
Organic matter, %	0.006	0.0368	–	–	–	–	–	–	–
Ash, %	0.013	0.0639	–	–	–	–	–	–	–
TC, mg/L	24.0	256	64.6	282	80.1	162.4	217	690	70.7
TOC, mg/L	1.85	135	64.2	278	79.5	69.6	54.9	66.6	25.4
IC, mg/L	22.1	121	0.47	4.39	0.59	92.8	162	624	45.2
TN, mg/L	0.51	101	9.48	3.53	6.53	6.41	5.96	4.43	3.5
NN, mg/L	0.15	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.0

[1] UW-aeration: This culture simulated the culture with experimental highest microalga growth at pH = 9 but without microalga inoculation and applied the same operating conditions: aeration rate = 0.5 mL/min during 469 h, agitation rate 200 rpm, artificial continuous illumination at intensity = at 395 µE cm^{-2} s^{-1}, and temperature = 25 °C. [2] Data not determined. [3] ND: not detected. Note: The values presented correspond to mean values of at least three samples with a coefficient of variation (CV = standard deviation ∗ 100/mean) of less than 2%.

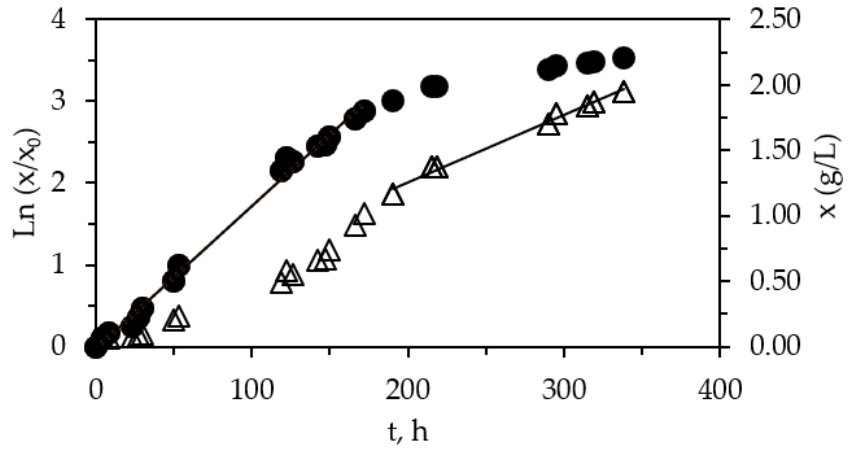
Figure 1A shows the growth curves of *C. vulgaris* in the P-UW at pH = 6. In this figure, it can be observed the exponential and the deceleration phase of growth and the calculation of the maximum specific growth rate, μ_m, and the volumetric biomass productivity, P_b, as indicated by Equations (1) and (2), respectively. Figure 1B represents the variation of the maximum specific growth rates and the volumetric biomass productivities versus the pH value of the culture media. Both μ_m and P_b experimental values are increased with the augment of the pH values until a pH value equal to 7, then these values decreased until pH equal to 11 for μ_m and 10 for P_b. No growth for *C. vulgaris* was observed at pH = 11 ($x_{average}$ = 0.0390 ± 0.0113 g/L during 337 h of culture).

The experimental values of $_m$ and P_b were fitted to a polynomial expression of Moser [31].

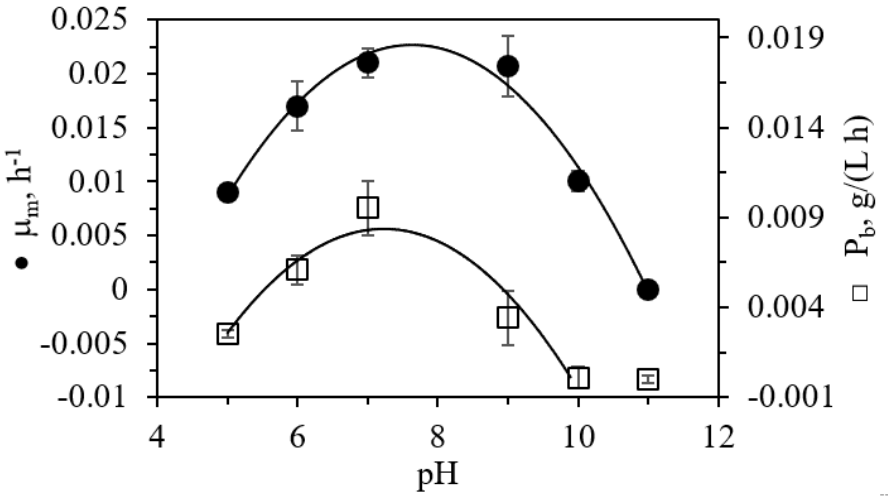
$$\mu_m = \mu_{m,\,max} (\pm \alpha_o \pm \alpha_1\ pH \pm \alpha_2\ pH^2 \pm \dots) \quad (4)$$

where '$\mu_{m,\,max}$' represents the maximum specific growth rate value achieved by *C. vulgaris* in P-UW, 'α_o', 'α_1', and 'α_2' are the parameters of the model. The value of $\mu_{m,\,max}$ determined by the model is equal to 0.0227 h^{-1} when the pH is equal to 7.65. The values of the parameters α_o, α_1, and α_2 were −4.198, 1.361, −0.0892, respectively. These data are consistent with those observed experimentally (Figure 1B). The parameters of the goodness of the fit were r^2 = 0.982 and residual sum of squares (SSE) = 5.95 × 10^{-6}. These results are similar to that obtained by Mayo [32] who studied the growth of *C. vulgaris*, in presence of

heterotrophic bacteria from activated sludge wastewater treatment plant (algal-bacteria system), in synthetic mineral culture medium enriched by glucose. In this work, the author determined the maximum growth rate of *C. vulgaris* equal to 0.0208 h^{-1}, which was obtained at a pH between 6.4 and 6.8. In addition, Rachlin and Grosso [33] studied the growth of *Chlorella vulgaris* (UTEX 30) at different pH values (3–9) in modified Bristol's medium [34] determining the optimal pH value for growth in the range of 7.5 to 8.0.



(**A**)



(**B**)

Figure 1. Growth curves of *C. vulgaris* growth in P-UW at pH = 6 (**A**) and the variation of the maximum specific growth rates and volumetric biomass productivities versus the pH value of the culture media (**B**). Common operating conditions: P-UW, aeration rate 0.5 L/min, mechanical stirring 200 rpm, illumination intensity 359 μE/(m^2 s) and temperature 25 °C. The error bars corresponding to the standard deviation.

Regarding the P_b values variation versus pH of the culture media, similar behavior was observed to that determined for the μ_m values variation. In this case, the P_b can be expressed as Equation (5),

$$P_b = P_{b,\,max}\,(\pm\,\alpha_o \pm \alpha_1\,pH \pm \alpha_2\,pH^2 \pm \ldots\,) \quad (5)$$

where '$P_{b,\,max}$' represents the maximum volumetric biomass productivity value achieved by *C. vulgaris* in P-UW, 'α_o', 'α_1', and 'α_2' are the parameters of the model. The value

of $P_{b,max}$ determined by the model is equal to 0.00836 g/(L h) when the pH is equal to 7.14–7.32. The values of the parameters α_o, α_1, and α_2 were −6.28, 2.01, −0.139, respectively. These data are consistent with that observed experimentally (Figure 1B). The parameters of the goodness of the fit were $r^2 = 0.925$ and SSE = 3.89×10^{-6}.

Blanco et al., [35] use wastewater from anaerobic digested gelatine industry as a culture medium for *Chlorella vulgaris* growth in bubble column photobioreactors (PBRs) in batch mode. In this work, different dilutions of the wastewater were enriched with modified Bold's Basal Medium (BBM) [36]. The maximum specific growth rate (μ_m) achieved was 0.0143 h^{-1}, and the biomass productivity was 0.00588 g/(L h), which are lower than that registered in this work.

In Figure 2 it can be see the net biomass generation during the cultures time when *C. vulgaris* was grown in P-UW at different pH values. The highest biomass generation ($x-x_o$ = 3.53 g/L) was obtained in cultures operated at pH = 7.

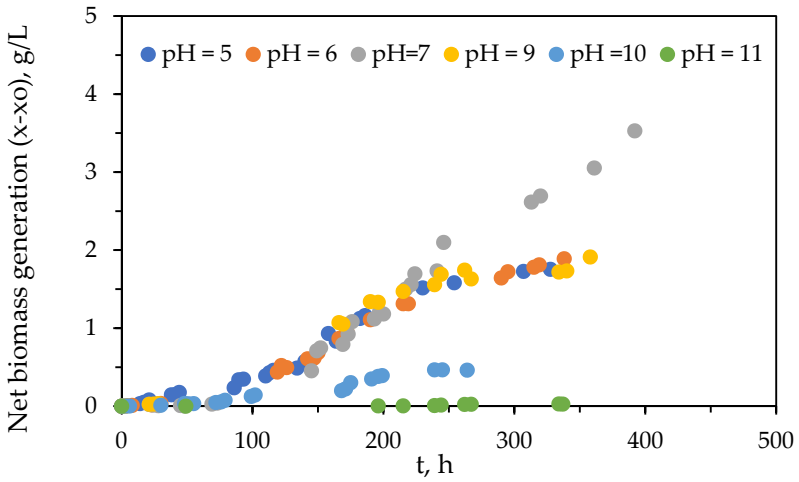


Figure 2. Net biomass generation ($x-x_o$, g/L) of *C. vulgaris* growth in P-UW at different pH values. Operating conditions: P-UW (without dilution), mechanical stirring = 200 rpm, aeration rate = 0.5 L/min, T = 25 °C and artificial illumination at 395 E/(cm^2 s). Note: The values presented correspond to mean values of at least three samples with a coefficient of variation (CV = standard deviation ∗ 100/mean) of less than 2%.

Cultures at pH = 5, 6 and 9 have the same harvested biomass at the end of each culture and an average value with standard deviation equal to 1.87 ± 0.0266 g/L can be considered. Work at pH = 10 implies a decrease in the final harvest biomass up to 0.530 ± 0.00121 g/L.

It is clear, from the data presented in Figures 1 and 2, that pH has an important effect on *C. vulgaris* growth since acid pH values in the range of 5.0 to 6.0 and 7.0 allow a biomass increase from 1.87 g/L to 3.55 g/L, respectively. Mackie [37] demonstrated the acidic pH effect on aquatic biota by showing an increase in the hydrogen ion content of water. Then, a reduction in the hydrogen ion content or an increase in OH^- ions in the culture media could result in a negative effect on population growth. At pH values of 8.3–9.0, population growth of *Chlorella vulgaris* cultures were reduced by 53.9–65.8%. However, pH values of 7.5 and 8.0 favored the population growth by 25 to 20% over control values, which indicates that a slight alkalinity may be favor the growth of this organism.

3.2. Carbon and Nitrogen Species Intercation between P-UW and C. vulgaris Biomass

Figure 3 shows the interaction/variation of carbon and nitrogen species between microalga biomas and P-UW. For P-UW, it can be observed a sharp decrease in the TC, IC and TOC values in the first hours of the culture, which corresponds to the exponential growth phase. In fact, the consumption velocities of total carbon and inorganic carbon during the exponential phase of growth determined by linear fitting were −1.15 and −1.24 mg/(L h) with a goodness of fit 0.990 and 0.983, respectively. In addition, this

decrease in TC, IC and TOC in P-UW indicated the mixotrophic metabolism of C. vulgaris in this phase of growth, in which air CO_2 and organic compunds are used as carbon source.

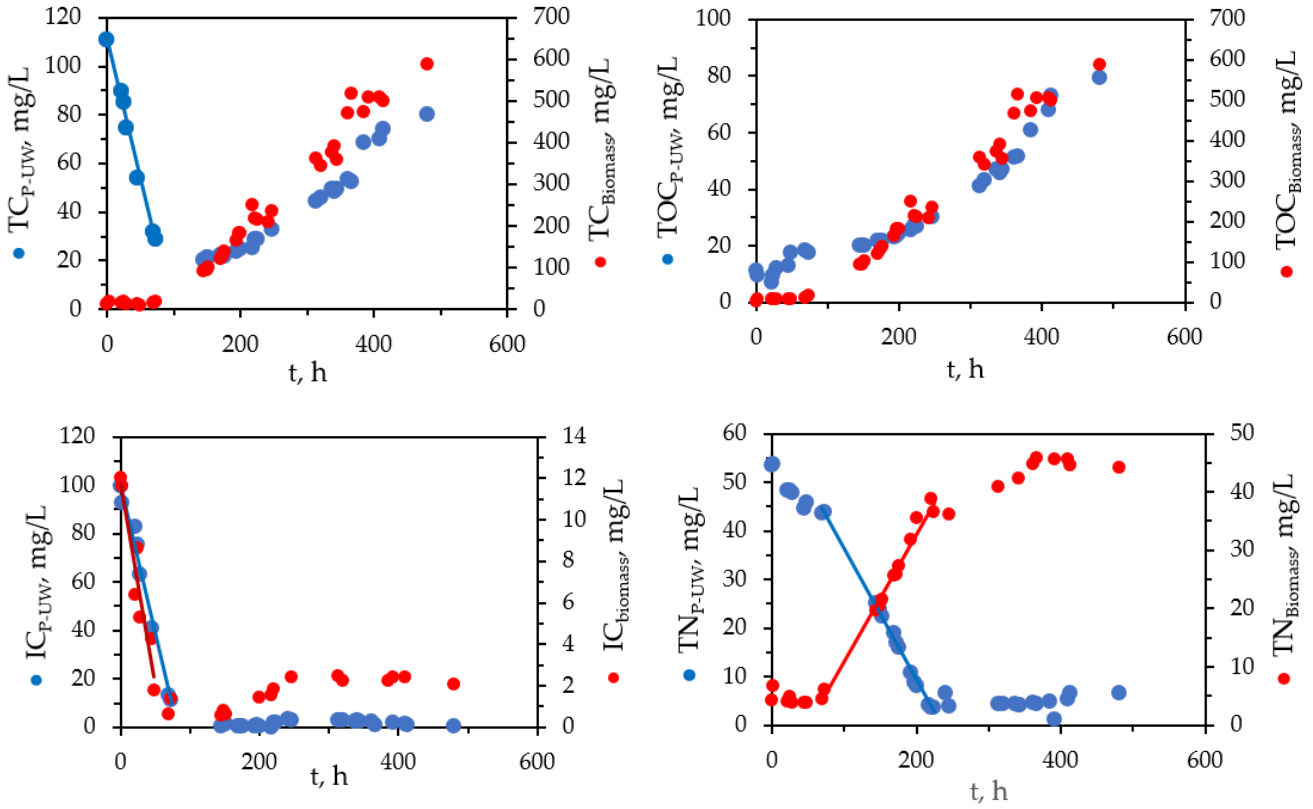


Figure 3. Carbon and nitrogen species behavior during *Chlorella vulgaris* growth on P-UW. Operating conditions: P-UW (without dilution), pH = 7, mechanical stirring = 200 rpm, aeration rate = 0.5 L/min, T = 25 °C and artificial illumination at 395 μE/(cm^2 s). Note: The values presented correspond to mean values of at least three samples with a coefficient of variation (CV = standard deviation ∗ 100/mean) of less than 2%.

Energy and carbon sources for microalgae in real culture media vary depending on the culture conditions, and therefore, their metabolism mode (autotrophic, mixotrophic and heterotrophic) is oriented in accordance with them. In photoautotrophic culture, light (natural or artificial) is utilized as the energy source and air CO_2 is used as the carbon source [38]. In heterotrophic culture, the organic carbon used by microalgae is degraded, similar to bacteria. Mixotrophic culture is the combination of both previous metabolisms menstioned [39]. In this sense, mixotrophic microalgae species such as Chlorella, Chlamydomonas and Scenedesmus can simultaneously present both photoautotrophic and heterotrophic metabolic activities by assimilating CO_2 and organic carbon compounds. P-UW is a mixture constituded by small and large molecules as well as some polymers ranging from <500 to >5000 Da. Carbon sources are mainly available for bacteria and only simple carbon molecules (glucose, fructose, disaccharides or sucrose, glycerol and volatile fatty acids) can be used by microalgae [40–42].

The increase in the TC and TOC values of P-UW after exponential phase of growth (Figure 3) is due to the huge biomass concentration increase in the culture and the non-efficient separation of biomass by centrifugation for 5 min.

In Figure 3 and regarding the interaction/behavior of the TN in the P-UW and the algal biomass, it can be clearly seen how the TN of the P-UW decrease and is incoporated to the algal biomass. In addition, it can be observed in this Figure a linear consumption of TN from the P-UW during the deceleration growth phase (consumption velocity = −0.271 mg of n/(L h), R^2 = 0.996) and lineal incoporation of this nitrogen to the algal biomass (nitrogen

incoporation velocity = 0.218 mg of n/(L h), R^2 = 0.987) that means the conversion degree of nitrogen is equal to 80.4%.

Figure 4 shows for all cultures studied at different pH values, the TOC, IC, and TN behavior in the P-UW and during the algal biomass formation. For P-UW fraction, it was observed a similar behavior of TOC values during the different cultures to that observed in Figure 3. In all experiments, the IC values were decreased throughout the culture time. Only for the experiments at pH = 10 and 11 these values increased with culture time because the cells rupture and the cell content flows out into the P-UW. For TN values, similar behaviors were observed to those shown in Figure 3 in which the value of TN decreased slowly during the exponential growth phase and in linear mode during the deceleration phase of growth to finished with concentration less than 9.5 mg/L. For the biomass fraction, it was observed a normal behavior for TOC, IC, and TN values in which the concentrations of theses parameters in the biomass were increased with the culture time. Only, in the case of TN in the culture at pH = 11 this tendency is down due to the cells rupture. In addition, the experimental results obtained in this work demonstrated the linear relationship between the biomass concentration (g/L) with the TOC concentration when the biomass generation is varied between 0 and 2.5 g/L (Figure 5).

3.3. P-UW Treatment by C. Vulgaris Biomass

Table 1 shows the characterization of the treated P-UW after the different *C. vulgaris* cultures performed at different pH values. The first observation is that the dissolved oxygen in the raw P-UW was 3.07 mg O_2/L, and at the end of all the algal cultures studied was higher than 7.32 mg O_2/L, which means the algal cultures have the ability to re-oxygenate the P-UW. In addition, it is observed a reduction of 28.1% to 63.4%, 91.5% to 93.4%, and 60.0–95.6% on the values of COD, BOD_5, and TN, respectively. These high COD, BOD_5, and TN removal percentages registered will improve the capacity of the UWWTPs to comply with the European regulation [43], which requires a minimum reduction percentage for the whole treatment process of 70–90%, 75%, and 70–80%, respectively. Similar observation was made for the turbidity values, which were reduced to 77.5–91.9%, respectively. In the same way, TC, TOC, IC, and TN values were reduced for all algal cultures performed. Higher reduction in these values were detected in the algal culture at pH = 7, where the final values were 80.1 mg/L (68.7%), 79.5 mg/L (41.1%), 0.59 mg/L (99.5%), and 6.53 mg/L (93.5%), respectively. As a control experiment, UW was only aerated at the same aeration rate to understand the effect of the *C. vulgaris* on the UW. In addition, the values of the different parameters shown in Table 1 about the quality of the final treated water are after algal biomass separation by centrifugation during only 5 min, which is not enough to harvest the total algal biomass since this microalga is a round nonmotile reproductive cell with size from 2 to 10 μm [44]. In fact, the clear treated water obtained showed microalgal cells under the optic microscope. This fact implies that the quality of the final treated water is better than that shown in Table 1, if it had been applied that an effective separation technique such as natural sedimentation during large time or flocculation-sedimentation, etc.

Finally, it is worthy to indicate that it has been recently published in the last Regulation (EU) 2020/741 of the European Parliament and of the Council of 25 May 2020, on minimum water quality requirements (main parameters: turbidity $\leq$5 FTU and BOD_5 $\leq$10 mg O_2/L) for agricultural irrigation (water reuse), in which the treated water obtained in this work could be reused after the application of filtration and disinfection units [9].

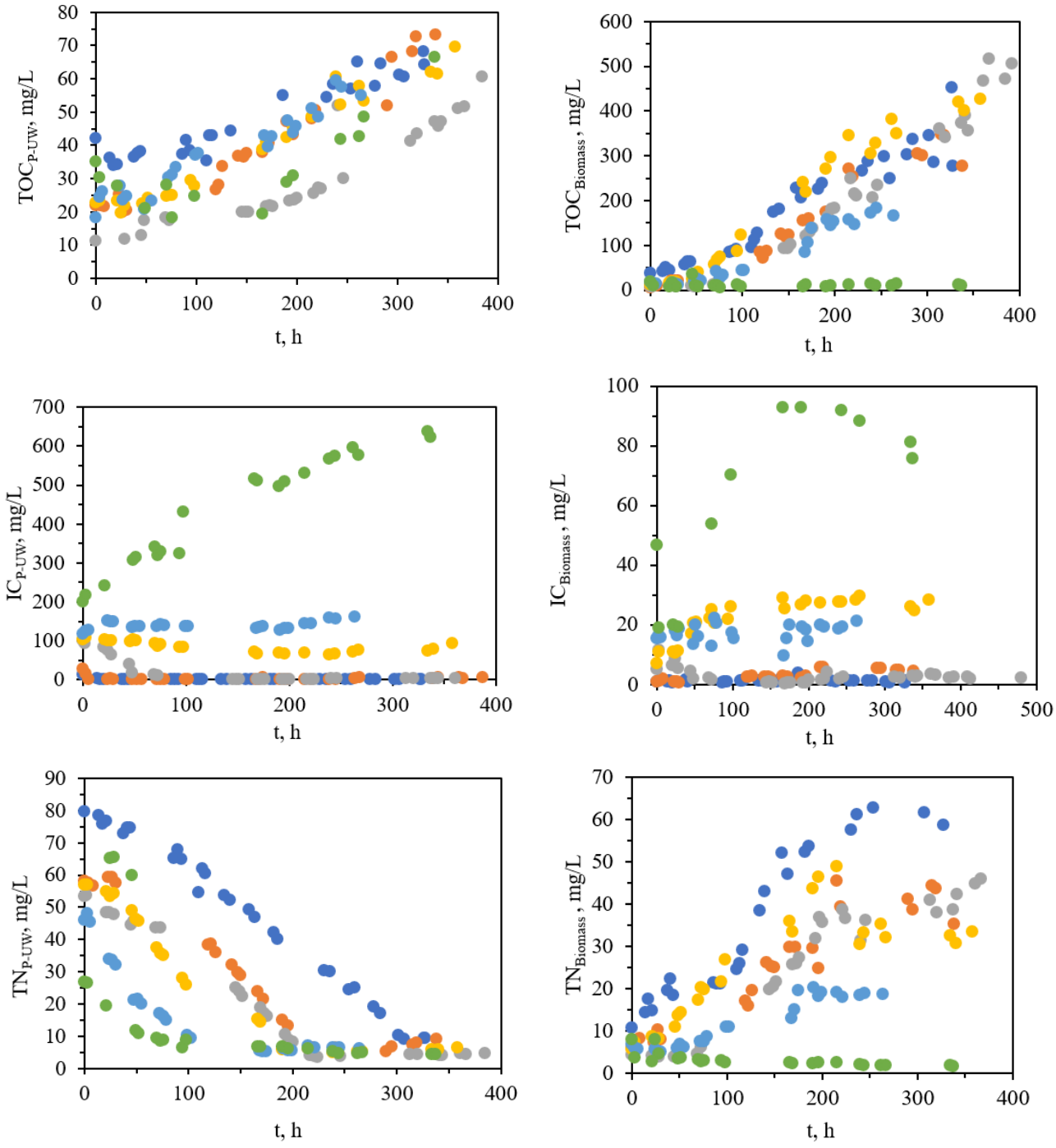


Figure 4. Carbon and nitrogen species behavior in the *C. vulgaris* cultures at different pH values. (**A**) Carbon and nitrogen species behavior in the treated P-UW (without microalgae) and (**B**) in *C. vulgaris* biomass produced in cultures. Operating conditions: pH = 7, agitation rate = 200 rpm, aeration rate = 0.5 L/min, T = 25 °C and artificial illumination at 395 E cm^{-2} s^{-1}. Note: The values presented correspond to mean values of at least three samples with a coefficient of variation (CV = standard deviation ∗ 100/mean) of less than 2%.

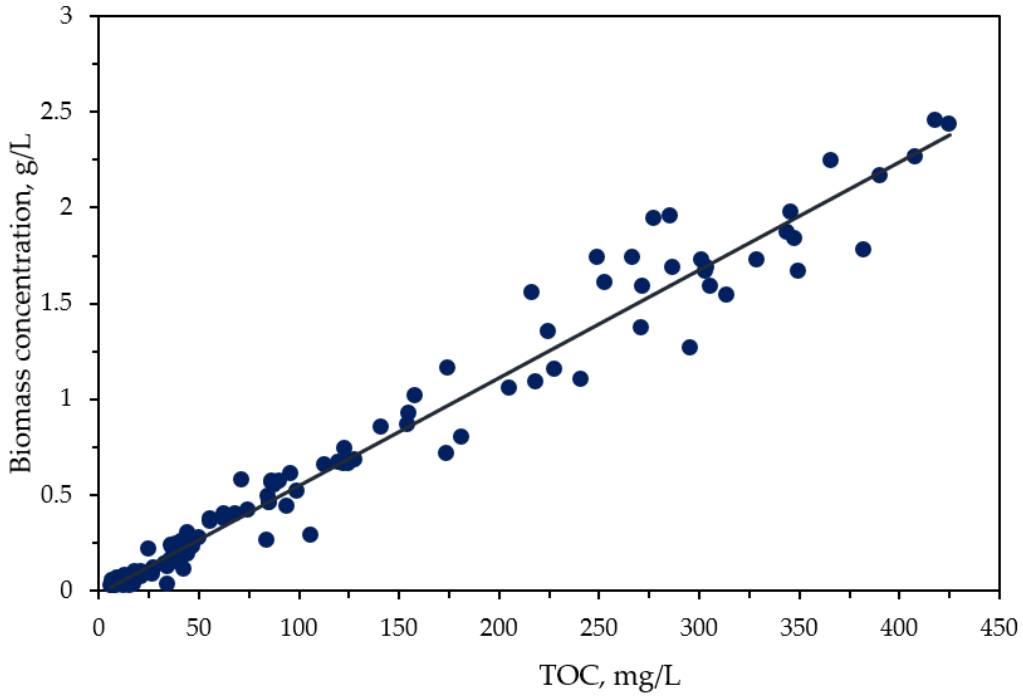


Figure 5. Linear relationship between biomass concentrations of the microalga *Chlorella vulgaris* versus total organic carbon (TOC) values.

3.4. Biochemical Composition of the Harvest Algal Biomass

Table 2 shows the biochemical composition of the biomass harvested at the end of *C. vulgaris* cultures as well as the composition of the biomass harvested after the control experiment (without microalga). In all cultures, the percentage of the total chlorophylls is higher than that registered for the carotenoids. Only in the case of the culture at pH = 6 was the concentration of carotenoids (0.202%) higher than that registered for total chlorophylls (0.0778%). This difference is normal since the percentages of total chlorophylls and pigments varied depending on the moment of the biomass harvested (more carotenoids at the end of the cultures). The total pigments (chlorophyll a, chlorophyll b, and carotenoids) percentages increased with the pH of the culture augment up to its maximum percentage at pH = 7 (0.598%), then these percentages were decreased with the pH increase to no pigmentation detection on the culture at pH = 11. In the control experiments, total pigment concentration up to 0.530% was registered, which is similar to that determined for *C. vulgaris* culture at pH = 7. In fact, microalgae (mainly from *Chlorella* sp.) have been detected in the control experiment due to the natural presence of microalgae in the UW. The highest and lowest value of the percentage in total chlorophylls, 0.0552% and 0.485%, are within the interval provided by the classical work of Milner [26] for another green microalga, *Chlorella pyrenoidosa*, of 0.01–6%. Clearly, the chlorophyll content depends on the culture conditions. Nakanishi and Deuchi [45] determined a chlorophyll concentration of 6.7% (D.M.) for *C. vulgaris* M-207A7 strain FC agar medium, which was prepared with seawater instead of distilled water.

Total protein percentages were varied in the range of 8.13% to 21.9%. These low percentages are consistent with the P-UW nitrogen content (3.53–9.48 mg/L, Table 1). The energetic compounds for the biofuel production (carbohydrates and lipids = 70.1–88.3%) represent the main fractions of the *C. vulgaris*-harvested biomass. In fact, carbohydrates represent the highest percentages in the harvested biomass and varied from 63.6% to 82.8% reaching the higher value in the culture at pH = 11, in which no lipids were detected in the biomass. This last culture is not feasible at industrial level since no significant biomass generation was observed ($x-x_o$ = 0.0268 ± 0.0135 g/L, Figure 2). Regarding the lipid fraction in the biomass, it was observed an increase in the lipid content with the rise of

the culture medium pH value up to pH = 9 and then decreased. The best pH values for lipid production are in the range of pH 7–9 (11.5–13.3%). In the control experiment, high percentage value of carbohydrates (75.8%) was registered, which is in the same order of *C. vulgaris* cultures, but the percentage of total lipids was higher in *C. vulgaris* cultures (at pH = 7–9) than in the control experiment. In this sense, we can indicate that the efficiency of the *C. vulgaris* culture is higher than in the control experiment considering the great number of microalgae applications. Becker [25] indicated that under optimal conditions, the lipid content of *C. vulgaris* is about 5–40% (dry weight), but it can increase up to 58% under unfavorable growth conditions, when produced lipids are mainly composed of triacylglycerols (TAGs). In this sense, Sakarika and Kornaros [46] have reported the growth of *C. vulgaris* in synthetic medium of BG-11 supplemented with approximately 10 g/L glucose, and the pH was adjusted to the following values: 3.0, 4.0, 5.0, 6.0, 6.5, 7.0, 7.5, 8.0, 9.5 and 11. Experiments were developed in 1L Erlenmeyer flasks with a working volume of 0.7 L with 1 v/v/min aeration rate and controlled temperature at 25 $\pm$ 1 °C on an orbital shaker bath adjusted at 90 rpm. The total lipids at pH = 8.0 (34.8%) was lower than at pH = 7.5 (53.43%). In other words, the optimal pH for lipid accumulation in *C. vulgaris* was 7.5.

Table 2. Biochemical composition of the biomass obtained from *Chlorella vulgaris* growth in primary urban wastewater at various pH values and in the control experiment at pH = 9 without microalga.

***C. vulgaris* Biomass**	**Parameter**	**Control Experiment**	***C. vulgaris* Cultures at Different pH Values**					
			5	**6**	**7**	**9**	**10**	**11**
Biochemical composition	Total chlorophylls, %	0.400	0.0552	0.0778	0.485	0.268	0.255	0.000
	Carotenoids, %	0.130	0.0174	0.202	0.113	0.0995	0.0876	0.000
	Total pigments, %	0.530	0.0726	0.280	0.598	0.368	0.343	0.000
	Crude Proteins, % *	8.39	20.5	13.5	8.13	10.9	21.9	17.8
	Carbohydrates, %	75.8	63.6	70.6	73.4	75.0	67.6	82.8
	Lipids, %	6.56	6.50	7.45	11.5	13.3	6.16	ND
	Total biomass composition, %	91.3	90.7	91.8	93.1	99.6	96.0	100
Biomass in terms of carbon and nitrogen species	TC, mg/L	188	278	282	589	455	187	85.8
	TOC, mg/L	175	276	278	587	426	166	10.2
	IC, mg/L	13.4	1.25	4.39	2.07	28.4	21.2	75.6
	TN, mg/L	11.9	58.7	35.3	44.1	33.4	18.6	1.48
	IC, mg/L	13.4	1.25	4.39	2.07	28.4	21.2	75.6

* Crude protein = 6.25 × % total nitrogen in the biomass [25]. Note: The values presented correspond to mean values of at least three samples with a coefficient of variation (CV = standard deviation * 100/mean) of less than 2%.

Based on the fatty acid profiles (FAME analysis, [4]) the fatty acids detected in *C. vulgaris* growth in P-UW at different pH values varied from C14:0 to C24:0. The main fatty acids detected in the biomass were palmitic acid (C16:0), oleic acid (C18:1), and linoleic acid (C18:2n6). However, in the control experiment, the main fatty acids were palmitic acid (C16:0), oleic acid (C18:1), and arachidic acid (C20:0). The lipid content registered for saturated, monosaturated, polyunsaturated, and essential fatty acid fraction (%ESE) values varied in the range of (32.4% to 42.0%), (26.0% to 33.6%), (18.3% to 32.1%), and (7.43% to 30.3%), respectively. Similar percentages (46.2%, 42.6%, 5.89%, and 3.57%, respectively) were registered in the biomass harvested from control experiment but with higher percentages on saturated and monounsaturated fatty acids and lower percentages on polyunsaturated and essential fatty acids.

Finally, biomass content in carbon and nitrogen species are shown in Table 2. These values are consistent with those observed on the conventional biochemical composition discussed above.

4. Conclusions

This research work has addressed the use of *Chlorella vulgaris* culture as a secondary treatment instead of or in parallel with the traditional use of an aerobic biological reactor currently used in WWTPs. The achieved performance in terms of COD (up to 63.4%), BOD_5 (up to 93.4%), TOC (up to 59.3%), and TN (up to 95.6%) removal are better than those currently achieved in the real conventional WWTPs. The application of this treatment allows high nitrogen removal levels of more than 95%, which eliminates the need for the introduction of physical and chemical tertiary treatment in WWTPs. The optimal growth (maximum specific growth rate and highest volumetric biomass productivity) was determined when the pH of the culture media had values in the range of 7–9. The use of *C. vulgaris* culture as secondary treatment with *C. vulgaris* concentrations up to 3.5 g/L allows the reduction of generated sludge on the conventional secondary treatment and obtained biomass that can be transformed into biofuels. The biochemical composition of the harvested biomass is rich on energetic compounds, which allows high conversion yields to biofuels. The final treated water quality after *C. vulgaris* growth complies with the current regulations established for direct discharges to waterways or for irrigation (standard requirements to discharge, COD < 125 mg/L, BOD_5 < 25 mg/L, and n < 10 mg/L). These results allow us to say that the hybrid use (conventional and microalgae) allows to improve the overall degradation efficiency in WWPTs, the production of a high added- value algal biomass considering the different applications of microalgae, and furthermore, that this mixed system could work with excellent results in different geographical areas worldwide.

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Nomenclature

A_{600}	Absorbance of the cell suspension at 600 nm
a	Parameter of Equation (1)
b	Parameter of Equation (2)
BOD_5	Biological oxygen demand during 5 days at 20 °C, mg O_2/L
COD	Chemical oxygen demand, mg O_2/L
D.M.	Dry matter
%ESE	Percentage of essential fatty acids, C16:3n4, C20:5n3, C22:6n3, C18:2n6, and C18:3n3.
IC	Inorganic carbon, mg/L
I_o	Initial light intensity, μE m^{-2} s^{-1}
P_b	Volumetric biomass productivity determined at deacceleration phase of growth, g/(L h)
T	Temperature, °C
TC	Total carbon content, mg/L
TN	Total nitrogen content, mg/L
TOC	Total organic carbon content, mg/L
t	Time, h
UW	Urban wastewater
P-UW	Wastewater collected at the outlet of the settling tank in the primary treatment
WWTPs	Wastewater treatment plants
x	Biomass concentration, g/L
x_o	Initial biomass concentration at the beginning of the experiment t = 0 h, g/L
α_o	Parameter of Moser model Equation (4)
α_1	Parameter of Moser model Equation (4)
α_2	Parameter of Moser model Equation (4)
μ_m	Maximum specific growth rate determined at exponential phase of growth, h^{-1}

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