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Municipal Wastewater: A Sustainable Source for the Green Microalgae *Chlorella vulgaris* Biomass Production

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Abstract: The need to reduce the costs associated with microalgae cultivation encouraged scientific research into coupling this process with wastewater treatment. Thus, the aim of this work was to assess the growth of *Chlorella vulgaris* (Chlorophyta) in different effluents from a municipal wastewater treatment plant (WWTP), namely secondary effluent (SE) and sludge run-off (SR). Assays were performed, under the same conditions, in triplicate with 4 dilution ratios of the wastewaters (25%, 50%, 75% and 100%) with the standard culture medium bold basal medium double nitrated (BBM2N) as a control. The capability of *C. vulgaris* for biomass production, chlorophyll synthesis and nutrients removal in the SE and SR was evaluated. The 25% SE and 25% SR showed increased specific growth rates (0.47 and 0.55 day^{-1}, respectively) and higher biomass yields (8.64×10^7 and 1.95×10^7 cells/mL, respectively). Regarding the chlorophyll content, the 100% SR promoted the highest concentration of this pigment (2378 µg/L). This green microalga was also able to remove 94.8% of total phosphorus of SE, while in 50% SR, 31.2% was removed. Removal of 73.9% and 65.9% of total nitrogen in 50% and 100% SR, respectively, was also observed. *C. vulgaris* growth can, therefore, be maximized with the addition of municipal effluents, to optimize biomass production, while cleansing the effluents.

Keywords: microalgae cultivation; biomass production; nutrient removal; wastewater treatment; bioactive compounds

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1. Introduction

Aquatic and coastal ecosystems are essential, providing physico-chemical conditions that support diversified communities and several ecosystem services, such as shoreline stabilization, nutrient regulation, carbon sequestration, as well as the supply of food and energy resources [1–3]. These ecosystems are often associated with highly populated areas, making these habitats even more susceptible, due to the increasing anthropogenic activity [4]. The discharge of urban and industrial effluents, as well as the intensification of agriculture or aquaculture, are some examples of the increasing anthropogenic stressors that endanger these habitats [5]. The sum of these stressors often causes nitrogen and phosphorus enrichment of estuarine areas, leading to algal blooms–eutrophication—and consequently the deterioration of water quality [4–7].

For this reason, the Directive 91/271/EEC [8], which was amended by the Directive 98/15/EC [9], establishes that discharges from urban wastewater treatment plants (WWTP) in sensitive areas to eutrophication (such as estuaries) must present a minimum percentage

of 80% (for total phosphorus) and 70–80% (for total nitrogen) reduction in relation to the influent load. Moreover, the Directive 98/15/EC [9] aims to protect the aquatic environment, through a set of measurements leading to the significant decrease of the organic and inorganic load discharged into the water bodies.

In WWTP, the effluent must complete four processes, namely the preliminary (harrowing and degreasing) [10], primary (biological treatment with nitrification/denitrification bacteria) [11], secondary (settling and sedimentation) [12] and tertiary (pathogens elimination) treatments [13]. Furthermore, the originated sludge is treated with heating and gravitational thickening, resulting in a liquid fraction, corresponding to the sludge run-off or centrate, and a solid fraction, the sludge, which is often incinerated or can be valorized for agricultural purposes [14]. Nevertheless, the treated effluents and sludge run-off still often present considerable high nutrient loads.

The need to reduce costs associated to microalgal biomass production led to a set of studies aiming at the evaluation of wastewaters application for microalgae cultivation. In fact, the coupling of both processes could be advantageous, since microalgae are able to reduce the organic and inorganic load of wastewaters and sequester atmospheric carbon dioxide [15–19]. Thus, microalgae provide a sustainable biological treatment for wastewaters and the microalgal biomass can be further valorized for energetic or agricultural purposes [20–22].

Microalgae cultivation is, however, challenging since growth can be affected by several biotic factors—light intensity [23], temperature, pH [21], aeration rate [24], nutrients and carbon dioxide availability [25]—and abiotic factors, such as the presence of pathogenic or predatory organisms [26,27]. Moreover, there are several systems (open or closed) [28–30], and strategies (batch, continuous or semi-continuous) [21,31–33] that can be employed in order to optimize microalgal culture conditions.

Closed systems or photobioreactors are distinguished by the absence of exchange between the community of microalgae and the surrounding environment, offering various designs, such as tubular, plastic, flat-plate and bubble-column bags [34]. Some of the difficulties faced by open systems, such as low efficiency, nutrient evaporation and contamination, are solved by closed-culture systems [30]. Nevertheless, these systems present a high demand for electricity and their implementation is costly [34].

In terms of cultivation strategies, the batch strategy is a low-cost strategy because it does not need much control. This strategy is distinguished by no renewal of the culture medium, which means that the culture of microalgae grows until it reaches the decline period. Nevertheless, in practice, the microalgal culture may crash for several reasons, such as nutrient or oxygen depletion, self-shading, pH variation or harmful compounds concentration [21,31].

Most microalgae are photoautotrophic, meaning that their growth requirements are: illumination, inorganic carbon and minerals dissolved in the culture medium [35]. However, some microalgae are capable of growing under heterotrophic or mixotrophic conditions [36]. For instance, in heterotrophic conditions, the growth occurs in the absence of light and microalgae uses organic carbon through aerobic respiration [37]. While under mixotrophic conditions, microalgae assimilate organic carbon (i.e., glucose or glycerol) through aerobic respiration [38]. Thus, the microalgal species must be wisely selected according to the wastewater type and the final metabolic product targeted. *Chlorella vulgaris* is commonly found in municipal wastewaters [18,39–41], as a unicellular, eukaryotic and green microalgae (Chlorophyta). This is a resilient and freshwater species, characterized for having a coccoid shape, often exhibiting a diameter of 2 to 10 µm [25,42]. This microalgae species holds an important economic value, thus several studies were conducted to optimize the microalgal growth [25,43–46]. Previous research showed that the optimal conditions for *C. vulgaris* growth is observed under mixotrophic conditions [45], within a temperature range of 25 to 35 °C [44], an alkaline pH (9–10) [47], a 16 h (day): 8 h (night) photoperiod, with a light intensity of 5–7 klux [44] and an aeration rate of 200 mL/ min [25].

Moreover, all the aforementioned abiotic parameters will affect the capability of *C. vulgaris* for nutrient uptake, impacting the growth efficiency. The microalgae *C. vulgaris* is one of the species that, according to the carbon source and concentration, can change their metabolism [48,49]. According to the abiotic parameters, particularly the carbon source, *C. vulgaris* can exhibit a photoautotrophic, heterotrophic or mixotrophic metabolism [45,50–52].

Microalgae growth optimization is pivotal for the research of their biotechnological applications. Furthermore, the application of wastewater as a culture medium is an important tool to reduce the costs associated with microalgae growth, as well as, to promote Circular Economy and develop innovative products and techniques. For instance, microalgal biomass can be used for pharmaceutical, agricultural or energy purposes.

Chlorella pigments, such as chlorophyll and carotenoids, have also been researched for use as a natural colorant that can be employed as antioxidants in the biomedical industry [53,54], and as a dye in textile industry [55,56].

Microalgal biostimulants include macro and micronutrients, phytohormones, carotenoids, amino acids, antifungal agents, hormones, polyamines, carbohydrates, proteins and vitamins essential to improve plant growth, nitrogen fixation and solubilize phosphate solubilization, important for plant health [57,58].

Chlorella is also considered a potential resource for biofuel production due to its high lipid content (ranging from 14 to 63% of dry weight). Following lipid transesterification for biodiesel, the residual biomass can be used to generate other biofuels such as methane, bio-oil and ethanol [46,59,60]. Nevertheless, the high costs associated with microalgae culture medium hamper microalgal production. For this reason, this work aims to assess the growth of *C. vulgaris* in different effluents from a municipal WWTP, namely secondary effluent (SE) and sludge run-off (SR), as possible substitutes of synthetic growth media, and evaluate the chlorophyll synthesis, as well as the nutrient removal.

2. Materials and Methods

2.1. Microalgae Culture Preparation

The green microalgae *Chlorella vulgaris* (ACOI-879) was provided by Coimbra Collection of Algae (ACOI). It was inoculated in a standard growth medium, bold basal medium double nitrated (BBM2N) [61], formulated as described in Table 1, being the pH rectified to 7.5, through the addition of NaOH (1N). The inoculate was grown for one week under the conditions mentioned in Table 2 in order to produce the inoculum for the experiments. The reagents used for the culture medium BBM2N formulation were all analytical grade (Panreac; VWR Chemical; Enzymatic; Merck; Chem-Lab and Sigma-Aldrich, Portugal).

Table 1. Chemical composition of the culture medium bold basal medium double nitrated.

Chemical Compound	Concentration (g/ L)
$NaNO_3$	0.25
$MgSO_4 \cdot 7H_2O$	0.075
NaCl	0.025
K_2HPO_4	0.075
KH_2PO_4	0.175
$CaCl_2 \cdot 2H_2O$	0.025
$ZnSO_4 \cdot 7H_2O$	0.00882
$MnCl_2 \cdot 4H_2O$	0.00144
$(NH_4)6Mo_7O_{24} \cdot 4H_2O$	0.00176
$CuSO_4 \cdot 5H_2O$	0.00157
$Co(NO_3)_2 \cdot 6H_2O$	0.00049
H_3BO_3	0.01142
EDTA	0.05
KOH	0.031
$FeSO_4 \cdot 7H_2O$	0.00498
H_2SO_4	0.001

Table 2. Microalgae culture conditions.

Typology	Method
Inoculum size	1.28×10^5 cells/ mL
Operation mode	Batch
Temperature	25 ± 3 °C
Light	White fluorescent
Light Intensity	5 klux
Photoperiod	16 h day; 8 h night
Working volume	250 mL
Aeration	Continuous
Agitation	Mechanically, daily
Aeration rate	0.05 L/ min

2.2. Wastewater Collection

Secondary wastewater and sludge run-off were collected from Vila Verde WWTP (Figueira da Foz, Portugal), located at an estuarine area, namely Mondego estuary.

The SE and SR were transported in plastic bottles to the laboratory in cool boxes, where they were sterilized, at 121 °C for 15 min, and allowed to cool to room temperature and settle by night, for suspended particle sedimentation.

2.3. Experimental Design

Chlorella vulgaris was cultivated on two types of municipal effluents: (a) secondary effluent (SE) (Figure 1a) and (b) sludge run-off (SR) (Figure 1b).

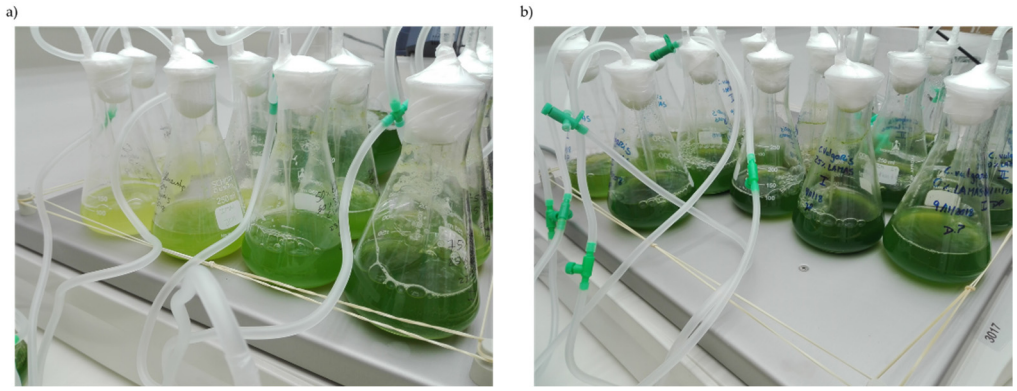

Figure 1. *C. vulgaris* cultivation in different concentrations of (**a**) secondary effluent (SE) and (**b**) sludge run-off (SR).

The assays were performed in Erlenmeyer flasks (V = 250 mL), previously sterilized in an autoclave for 15 min. at 121 °C, in order to prevent any contamination. The experiment was performed under aseptic conditions, in triplicate, with 4 dilution levels of the wastewater (25%, 50%, 75% and 100%) with the standard culture medium BBM2N, also used as a control. The assays were conducted under the conditions mentioned in Table 2, for 12 days. Sampling was performed every 2 days, 25 mL of sample being retrieved for growth, chlorophyll, and nutrient analysis.

2.4. Microalgae Growth Assessment

Microalgal cells were counted by placing 20 µL in a Neubauer chamber, in a light microscope (Kern and Sohn Gmbh, Germany) at 40× magnification. The optical density was measured in a 6715 ultraviolet/visible (UV/VIS) spectrophotometer (Jenway, UK) at the wavelength of 670 nm.

The correlation of the algal number of cells with optical density is shown in Figure 2, where is possible to observe a linear relationship. The optical density in the culture can be used as proxy measure of algal cellular density (expressed in number of cells/ mL), only if

they are linearly related [62]. In this study, the correlation equation was used to estimate the algal biomass concentration in the cultures.

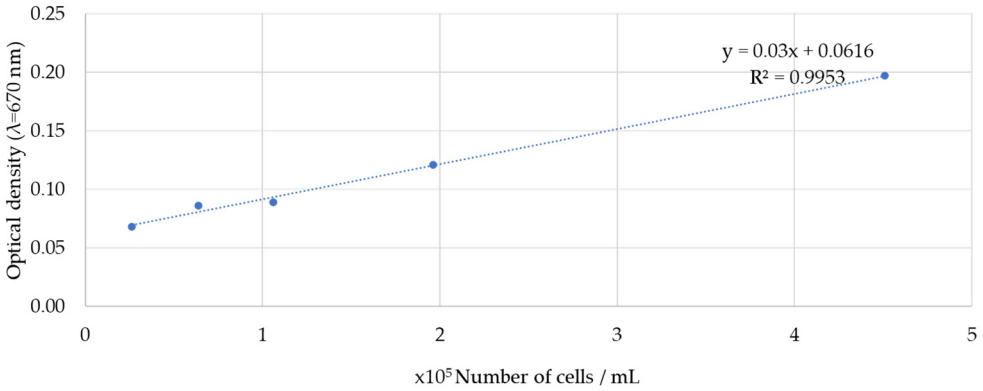

Figure 2. Correlation between microalgal number of cells per milliliter and optical density of the microalgal culture.

The specific growth rate (μ) was calculated through the formula [63,64]:

$$\mu = (\ln (N_L) - \ln (N_0))/(t_L - t_0)$$

N_L—the biomass concentration at time L
N_0—the biomass concentration at time 0
t_L—is the moment time for the end of the period L
t_0—is the moment time for the start of the period 0

2.5. Determination of Physical and Chemical Parameters

In the sampling days, chlorophyll content was measured through a fluorimeter (Turner Designs, CA, USA) and the pH was analyzed through a pH meter (WTW Inolab, Germany).

2.6. Nutrient Analysis

Ammonia (NH_3-N), total nitrogen (Total N) and total phosphorous (Total P) were measured in the collected growth medium at the beginning of both assays and at every sampling day for 0% SE (control) or SR, 50% SE or SR and 100% SE and SR. Aliquots of growth medium were firstly centrifuged for 30 min at 5000 rpm and filtrated with a polycarbonate filter of 0.4 of μm porosity prior analysis.

Nutrient analyses were performed in a continuous flow autoanalyser (SAN++ System, Skalar, Netherlands). Working standards solutions were used for quantification by external calibration. The working standards were prepared by dilution of stock standard solutions, prepared monthly using analytical grade reagents (NH_4Cl, $NaNO_2$, KH_2PO_4). Limits of detection were 0.04 mg/L for NH_3-N, 0.14 mg/L for Total N and 0.06 mg/L for Total P.

2.7. Statistical Analysis

The statistical analysis was performed with the software Sigma Plot v.14. Data was checked for normality (Shapiro–Wilk test) and homogeneity (the equal variance test Brown-Forsythe). Two-way analysis of variance (ANOVA) was then performed to assess statistically significant differences among the cellular density, the specific growth rates, the chlorophyll and the nutrients concentration within the time and the effluent concentration. The Holm–Sidak multiple comparison *t*-test was used after the rejection of the two-way ANOVA null hypothesis.

3. Results

3.1. Microalgae Growth

Generally, *C. vulgaris* was able to grow in every tested concentration on both effluents (Figure 3), exhibiting a typical growth curve by presenting a lag and exponential phase.

In both experiments with SE and SR, it was observed the presence of lag phase, however with different time intervals. For instance, in the 50% SR, it was possible to observe the stationary phase between days 8 to 10, and the declining phase until day 12 of the assay. On the other hand, in the 75% SE, the declining phase was observed from day 8 until the end of the assay. Regarding cultivation in different SE concentrations (Figure 3a), no statistically significant differences were found between the number of cells per milliliter until day 8 of the experiment. From that day on, the 100% SE stands out ($p < 0.05$) from the other treatments, showing the lowest number of cells (day 12; 2.37×10^7 cells/ mL). Since days 8 to 12, no statistically significant differences were observed between the control, the 25 and 50% SE.

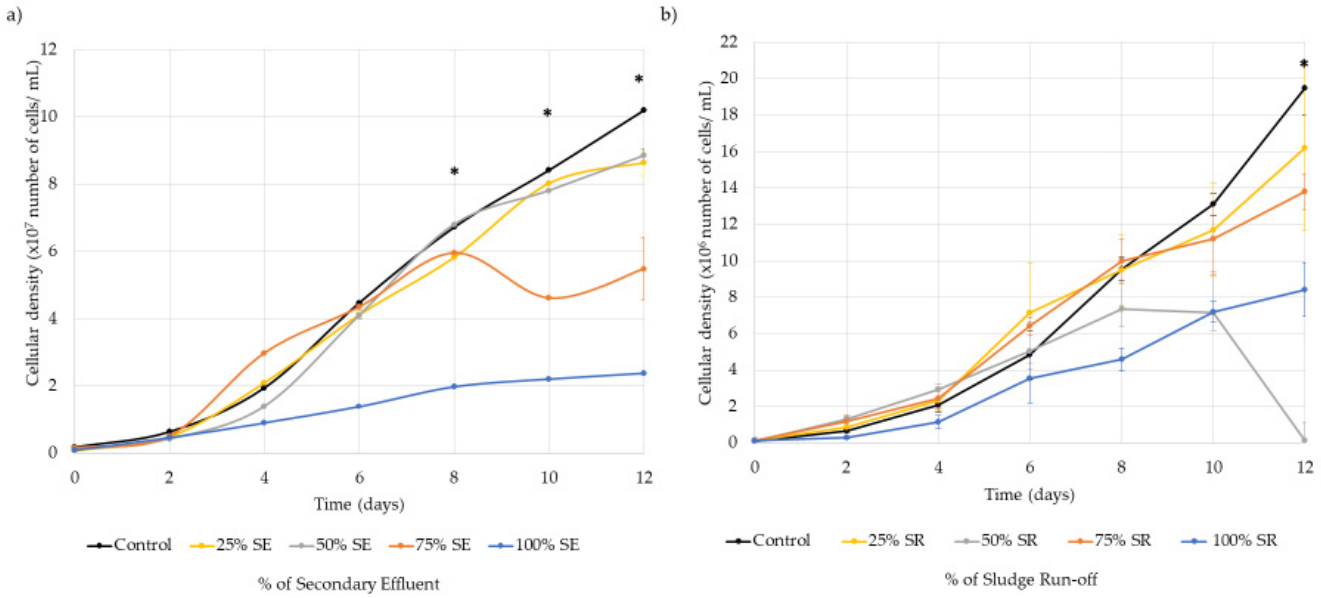

Figure 3. Microalgal growth in the different concentrations of (**a**) secondary effluent (SE) and (**b**) sludge run-off (SR) for 12 days (mean ± standard deviation; n = 3). The symbol * indicates statistical differences among data (p-value < 0.05).

Regarding the cultivation of *C. vulgaris* in SR (Figure 3b), statistically significant differences ($p < 0.05$) in the cellular density were only observed at day 12 of the experiment. In this sampling day, the 100% SR (8.42×10^6 cells/mL) stands out, by differing from all the other treatments ($p < 0.05$). However, for day 12, no statistical differences were detected when comparing control (1.95×10^7 cells/mL), the 25% (1.62×10^6 cells/mL) and the 75% SR treatments (7.91×10^6 cells/mL) ($p > 0.05$).

The specific growth rate is an important parameter to assess the dynamic behavior of the microalgae culture (Figure 4). Except for the 50% SR, it was possible to visualize an overall increase of the specific growth rate using the SR as a growth medium, compared to the utilization of the SE. However, the 75% concentration of both SE and SR stands out from the other treatments (p-value < 0.05).

For the SE assay, the highest specific growth rates, and showing statistically significant differences (p-value < 0.05) from the remaining treatments, was recorded for the 25% and 50% SE concentration (0.47 and 0.46 day^{-1}, respectively), while the control unveiled a value of 0.39 day^{-1}. Comparatively, the 75 and 100% SE exhibited the lowest specific growth rate (0.33 and 0.32 day^{-1}, respectively).

In the SR assay, the control and 50% SR showed the highest specific growth values (0.53 and 0.56 day^{-1}, respectively), contrasting with the remaining treatments, where a decreasing trend was observed in the percentages SR, namely 75, 50 and 100, exhibiting respectively the values of 0.46, 0.43 and 0.42 day^{-1}. No statistically significant differences were observed among different SR concentrations.

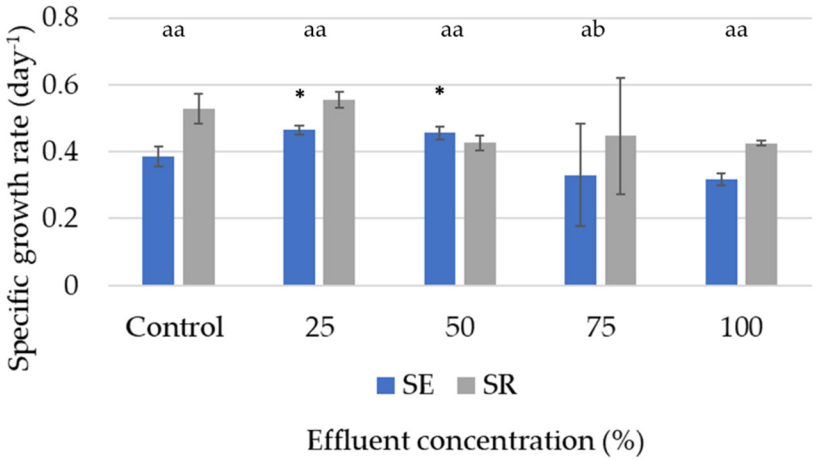

Figure 4. *C. vulgaris* specific growth rate in the different concentrations of the secondary effluent (SE) and sludge run-off (SR) (mean ± standard deviation; n = 3). * *p*-value < 0.05, when compared with the 75% SE. Statistically significant differences found between the two types of effluent (SE and SR), comparatively with the control, are expressed by different letters.

3.2. Physical and Chemical Parameters of Cultivation

Generally, an overall increase in the chlorophyll concentration for every treatment was registered (Figure 5). In the SE assay, the control was the treatment in which *C. vulgaris* exhibited the highest content of chlorophyll (day 12; 2301.67 µg/ L), throughout the experiment (Figure 5a). Nevertheless, from day 4 until the end of the experiment, it was possible to establish two statistical different groups, namely the group that exhibits higher—control, 25% and 50% SE—and lower chlorophyll content—75% and 100% SE (Figure 5).

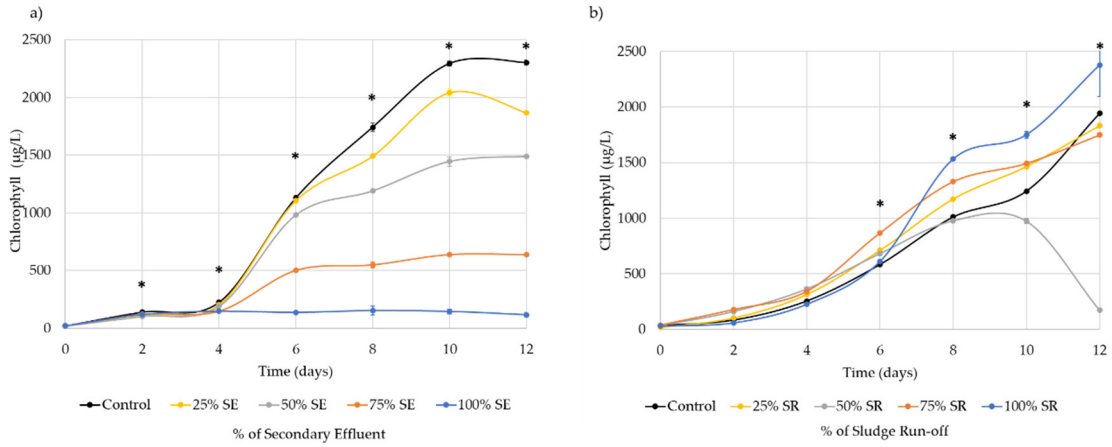

Figure 5. Chlorophyll concentration of *C. vulgaris* grown in the different concentrations of (**a**) secondary effluent (SE) and (**b**) sludge run-off (SR) for 12 days (mean ± standard deviation; n = 3). The symbol * indicates statistical differences among data (*p*-value < 0.05).

However, the cultivation of *C. vulgaris* in 100% SR significantly enhanced the chlorophyll synthesis comparatively with control (Figure 5b). After day 6, it was possible to observe differences (*p*-value < 0.05) between the SR treatments. On day 6, the highest chlorophyll concentration was found for 75% SR (866.67 µg/L), and was statistically different from all the other treatments. From day 8 until day 12, 100% SR stood out positively from all the other treatments (*p*-value < 0.05), achieving a maximum chlorophyll content of 237,834 µg/L (day 12).

In both assays (Figure 6a,b), it was observed an increase of pH values in the control and 25% treatment, ranging between 7 and 9.5. Despite the slight oscillation in pH values for 50%, 75% and 100% treatments, pH seems to stabilize around 8.

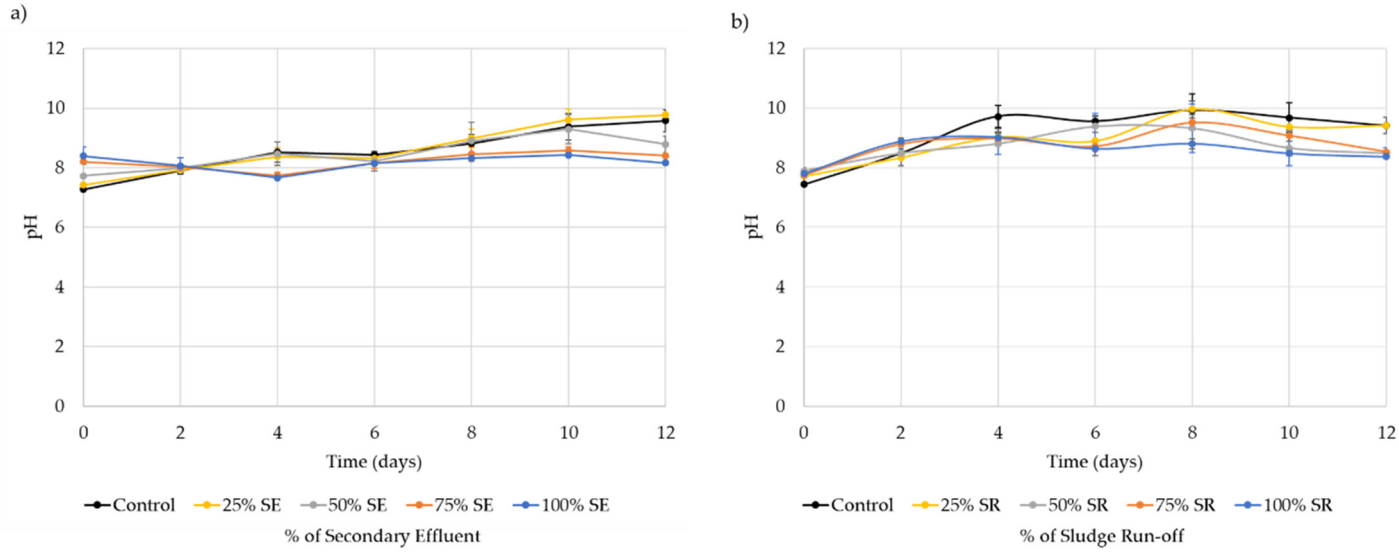

Figure 6. pH value of the growth medium, in which *C. vulgaris* was grown for 12 days. Different concentrations of (**a**) secondary effluent (SE) and (**b**) sludge run-off (SR) were tested (mean ± standard deviation; n = 3).

Regarding nutrients availability, in the SE assay (Table 3) a tendentious increase of N-NH_3 concentration was observed in control, 50% and 100% treatments. Concurrently, total P concentration decreased, significantly (p-value < 0.05) in the 50% and 100% SE concentrations, achieving removal rates of 45.4% and 94.8%, respectively.

Table 3. Nutrient availability in each treatment with secondary effluent (SE) (mean ± SD; n = 3). The symbol * indicates statistical differences (p-value < 0.05) among the different effluent concentrations, in each sampling day, in comparison with the control.

	N-NH_3 (mg/ L)			Total P (mg/ L)		
Time (Days)	**Control**	**50% SE**	**100% SE**	**Control**	**50% SE**	**100% SE**
0	0.12 ± 0.09	0.05 ± 0.00	0.04 [a]	52.23 ± 1.57	32.62 ± 4.99 *	18.68 ± 8.57 *
2	0.12 ± 0.04	0.08 ± 0.02	0.19 ± 0.04	51.33 ± 2.74	27.34 ± 5.34 *	0.97 ± 0.31 *
4	0.15 ± 0.02	0.11 ± 0.02	0.2 ± 0.07	46.5 ± 1.76	20.09 ± 1.75 *	1.16 ± 0.11 *
6	0.171 [a]	0.22 ± 0.03	0.2 ± 0.03	43.76 ± 4.25	19.44 ± 7.63 *	1.06 ± 0.35 *
8	0.28 ± 0.01	0.27 ± 0.02	0.15 ± 0.02	49.7 ± 4.59	15.03 ± 0.92 *	1.82 ± 0.28 *
10	0.28 ± 0.06	0.26 ± 0.04	0.26 ± 0.04	52.5 ± 5.66	17.23 ± 2.67 *	1.68 ± 0.47 *
12	0.48 ± 0.18	0.26 ± 0.02	0.66 ± 0.41	52.64 ± 5.18	17.81 ± 0.34 *	0.97 ± 0.17 *

[a] only 1 replicate analyzed. Note: The analysis of Total N was not possible.

For the SR experiment (Table 4), the content of N-NH_3 increased in control, whereas it significantly decreased in the other treatments, removal rates of 78.7% and 95% being achieved, respectively, for 50% and 100% SR concentrations. Furthermore, a decrease in total N concentration were found for all treatments. Control, 50% and 100% SR exhibited removal rates of 52.1%, 73.9% and 65.9%, respectively. For total P, a reduction of total P concentration was registered in control (14.7%) and 50% SR (31.2%), but an increase of this nutrient in the 100% SR was observed.

Table 4. Nutrient availability in each treatment with sludge run-off (SR) (mean ± standard deviation; n = 3). The symbol * indicates statistical differences (p-value < 0.05) among the different effluent concentrations, in each sampling day, in comparison with the control.

Time (Days)	N-NH_3 (mg/ L)			Total N (mg/ L)			Total P (mg/ L)		
	Control	50% SR	100% SR	Control	50% SR	100% SR	Control	50% SR	100% SR
0	0.01 [a]	22.62 ± 1.53 *	28.14 ± 1.88 *	15.13 ± 2.35	76.49 ± 21.36 *	22.95 ± 1.80	59.86 ± 7.54	47.94 ± 3.39	17.11 ± 0.84 *
2	0.34 ± 0.02	11.62 ± 1.76	21.42 ± 4.23 *	11.72 ± 1.03	48.36 ± 3.52 *	21.92 ± 4.16	53.29 ± 5.55	53.09 ± 2.57	12.24 ± 1.38 *
4	0.06 ± 0.02	2.86 ± 1.06	16.91 ± 3.13 *	13.57 ± 2.63	31.45 ± 1.88 *	16.52 ± 2.08	41.06 ± 10.15	43.3 ± 2.33	18.58 ± 0.34 *
6	1.46 ± 0.54	5.51 ± 3.69	12.05 ± 4.07	13.31 ± 2.21	27.35 ± 6.14	15.07 ± 2.85	65.65 ± 9.92	42.54 ± 3.86 *	31.36 ± 4.11 *
8	1.28 ± 0.97	n.a.	2.43 ± 0.75	8.17 ± 1.24	n.a.	10.09 ± 0.33	44.82 ± 11.61	n.a.	20.44 ± 4.07 *
10	1.72 ± 0.35	4.42 ± 3.15	2.03 ± 0.48	8.84 ± 1.40	24.26 ± 2.36	9.26 ± 1.03	51.08 ± 0.87	42.18 ± 0.45	21.77 ± 0.84 *
12	2.28 ± 0.37	4.82 ± 1.72	1.39 ± 0.27	7.24 ± 1.63	19.97 ± 4.05	7.82 ± 1.86	50.44 ± 13.40	32.97 ± 2.90 *	20.21 ± 1.42 *

n.a.—not analysed. [a] only 1 replicate analysed.

4. Discussion

Wastewater application for microalgae culture is a sustainable and low-cost culture medium that can be used for microalgae biomass production. Municipal wastewaters are a complex mixture of organic and inorganic compounds [17]. They are a source of nutrients (i.e., nitrate, nitrite, phosphorus), but can also contain noxious compounds (i.e., pesticides, household chemicals, hormones) that can affect *C. vulgaris* growth and compromise the microalgae growth [65]. Moreover, previous studies showed that the inoculation of the microalgae from a standard culture medium directly to wastewater can extend the lag phase, resulting in a lower *C. vulgaris* growth [66].

The existence of lag phases for all the treatments suggested that *C. vulgaris* needed a period of adaptation to the SE and SR [63]. The duration of the lag phase for each microalgae culture was, however, different. The higher cell density observed in the SE control was expected, because the growth medium used (BBM2N), is an optimized culture medium that is enriched in nutrients that are pivotal for *C. vulgaris* metabolic processes and growth [67]. As a counterpart, throughout the experiment period, we observed a microalgae growth reduction in the 25%, 50% and 75% SE concentrations, probably due to the lack of limiting nutrients or due to the self-shading effect caused by the cell density [21,25].

In SE assay, *C. vulgaris* achieved a lower specific growth rate (0.32 day^{-1}) in comparison with the studies conducted by Sydney et al. [68], Almomani and Örmeci [63] and Znad et al. [65], in which *C. vulgaris* exhibited a value of 0.64, 0.52 and 0.62 day^{-1}, respectively. Concomitantly, Almomani and Örmeci achieved a specific growth rate of 0.25 day^{-1} in centrate [63], while in this study we registered a higher value of 0.42 day^{-1} in the SR assay. The enhanced specific growth rates in the 25% SR, 25% SE and 50% SE, compared to control, can be an effect of the organic carbon present in the SE and in the SR [65]. For instance, the presence of these organic nutrients can promote the mixotrophic growth of *C. vulgaris*, enhancing the specific growth rate [69].

The chlorophyll content expresses photosynthetic activity, which varied within the different effluent ratios tested, and is in concordance with the cellular density of the cultures in each treatment. The chlorophyll is an essential pigment that acts as a light converter into energy, through the process of photosynthesis [70]. Furthermore, this pigment has several biotechnological applications for several industries, such as food, feed or pharmaceutical [71–73]. In this context, several studies have been conducted in order to optimize microalgae chlorophyll production [22,74–76]. Growth media with lower

nitrogen and phosphorus content have been shown to decrease chlorophyll production, while phosphorus-enriched growth media have revealed to be a chlorophyll production promoter [77,78]. However, it is necessary to consider that the microalgae growth is not only affected by nutrient concentrations, but also by the ratios of C, N and P in the wastewater [63].

Nitrogen is an important and limiting nutrient for microalgae, being necessary for protein, nucleic acid and chlorophyll molecules synthesis [79]. Consequently, lower contents of nitrogen can lead to a significant reduction of chlorophyll production [80,81]. Phosphorus is equally a pivotal and limiting nutrient for microalgae growth and development. This nutrient plays an important role in several metabolic pathways, such as the Calvin cycle, phosphorylation processes and ATP synthesis [82,83]. Thus, growth media with low phosphorus content will directly affect the microalgae growth and chlorophyll production [65,80]. In this context, the higher phosphorus concentration found in the SR in comparison to SE can explain the highest chlorophyll content found in *C. vulgaris* cultivated in SR.

According to some authors, the carbon source also influences *C. vulgaris* metabolism, that can present a photoautotrophic, heterotrophic or mixotrophic metabolism [84]. Due to the absence of a carbon source in the control (with BBM2N growth medium), *C. vulgaris* show a photoautotrophic metabolism and carbon is provided through the aeration (atmospheric carbon dioxide) [45]. When municipal wastewater is added to the growth medium, an organic carbon food source is also added [65,85]. In the presence of an organic carbon source, *C. vulgaris* can present a mixotrophic metabolism. According to Znad et al. [65], during the photoautotrophic growth pH values often increase, whereas in the mixotrophic metabolism a low pH range variation is observed. Based on these findings, the pH range variation and *C. vulgaris* growth registered herein suggest that the SE and the SR are also an organic carbon source. Ammonia concentration can also affect pH values [86]. Ammonia is a critical nutrient, but in high concentrations can be toxic for microalgae [87]. In fact, a previous study shown that an ammonia concentration higher than 28 mM can reduce the microalgae culture viability [88]. Here, the reported ammonia concentration was significantly lower and did not present a toxic risk for *C. vulgaris* growth.

In light of the results presented here, the application of wastewater for microalgae cultivation revealed to be advantageous, maximizing the growth of *C. vulgaris*, particularly for 25% and 50% SE and 25% SR treatments. The addition of effluent to growth media will reduce the costs inherent to microalgae biomass production enhancing the economic viability of the process.

The present study showed that the cultivation of *C. vulgaris* can be coupled with bioremediation of effluents since removal of nutrients was verified during the 12 days of cultivation. No differences were observed in the total P of the control, which could be caused by the nitrogen phosphorus ratio, whereas high levels of nitrogen are required to ensure the efficient removal of phosphorus [89]. On the other hand, in effluent, phosphorus removal (94.8%) occurred and in this study a higher removal was registered in comparison with the study conducted by Singh and Dhar (2007), where *C. vulgaris* presented a removal rate of 49.6% [90]. Higher phosphorous removal has also been reported by other works. For instance, an 85% decrease in phosphorus content was observed after two days of *C. vulgaris* cultured in secondary effluent [91]. The cultivation of *C. vulgaris* in SR also showed to remove considerable amount of nitrogen. Previous research, using centrate as culture medium, demonstrated a total nitrogen and total phosphorus removal of 33.6 and 25.8%, respectively [63]. Furthermore, high removal rates of ammonia and nitrogen, 78.3% and 82.8% respectively, were registered by Wang et al. when *C. vulgaris* was cultivated in sludge run-off [20]. This approach combining microalgae cultivation-bioremediation shows potential for further application at industrial scale, possibly by integrating algal farms with wastewater treatment. The application of such practice will promote the production of biomass, reducing the cost of the cultivation process, while enhancing the removal of nutrients from effluents improving the efficiency of wastewater treatments.

Nevertheless, upscaling the usage of municipal wastewaters for microalgae cultivation currently faces several drawbacks from an economic perspective. Despite close systems providing higher control under the abiotic culture conditions, the costs associated to illumination and sterilization hinders its industrial application [92–94]. Moreover, the suspended microalgal biomass harvesting is still expensive, so techniques have been being developed to tackle this problem, such as microalgae immobilization [94]. Furthermore, the added-value product can be valorized for different applications. For instances, microalgae cultivation in municipal wastewater can be used for bioactive compounds extraction if the safety and quality of the biomass is guaranteed. In fact, recent studies showed the potential of microalgae wastewater cultivation for polyhydroxyalkanoate extraction [95], which is a compound with several biomedical applications [96–98]. Another bioactive compound produced by microalgae is chlorophyll, a well-known antioxidant, and a pigment, that can be employed in the textile industry as a natural dye [55].

In addition, microalgae grown in municipal wastewater can contribute to human health through the production of metabolic by-products during the wastewater treatment process, making them renewable sources for sustainable agriculture, highlighting three main objectives: environment, economic feasibility, and socioeconomic net patrimony. Since microalgae produce high levels of important micronutrients and macronutrients for plant growth, they can be used as biofertilizers. Several studies have identified an association between higher nutrient absorption, higher biomass accumulation and higher cultivation yield through the incorporation of microalgae-based fertilizers [99–101]. In a study that examined the effects of algae extracts on plant growth, faster germination and greater rice seed growth were observed [102]. The use of the green microalga *Chlorella* sp. is reported in some studies of microalgae-based fertilizers spread application on agricultural land, for instance, liquid extract application of *C. vulgaris* as a foliar spray has promoted the germination percentage, the radicle and plumule length of three plant species, *Lepidium sativum* (garden cress), *Eruca sativa* (arugula) and *Vigna radiata* (mung bean) [103–106]. Moreover, dried *C. vulgaris* can be used as a soil conditioner and natural fertilizer, enriching the soil with nutrients, and promoting *Zea mays* (maize) seedling [107].

Microalgae production in municipal wastewaters has been studied for energy production [108,109]. Microalgal lipidic profile, particularly the triacylglycerides (i.e., C14:0, C16:0, C16:1, C18:0, C18:2 and C18:3), through transesterferication have been shown to be suitable for biofuel production. For instance, researchers demonstrated that *Chlorella* sp. cultivated in municipal wastewater exhibited a 27.4% lipid content [110], while *C. vulgaris* produced 18.6% [111] and *Auxenochlorella protothecoides* (formerly *Chlorella protothecoides*) presented a lipidic concentration of 33.4% [112].

After lipids extraction, the residual biomass still contains carbohydrates, which are also interesting for bioethanol production [113]. The content of lignin and cellulose that is synthesized by microalgae can be converted into methane-rich biogas through anaerobic digestion. For example, previous research demonstrated that through this method, *Chlorella sorokiniana* and *Chlorella vulgaris* were able to achieve a biogas yield up to 40–73% [114]. Another study points out that the biomass of *C. vulgaris* cultured in urban sewage allowed the production of biogas, presenting a yield of 442 mL/g Volatile Solids (VS), in batch reactors [115]. Furthermore, *C. vulgaris* can also be used as a feedstock for bio-hythane, an alternative to fossil fuels, by coupling dark fermentation with anaerobic digestion [60].

5. Conclusions

This study shows that the municipal effluents' application for *C. vulgaris* growth is feasible, it being possible to achieve higher specific growth rates, biomass yields and chlorophyll content through the addition of these wastewaters into conventional nutrient-rich growth media. Hereby, the highest nutrient concentration was found in the sludge run-off, resulting in a higher chlorophyll content, particularly in the 100% SR. The application of wastewater for microalgae growth was shown to be advantageous, particularly in 25% and 50% SE and 25% SR, where higher specific growth rates were observed.

However, microalgae cultivation in municipal wastewater, still faces several bottlenecks, which hampers the upscaling of this technique. Several up and downstream processed need to evolve, in order to reduce the costs associated with electric, harvest and biorefinery processes.

Currently, research on the application of microalgae to wastewater bioremediation from different sources has evolved and has been focused on coupling bioremediation with biomass growth and further valorization of microalgal feedstock, with novel photobioreactors design, in order to improve its economic viability at an industrial scale. Furthermore, this coupling represents a multipurpose solution with widespread applications.

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