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Municipal Wastewater Effects on the Performance of Nutrient Removal, and Lipid, Carbohydrate, and Protein Productivity of Blue-Green Algae *Chroococcus turgidus*

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Abstract: The use of microalgae in wastewater treatment (WWT) is seen as a promising and sustainable alternative to conventional WWTs, and the obtained biomass is gaining importance as a bio-product. The present study aimed to investigate the effectiveness of using municipal wastewater (MWW) as a nutritional supplement for the cultivation of the cyanobacteria *Chroococcus turgidus* (Kützing) Nägeli 1849 and the pollutant removal potential of the microalgae. The WW received from the different treatment stages (primary, secondary, and final effluent) was applied to the microalgae culture, and algal growth was compared with regard to growth rate, nutrient removal efficiency, and final algal lipid (%) and protein (%) content. In 7-day batch experiments, except for BOD_5 analysis, COD, PO_4-P, and N forms analyses were carried out daily in parallel with in vivo Chl-a and Chl-b, DO, pH, temperature, and conductivity measurements. The growth rates and Chl-a quotas of the microalgae grown in trials were different, and the highest growth rate was with a 1.03 ± 0.06 d^{-1} in the primary effluent (PE). The highest Chl-a and Chl-b quotas among WW trials of microalgae were obtained from the PE trial as 252.4 ± 2 μg L^{-1} and 112 ± 18 μgL^{-1}, respectively. NH_4-N, NO_3-N, NO_2-N, PO_4-P, BOD_5, and COD treatment efficiencies were in the ranges of (74.6–83%), (16–71.2%), (22.2–63.6%), (89–95.3%), (50–76.2%), and (70.3–78.6%), respectively. The microalgae were observed to accumulate the highest lipid (28.05 ± 2.26%DW) content in secondary effluent (SE), the highest carbohydrate (43.93 ± 1.02%DW) content in the effluent (E), and the highest protein content (35.25 ± 1.22%DW) in the PE. The results of this study suggested that *C. turgidus* is a new candidate for bioremediate pollution load of MWW, and its biomass has the potential to offer options in bio-product applications.

Keywords: *Chroococcus turgidus*; nutrient removal; organic matter removal; Chl; lipid; protein; carbohydrate; phycoremediation; municipal wastewater

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1. Introduction

Due to rapid population growth and industrialization, the rapid increase in water pollution and water demand are the two main water-related global problems. By 2050, global municipal wastewater (MWW) production is indicated to increase by 51 percent from current levels [1], while global water demands for industrial and domestic use are projected to be 20 to 30 percent higher than current water use levels [2]. Since most countries are not rich in water resources and existing water resources are not evenly distributed, developing technologies to increase the treatment and reuse of domestic and industrial WW is vital for a sustainable water supply. Therewithal, cost-effective WW management and continuous improvement provide opportunities for reducing pollution and increasing the clean water supply; they promote sustainable development and support the transition to a circular economy [3].

Conventional WWT systems are processes that can be conducted with different combinations of physical, chemical, and biological processes. However, in most cases, they

provide insufficient treatment efficiency [4], cause intense energy consumption and greenhouse gas emissions [5,6], and may continue adverse effects on aquatic life even if the treatment meets final WW discharge standards [6,7]. On the other hand, advanced treatment systems still do not seem to be an ecological and economic solution, as it is a costly, complex, and high-energy-requiring process. In this context, microalgae are expected to make significant contributions to sustainable WWT thanks to features such as effective cost, low energy requirement, beneficial biomass production, contribution to the reduction of sludge formation, success in heavy metal removal, and high treatment efficiency [8].

The phycoremediation process, briefly, is the use of microalgae, or more rarely macroalgae, for the removal or biotransformation of pollutants, including harmful chemicals, from food and WW [4,9]. The microalgae-based treatment system is an ecological, economic, and sustainable approach [10,11], and in recent years there has been a rapid increase in the number of microalgae-based treatment plants globally [12]. However, there may be difficulties in optimizing the system, as municipal and industrial WW mostly contain variable pollution loads [6], poor light penetration [13], and metal concentrations that are not suitable for algae growth [14]. Therefore, choosing the convenient algae species is the first of the key processes for treating WW effectively [4].

Most of the microalgae used in WWT belong to the classes Cyanophyceae (blue-green algae) or Chlorophyceae (green microalgae), and members of the Bacillariophyceae classes (diatoms) have only recently been used [4,15]. The Cyanophyceae members, photosynthetic autotrophic prokaryotes, have an important ecological role due to their contribution to global biological C-sequestration, O_2 production, and the N cycle [16]. Moreover, cyanobacteria species are widely produced commercially for purposes such as potential food, feed, and bioenergy sources [17]. On the other hand, they are extremely tolerant to contaminants and are abundant in almost any habitat [18,19]. Since cyanobacteria have high N and P requirements, their dominance and survival in adverse habitats result in improved water quality in polluted habitats, which makes them convenient candidates for WWT [20]. However, most cyanobacteria-based WWT research has concentrated on traditional cyanobacteria genera such as *Arthrospira*, *Anabaena*, *Oscillatoria*, and *Nostoc* [17,21]; the number of microalgae species known to perform the highest is still very limited. Therefore, the first of the main steps towards goals such as determining the best WWT, biofuel production, or valuable metabolite extraction, is to discover promising new microalgae species [22].

Although *Chroococcus turgidus* has been shown as an indicator of a low-impact environment or uncontaminated water in a previous study [23], many studies, conversely, report on on its dominance in WW [24]. *C. turgidus* is a species that can survive at high temperatures [25], in salt water [26], and even in the presence of highly toxic substances [27]; it has also been reported to be a promising species that can produce highly valuable molecules and antimicrobial-fungal bioproducts [28–30]. These characteristics of the strain have raised the question of whether it could be a potential candidate for both WWT and WW-fed biomass production. However, the number of bioremediation studies on this species is quite limited, mostly based on studies of Sivasubramanian et al. [31] with a strain of *C. turgidus*. The species has been used before for small numbers of bioremediation studies of different WW, such as those in tannery and pharmaceuticals [32], hypochlorite manufacturing [33], sewage [34], oil drilling effluent [31], and detergent industries [35]. Although MWW, a rich source for microalgae growth, is widely used for microalgae cultivation, microalgae-based treatment, or other biotechnological purposes [36–38], to the best of our knowledge, this cyanobacteria *C. turgidus* has not been previously studied in MWWT. The main reasons *C. turgidus* was brought to the focus of this study were that it is highly resistant to toxicity and pollutants [27,39,40], it can grow rapidly, and its biomass has the potential to be evaluated in different areas [28–30]. While *C. turgidus* has been previously used in a limited number of industrial WWTs, this study is the first in terms of the performance of the species in MWWT.

This study aimed to investigate the pollutants-removal potential and growth performance of the cyanobacteria *Chroococcus turgidus* (Kützing) Nägeli 1849 using different

stages of municipal WW as a nutritional supplement. Different stages of MWW were used to determine possible integration locations for phycoremediation systems employed at WWTs using a practic set-up. It also aimed to investigate the biomass content (lipid, protein, carbohydrate) to determine the potential to offer options in bio-product applications.

2. Material and Methods

2.1. Microalgae Culture and Experimental Setup

Microalgae *Chroococcus turgidus* (Kützing) Nägeli 1849, belongs to the phylum Cyanobacteria, was isolated by Sisman-Aydin in 2006 from a water sample taken from Bornova Stream, Izmir, Turkey, at coordinates 38°26′53.32″ N and 27°10′26.80″ E. The monographic and descriptive studies belonging to different scientists had been used for species identification, and a subject expert at Gazi University (Turkey) had the species identification checked and approved. Since 2006, stock culture has been maintained in the Culture Collection of Algae Ecotechnology Lab, Fisheries Faculty, Ege University. BG11 medium [41] was used in enrichment and in all control trials (Table S1). Drinking water was filtered (0.2 µ cartridge filter, Merck, Darmstadt, Germany) and sterilized (121 °C, 15 min) for the microalgae culture. The incubation and acclimation of the strain were carried out according to Simsek and Sisman-Aydin, 2018 [42]. The strain incubation was performed under autotrophic conditions (L:D-24:0) (20 ± 0.5 °C, 100 µmol $m^{-2}s^{-1}$ Illum. by using daylight fluorescent lamp, 400 mL min^{-1} airflow), and afterwards the stock culture was adapted to sterile-filtered WWs (5 L).

The MWW was provided by MMCWTP, Izmir, Turkey (38°28′41.81″ N, and 27° 0′14.59″ E). The WWTP comprises primary treatment units that consist of screens, grit chamber, parshall flume, 12 primary settling tanks, 6 bio-phosphorus tanks, 12 bio-aeration tanks, and 12 secondary settling tanks. The WW samples received from the different treatment stages (primary (PE), secondary (SE), and final (E) effluent) were brought to the laboratory promptly. Then, WW samples were filtered (FS) and sterilized as given above, their characteristics analyzed, and the samples stored at +4 °C until the trials started. The effluent samples (sterile-filtered) which were obtained from primary settling (PE), secondary sedimentation (SE), and discharge (E), and also the control group (C) (BG11-medium), were put into bottles (in triplicate-1.5 L). Then, *C. turgidus* samples, which were obtained from centrifuging at 4500× *g*, were added to each experimental group. Initial in vivo chlorophyll-a concentrations of the trials were approximately 20 ± 5 µ Chla L^{-1} in each trial group. Chlorophyll-a and Chlorophyll-b concentrations were measured daily by using a fluorometer (AquaFluor, Turner Designs, San Jose, CA, USA), and also fluctuation in T (°C), and pH (Orion, SA 729, Beverly, MA, USA) and DO (WTW Oxi 330, Weilheim, Germany) were monitored throughout the experiments. COD and BOD_5 were analyzed using the Merck Spectroquant® Cell Test (Darmstadt, Germany) on the first and last day of the experiment. The analyses of PO_4-P, NO_3-N, NO_2-N, and NH_4-N were performed by modifying the method proposed by [43]. Microalgae suspensions of 25 mL were centrifuged at 6000× *g* (10 min.), the supernatants were filtered (Whatman® nylon MF.-p.sz.0.45 µm, Merck, Darmstadt, Germany), then all parameters were analyzed using Merck-reagent and -cell tests following the Merck Spectroquant Multy© (Darmstadt, Germany) Standard Methods Manual.

2.2. Biochemical Composition

After the 7 day test duration, the microalgae biomass was analyzed for the total lipid, protein, and carbohydrate content.

The total lipid content of *C. turgidus* was established according to Bligh and Dyer (1959) [44]. Briefly, 0.2 g of dried *C. turgidus* samples were consecutively extracted with 7.5 mL of a chloroform/methanol mixture (1:2), 2.5 mL of chloroform, and 2.5 mL of water each for 10 min in a pre-weighed glass tube. For solid particulates of the microalgae, the samples were filtrated (Whatmann, 5951/2 185 mm, Merck, Darmstadt, Germany). The filtrate was centrifuged (1000 rpm, 5 min), and when a biphase (water and organic solvents)

occurred, the water phase was removed and the organic phase was recovered for the estimation of oil content, weighed on a precision balance with 0.001 mg sensitivity.

Lowry et al.'s [45] method was used for total protein content. Briefly, 0.1 mL of deoxycholate (DOC) solution was added to a 1 mL sample. After 10 min, 0.1 mL of TCA was added and the sample was centrifuged (7500 rpm-10 min) [45]. The supernatant was removed, and 1 mL of Lowry's solution (Table S2) was added to the precipitate. After the waiting period of 20 min, 1 mL of fooling reagent was added, and kept on hold for 30 min. The absorbance of the obtained sample at 750 nm was evaluated according to the standard curve.

Total carbohydrate was determined according to the phenol-sulfuric acid method [46]. Briefly, a 1 mL sample containing 1 mg biomass per mL^{-1} was reacted with 3 mL of concentrated sulfuric acid (72 wt%) and 1 mL of phenol (5%, *w*/*v*) in a water bath. After the waiting period of 5 min (at 90 °C), the absorbance was measured via a spectrophotometer (at 490 nm). The absorbances were compared to a standard glucose curve.

2.3. Equations and Statistical Analysis

The growth of the microalgae cultures was measured on a Chl-a and Chl-b basis and their exponential growth rates (μ) were calculated based on Equation (1).

$$\mu = \frac{\log_2(Chl_X/Chl_0)}{t_X - t_0} \quad (1)$$

where μ: specific growth rate (day^{-1}); Chl_0: the Chl-a measure at the beginning of the exponential growth phase, μg L^{-1}; Chl_x: Chl-a measure at the end of the exponential growth phase, μg L^{-1}; t_0: the time period during which Chl_0 was determined, t_x: the time period during which Chl_x was determined.

The removal efficiency of COD, BOD_5, NH_4-N, NO_2-N, NO_3-N, PO_4-P, and total inorganic nitrogen (TN = NH_4-N + NO_2-N +NO_3-N) was calculated for each WW type. Removal efficiency, R (%), was obtained from Equation (2).

$$R(\%) = \frac{C_0 - C_t}{C_0} \times 100 \quad (2)$$

where C_0 and C_t are the mean value of pollutant concentration at the initial time (t_0) and observed time (t), respectively.

The biomass DW (g L^{-1}) was determined using the method described previously [47]. A 50 mL microalgae suspension was collected, and microalgae suspensions (V) were sampled every 24 h and filtered through 0.45 μm membrane filters (Whatman GF/C 47 mm glass fiber) after being pre-dried at 105 °C for 24 h (W_1, g). The algae-containing filter membrane was also dried to constant weight (W_2) and the weight of deionized water (W_0) was used as a blank to deduct the errors caused by environmental factors in the measurement process. The dry weight (DW, gL^{-1}) and biomass productivity (BP, $gL^{-1}day^{-1}$) were calculated based on Equations (3) and (4), respectively, where T was the culture time.

$$DW = \frac{W_2 - W_1 - W_0}{V} \quad (3)$$

$$BP = \frac{DW}{T} \quad (4)$$

The lipid ratio (%) of microalgae cultures was calculated according to Equation (5).

$$\text{Lipid } (\%) = \frac{\text{weight of the oil extracted}}{\text{fresh weight of sample}} \times 100 \quad (5)$$

Significant differences were determined for each parameter (mean ± SD) itself (μ, Chl-a, and Chl-b, DW, BP, R%, biomas content %) with a one-way variance analysis (Fisher's LSD-ANOVA).

3. Result and Discussion

In this study, the primary objective of the study was to investigate the WWT potential of cyanobacteria (blue-green algae) *Chroococcus turgidus* (Kützing) Nägeli 1849 and the effect of the wastewater on the biomass and growth of the microalgae. *C. turgidus* species was chosen as it adapts to changing conditions easily and has the ability to grow quickly. This study, performed in a batch culture system, was limited to a 7-day period that would not cause microalgae death and decay.

3.1. Growth Performance

Chroococcus turgidus (Kützing) Nägeli 1849, a cyanobacterium species, is a bright blue-green prokaryote. *C. turgidus* colony has 2–8 oval or hemispherical-shaped cells ranging in size from 4 to 40 µm. The sheath thickness, covered with a layered gelatinous sheath, ranges from 2 to 6 µm. Its cells are bright blue-green, and also may have coarse-grained content. It can survive at high temperatures [25], in salt water [26], and even in the presence of highly toxic substances [27]; it also produces valuable molecules and anti-microbial-fungal bioproducts [28–30]. These features of the species may make it an appropriate candidate for both WWT and WW-fed biomass production.

The growth performance of *C. turgidus* in the different C:N:P ratios was evaluated, as max-Chl-a and Chl-b quota, specific growth rate, biomass dry weight, daily biomass yield, and lipid, protein, and carbohydrate content, and is summarized in Table 1. *C. turgidus* adapted well to all wastewaters, with a lag phase of 1 day (Figure 1). The growth model of the breed was the exponential model with a sharp slope. The strain entered the stationary phase for all experimental groups on different days in MWW over a 7-day retention period. For the same wastewater in all experimental groups, the Chl-b increase of the strain was similarly parallel to the Chl-a increase (Figure 1). As in this study, the stability of the Chl-a/Chl-b ratio shows that the photosynthetic apparatus of the species function was healthy [22]. The lowest amount of Chl-a obtained after the 7-day culture period was obtained from experimental group E with 107.65 ± 1.75 μgL^{-1} (Table 1). *C. turgidus* reached the highest amount of Chl-a among the experimental groups with a value in BG11 medium of (124.50 ± 0.02 μgL^{-1}), which has the highest TN concentration. Similarly, it was shown in a previous study that there was an increase in all photosynthetic pigments of *Isochrysis galbana* in parallel with the increase in the amount of N in the medium [48].

Table 1. *C. turgidus* growth performance depends on: the max. quota of Chl-a and Chl-b, specific growth rate, biomass dry weight, daily biomass yield, and percent lipid, protein, and carbohydrate content in different C:N:P ratios.

Parameters	Growth Medium			
	PE	SE	E	C (BG-11)
maxChl-a, μgL^{-1}	118.05 ± 2.45	114.80 ± 0.2	107.65 ± 1.75	124.50 ± 0.02
maxChl-b μgL^{-1}	93.07 ± 1.79	95.86 ± 0.97	88.37 ± 0.89	113.90 ± 3.27
µ, day^{-1}	1.03 ± 0.06	0.78 ± 0.03	0.73 ± 0.01	0.54 ± 0.02
µ, day^{-1}	0.88 ± 0.03	0.67 ± 0.01	0.65 ± 0.00	0.46 ± 0.01
DW, g L^{-1}	0.90 ± 0.1	0.81 ± 0.06	0.63 ± 0.09	0.5 ± 0.05
BP $gL^{-1}d^{-1}$	0.10 ± 0.02	0.09 ± 0.02	0.07 ± 0.02	0.06 ± 0.01
C:N:P	40.5:12.1:1.0	36.7:9.7:1	37.1:14.2:1	25.1:8.5:1.0
Lipid %	21.75 ± 3.04	28.05 ± 2.26	15.50 ± 1.53	10.03 ± 0.7
Protein %	35.25 ± 1.22	21.18 ± 0.83	28.97 ± 1.26	26.25 ± 3.27
Carbohydrate %	39.0 ± 2.1	43.05 ± 1.9	43.93 ± 1.02	17.80 ± 2.0

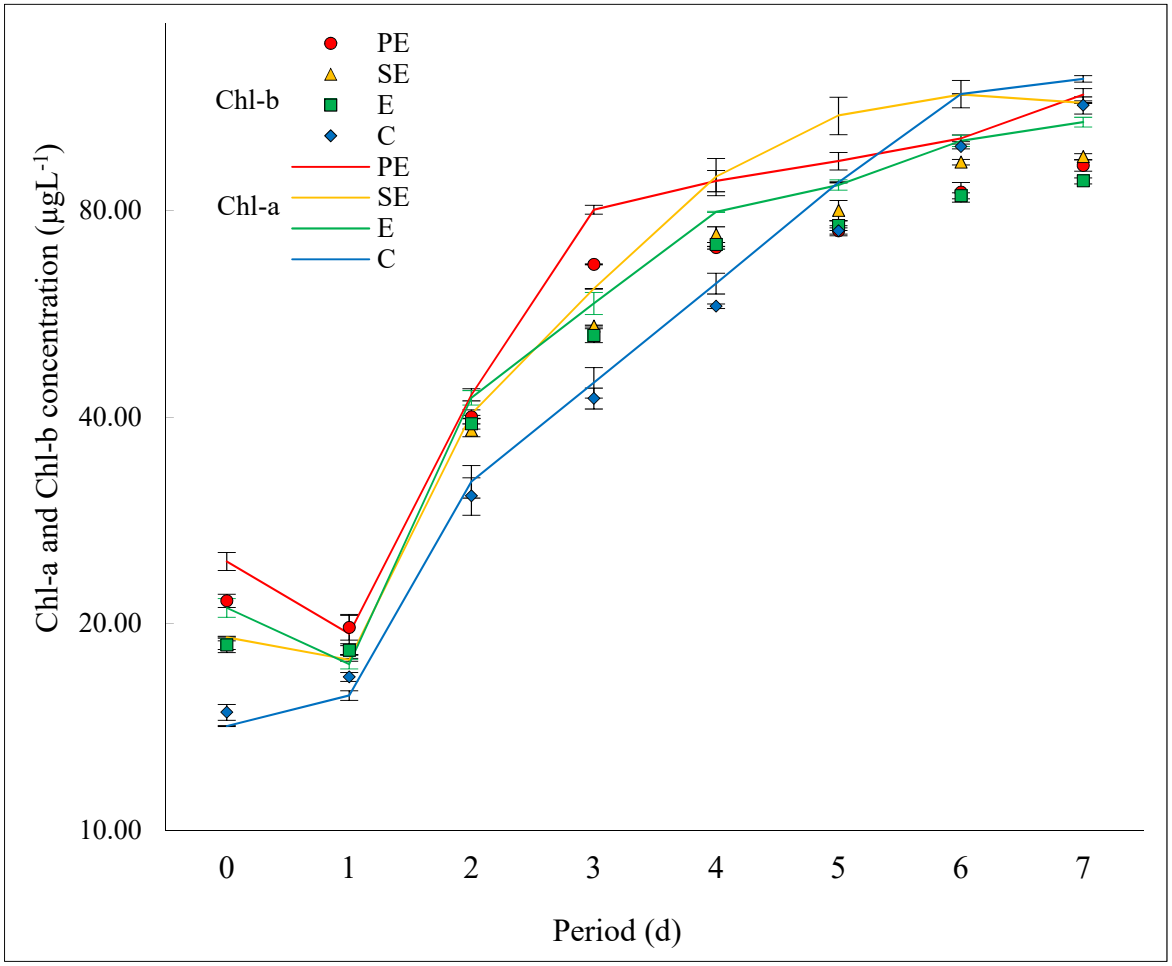


Figure 1. Chl-a and Chl-b-based logarithmic growth curves of *C. turgidus* species exposed to PE, Se, E, and C (BG11) mediums. Error bars show standard deviations (SD) of trials in triplicate.

For whatever purpose microalgae are cultured, the ability to adapt to changing conditions and high growth rate are the most important criteria for species selection for cultivation. According to the results obtained, the growth rates of *C. turgidus* were ordered from highest to lowest as PE, SE, E and C (BG11) experimental groups (Table 1). It was explained in previous studies that the C:N:P ratios, and also N:P ratios, of wastewater can affect microalgae growth rates [49,50]. The reason for this ordering may be that each experimental group had a different exponential phase duration, or different C:N:P ratios in the medium. The C:N:P ratios in the trials were highly variable, and the ratios in PE, SE, E, and C were 40.5:12.1:1.0, 36.7:9.7:1, 37.1:14.2:1, 25.1:8.5:1.0, respectively. While the highest growth rate of the strain was obtained with 1.03 ± 0.06 day^{-1} from the PE experimental group that had a C:N:P ratio of 40.5:12.1:1, the lowest growth rate with 0.54 ± 0.02 day^{-1} was obtained from the C (BG11) experimental group that had a ratio of C:N:P of 25.1:8.5:1.

One of the main goals in microalgae-based wastewater treatment is to obtain high biomass. Municipal wastewater is the most preferred wastewater type for microalgae growth and is seen as a good alternative to obtaining algal biomass. It is known that growth under nutrient-sufficient conditions can increase biomass [51]. In this study, *C. turgidus* biomass yield (DW), which was inoculated into different municipal wastewaters, increased depending on the increasing nutrient concentration in the environment (Table 1). The amounts of biomass in different culture media were significantly different from each other (Table 2).

Table 2. Biomass yields and percentage productivity of lipid, protein and carbohydrate of different microalgae species. IWW: industrial ww.

Medium	Microalgae	Dry Weight Biomass gL^{-1}	Percentage Biochemical Contents (%)			Culture Time (d)	Ref.
			Lipid	Carbohydrate	Protein		
P-MWW S-MWW E-MWW BG11	*Chroococcus turgidus*	0.90 ± 0.1 0.81 ± 0.06 0.63 ± 0.09 0.5 ± 0.05	21.75 ± 3.04 28.05 ± 2.26 15.50 ± 1.53 10.03 ± 0.70	39.00 ± 2.1 43.05 ± 1.9 43.93 ± 1.02 17.80 ± 2.0	35.25 ± 1.22 21.18 ± 0.83 28.97 ± 1.26 26.25 ± 3.27	7	This study
BG11	*Chroococcus turgidus*	-	3.28 ± 0.39	78.89 ± 0.26	17.80 ± 0.41	10	[52]
Chu 10 medium	*Chroococcus turgidus*	≈1.2	10.1 ± 0.52	-	-	28	[53]
Britol's medium	*Chroococcus turgidus*	0.35	6.25	-	-	15	[54]
MWW + IWW	Diatom consortium	0.9	30.13	-	-	10	[55]
MWW + 15% CO_2	*Ourococcus multisporus*	0.31	31	-	-	8	[56]
MWW	*C. humicola*	-	33.55	29.81	4.80	16	[57]
	Oscillatoria sp.	-	6.07	12.39	30.07	16	
	Oscillatoria sp.	-	11.76	27.36	32.9	5	[58]
	Scenedesmus obliquus	0.88	26.5	27.5	28.5	16	[59]
	Coelastrella sp.	1.46 g L^{-1}	30.80	-	-	13	[60]
	Synechocystis PCC6803	0.21 g L^{-1} d^{-1}	28	27.1	-	44	[61]
Aquaculture WW	*Auxenochlorella protothecoides* UMN280	1.51 $gL^{-1}d^{-1}$	28.9	-	-	4	[62]
	Chlorella sorokiniana	1.51	33.45	35.43	29.46	7	[63]
	Tetraselmis chuii	-	17.6	33	12	8	[64]
Agriculture runoff WW	*Cyanobacteria*	24 mg TSS·L^{-1}·d^{-1}	-	4.5–69	-	15	[65]
Wet market WW	*Scenedesmus* sp.	98.54 mgL^{-1}·d^{-1}	23.2	-	41.2	18	[66]

Biomass yields (DW) obtained from PE, SE, E, and C batch groups were 0.90 ± 0.1, 0.81 ± 0.06, 0.63 ± 0.09, 0.5 ± 0.05 gL^{-1}, respectively. In a previous study, 0.31 gL^{-1} biomass was obtained for *Ourococcus multisporus* strain at a retention time of 8 days [56]. Another previous study with municipal wastewater reported that *Scenedesmus obliquus* provided biomass of 0.88 gL^{-1} at the end of 16 days [59]. Differences between the biomass yield results from previous studies and results of the current study may be due to differences in species-specific characteristics in initial concentrations of inoculated microalgae at the start of experiments or nutrient levels in the medium [6]. As a matter of fact, in a previous study, it has been reported that the highest biomass which was under the same conditions was obtained from *Sirogonium sticticum* (0.67 gL^{-1}) and *Temnogyra reflexa* (0.66 gL^{-1}) species, while the lowest biomass was obtained in *C. turgidus* (0.20 gL^{-1}) [54]. The amount of DW obtained in a 7-day culturing process and the biomass yields of other species in similar wastewater are compared in Table 2. As microalgae have the ability to regulate their biomass volumes according to the amount of nutrient uptake from the environment low biomass (DW) can, therefore, be achieved when nutrient levels in wastewater are low [67]. According to the results obtained, *C. turgidus* may provide significant results in terms of biomass yield in municipal wastewater.

3.2. Performance in Pollutant Removal

In this study, for determining the treatment performance of the species, the main forms of nutrients were focused on NH_4^-, NO_2^-, NO_3^-, and PO_4^{-3}. These parameters are the primary nutrient forms in MWW [37,68], so they are frequently used in biological, chemical, or microalgal treatment performance evaluation. In addition, T (°C), DO, S%, Cond., and pH parameters were also monitored. No significant changes were observed in S% and conductivity values in all trial groups during the trial period (Figure 2d,e). The fluctuation observed in T (°C), DO, and pH during the trial period is given in Figure 2a–c. The initial and final characteristics of growth mediums (PE, SE, E, and C) are given in Table 3. MWW was filtered and sterilized in this study to avoid suspended solids and possible bacterial and zooplankton contamination [69]. However, sterilization is not economical for large-scale applications, so future studies should investigate this strain's performance in non-sterile wastewater.

Trial groups inoculated with *C. turgidus* strain started to increase rapidly due to photosynthetic activity towards the peak value in DO as soon as the batch system test started, and at the end of the 1st day reached its highest value (approximately 9.69 mgL^{-1}) by the E trial. A decrease in DO values was observed for all trials in the range of 8.67–8.98 mgL^{-1} on day 5, which coincides with the period when the species entered the stationary phase in all trials (except group C). It was then recovered in the range of 9.2–9.69 mgL^{-1}, with the increase of Chl-a and -b. As a general trend in the microalgae cultures, pH tended to increase in all experimental groups, reaching a peak value in the range of 2–4 days in all experimental groups. The highest measured pH value was obtained from experiment C (BG11) on day 5, with a value of 9.56. While there was no statistically significant change in salinity and conductivity ratios throughout the experiments, PE, SE WW, and C (BG11) media showed similar characteristics.

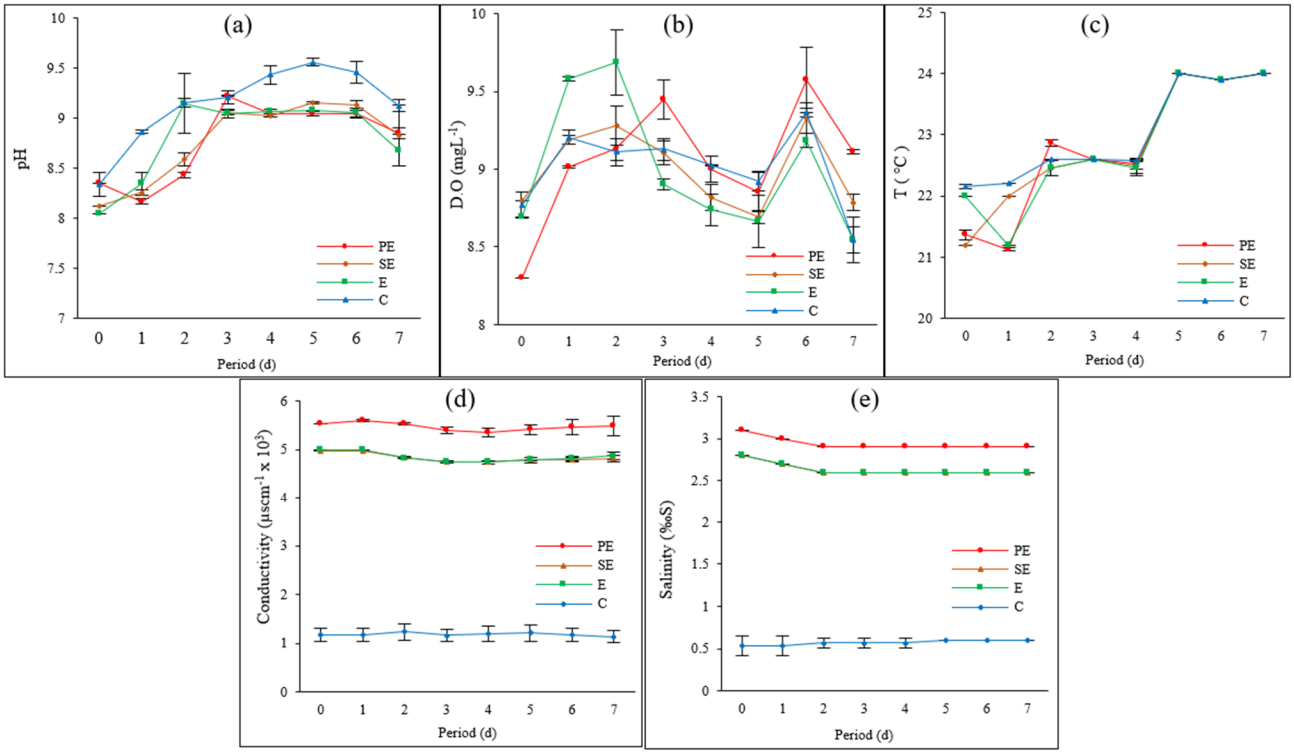


Figure 2. The fluctuation observed in (**a**) pH, (**b**) DO, (**c**) T (°C), (**d**) Cond., and (**e**) S% during the test period.

Table 3. The initial and final of characteristics of growth mediums.

Parameters	Initial Concentration of Growth Medium				Final Concentration of Growth Medium			
	PE	SE	E	C (BG-11)	PE	SE	E	C (BG-11)
Chl-a quota, μgL^{-1}	24.65 ± 0.74	19.11 ± 0.07	21.09 ± 0.66	14.18 ± 0.02	118.05 ± 2.45	114.80 ± 0.2	107.65 ± 1.75	147.15 ± 7.77
Chl-b quota, μgL^{-1}	21.59 ± 0.48	18.63 ± 0.49	18.61 ± 0.28	14.86 ± 0.4	93.07 ± 1.79	95.86 ± 0.97	88.37 ± 0.89	113.90 ± 3.27
NH_4-N mgL^{-1}	53.00 ± 0.0	35.00 ± 0.0	21.00 ± 0.0	9.00 ± 0.01	9.01 ± 0.01	6.03 ± 0.02	5.33 ± 0.33	1.88 ± 0.1
NO_2-N mgL^{-1}	0.04 ± 0.0	0.09 ± 0.0	0.11 ± 0.0	0.08 ± 0.0	0.16 ± 0.0	0.07 ± 0.0	0.04 ± 0.0	0.30 ± 0.0
NO_3-N mgL^{-1}	49.60 ± 0.1	41.66 ± 1.0	49.66 ± 0.3	127.33 ± 20.2	20.00 ± 2.0	13.33 ± 0.58	14.33 ± 1.17	107.00 ± 4.77
TN mgL^{-1}	102.64 ± 0.1	76.75 ± 1.0	70.77 ± 0.3	136.41 ± 20.2	29.17 ± 1.99	19.43 ± 0.56	19.70 ± 0.84	109.18 ± 4.82
PO_4-P mgL^{-1}	8.50 ± 0.0	7.90 ± 0.0	5.00 ± 0.0	16.00 ± 0.1	0.40 ± 0.01	0.60 ± 0.26	0.55 ± 0.25	1.53 ± 0.01
BOD_5 mgL^{-1}	201 ± 0.1	148.01 ± 0.2	48 ± 0.0	120 ± 0.0	48.84 ± 0.4	41.26 ± 0.1	24.0 ± 0.85	36.0 ± 0.9
COD mgL^{-1}	344 ± 2.25	290 ± 1.61	185.57 ± 2.41	402.55 ± 3.11	59.99 ± 0.01	121.74 ± 0.1	49.71 ± 1.0	91.66 ± 0.06
pH	8.35 ± 0.0	8.12 ± 0.0	8.05 ± 0.0	8.47 ± 0.34	9.18 ± 0.1	9.13 ± 0.13	8.46 ± 0.44	8.99 ± 0.55
DO mgL^{-1}	8.30 ± 0.0	8.80 ± 0.0	8.69 ± 0.0	8.77 ± 0.09	9.12 ± 0.02	8.79 ± 0.06	8.55 ± 0.15	8.54 ± 0.09
T °C	21.36 ± 0.08	21.20 ± 0.0	22.00 ± 0.0	22.15 ± 0.04	24.00 ± 0.0	24.00 ± 0.0	24.00 ± 0.0	24.00 ± 0.0
$S_{‰}$	3.10 ± 0.0	2.80 ± 0.0	2.80 ± 0.0	0.53 ± 0.12	2.90 ± 0.0	2.60 ± 0.0	2.60 ± 0.0	0.60 ± 0.0
Cond μscm^{-1}	5.53 × 10^3 ± 0.0	4.98 × 10^3 ± 0.0	5.00 × 10^3 ± 0.0	1.17 × 10^3 ± 0.0	5.49 × 10^3 ± 200	4.82 × 10^3 ± 67	4.87 × 10^3 ± 82	1.13 × 10^3 ± 123

Nitrogen is one of the essential nutrients that regulates the growth and biochemical content (protein, lipid and carbohydrate) of microalgae [22]. Most microalgae can assim-

ilate different forms of nitrogen, namely NO_3, NO_2, NH_4 and urea, but prefer NH_4-N as a nitrogen source because less energy is required for cellular uptake [4,70]. In NO_3 and NO_2 uptake, energy (NADH) is needed for the active transport of nitrate and reduction to nitrite by the reductase enzyme [71]. The percentages of NH_4-N removal for PE, SE, E, and C experimental groups treated with *C. turgidus* were 83.01%, 82.78%, 74.62%, and 79.07%, respectively (Table 4.). The amounts of NH4-N removed for the PE, SE, E, and C experimental groups were 43.99 ± 0.01, 28.97 ± 0.02, 15.67 ± 0.33 and 7.12 ± 0.1 mgL^{-1}, respectively. The NH_4-N concentration continued to decrease steadily in all trial groups during the 7-day trial period (Figure 3a–d). The fact that the pH did not rise above 9.25 except C (BG11) or the temperature above 24 °C during the trial period suggested that the microalgae took up all the NH_4-N, because, besides microalgal uptake, over pH 9.26, the NH_3-N stripping effect from the alkalinization of WW may be combined with NH_4-N reduction [72,73]. On the other hand, at 25 °C and pH < 9.25, the dominant form in WW is NH_4^+.

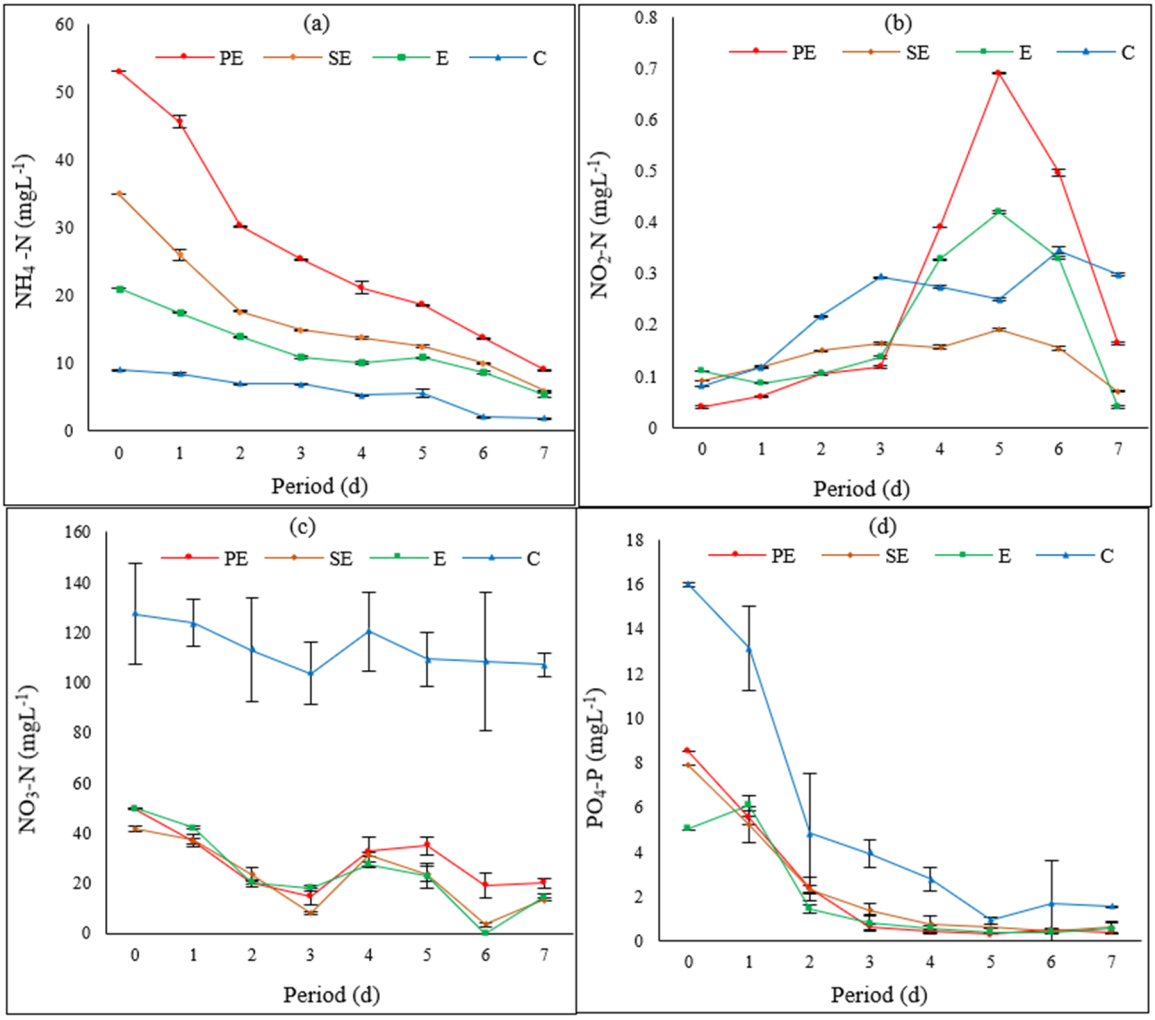


Figure 3. NH_4-N (**a**), NO_2-N (**b**), NO_3-N (**c**) and PO_4-P (**d**) of concentrations values.

Among the microalgae trials, the highest TN removal rate was obtained from the SE trial, with 74.68%, while the lowest removal efficiency was obtained from the BG11 (C) trial, with 19.96%. In terms of removal efficiency, the experimental groups were SE > E > PE > BG11 from high to low, respectively. On the other hand, the amounts of TN removed by the species for the PE, SE, E, and C trials were 73.47 ± 1.99, 57.32 ± 0.56, 51.07 ± 0.84 and 27.23 ± 0.84 mgL^{-1}, respectively. Although the decrease in residue amounts of N in the form NH_4-N continued throughout all trials, it was observed that there was a pause in

all experimental groups on the fourth day in terms of TN removal efficiencies (Figure 4.). The probable reason for this pause is the NO_3-N-NO_2-N balance in the medium, which changes depending on the ambient pH [74]. On the same days, in parallel with the increase in pH (Figure 2a), the amount of NO_2-N in the environment peaked in all experimental groups, and then the amount of NO_2-N decreased with decreasing pH (Figure 3b). *C. turgidus* has been used previously for pH balancing of acidic WWs, adapted to pH = 6 levels, increasing the pH up to 8.2 levels in WW [33]. In this study, pH during the growth of *C. turgidus* similarly increased in all experimental groups and remained relatively similar until six days later (Figure 2a). After day 6, a sharp decrease in all experimental groups resulted in NO_3-N increasing in all experimental groups (Figure 3c).

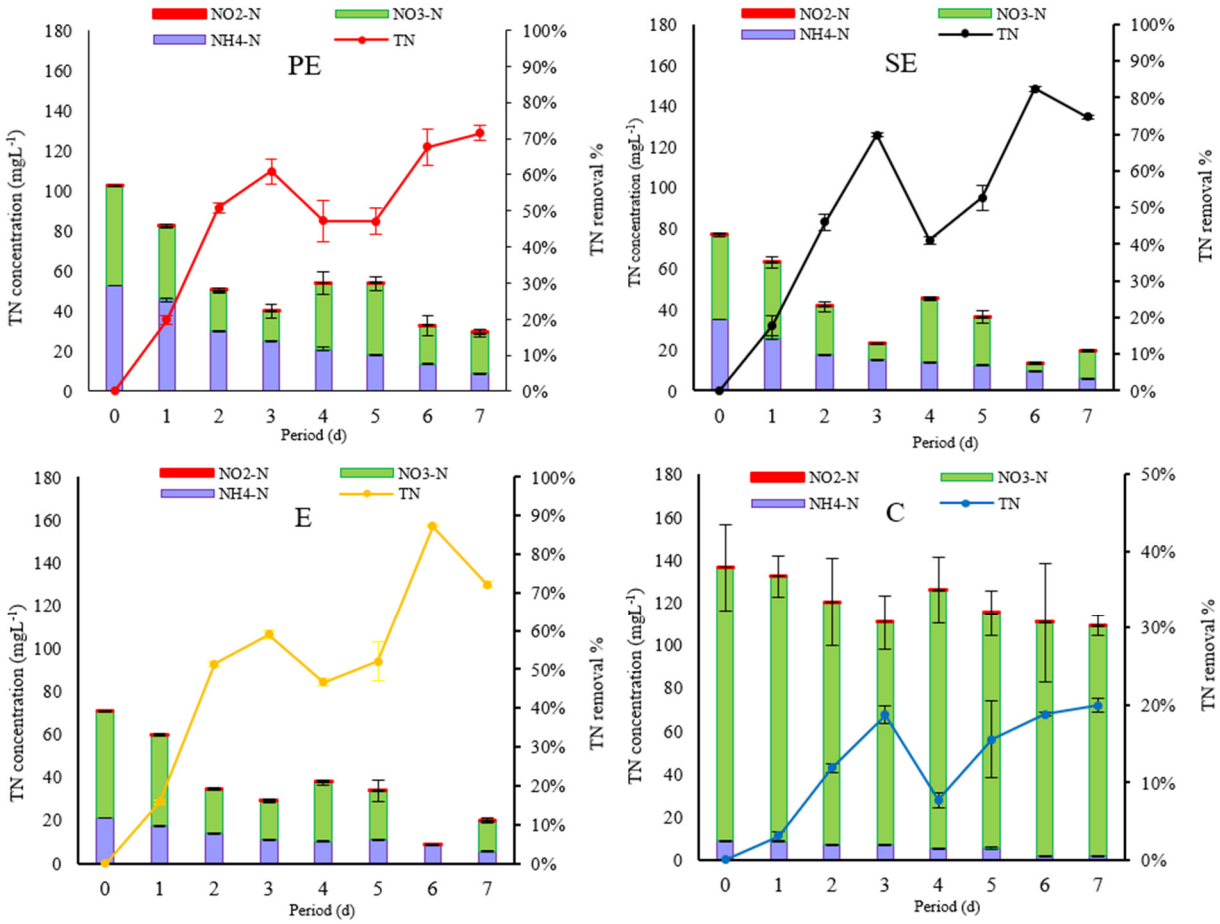


Figure 4. Nitrogen forms concentrations and removal ratios (TN%) in PE, SE, E, and C (BG11) (mean ± SD).

NO_2-N production occurred in the C (BG11) group starting from the first day. After the third day, the highest nitrite was produced in the PE and E groups during the removal of NH_4-N and NO_3-N, and a peak value of NO_2-N was achieved on day 5. The nitrification-denitrification process was observed in all experimental groups. Microalgal NO_3-N assimilation consists of two steps each, both transport and reduction to produce ammonium in the chloroplast [75]. The main reason for this increase in NO_2-N was that NO_3-N was produced in the process of reducing nitrate to ammonium, and some of the nitrite produced was transferred to the environment [70]. However, the NO_2-N concentration in the experimental groups did not pass over 0.69 mgL^{-1} for PE, 0.19 mgL^{-1} for SE, 0.42 mgL^{-1} for E, and 0.35 mgL^{-1} for C (BG11). A previous study showed a similar peak value and subsequently reduced NO_2-N and NO_3-N values for cyanobacteria *Phormidium* sp [76]. It was stated that 6.8% (1.82 ± 0.87) of the nitrogen removed is in the form of N_2 or N_2O by the nitrification-denitrification process or lost by ammonia volatilization for the cyanobacteria. As a matter of fact, in another

previous study, cultivation of *Chlorella* sp in WW was noted with an increase in NO_2-N and a corresponding decrease in NO_3-N [70].

Microalgal P uptake is predominantly related to the stocking into ribosomal RNA [50], and sufficient N in the medium is needed to achieve this. Therefore, the N:P ratio is also very important in the P uptake mechanism of microalgae. It has been shown in previous studies that P uptake was limited in N-limited conditions, regardless of P concentration [77]. At the initial time of the study, the E trial group had the highest N/P ratio among the trial groups (Table 1), with an N/P ratio of 14.15:1. Still, also, the lowest P removal ratio (89%) was achieved from this group (Table 4). Considering that the TN concentration of the trial medium was not at a concentration (29.17 ± 1.99 mgL) which inhibited the microalgae growth, this result may be attributed to the fact that the E trial group had the lowest PO_4-P concentration (5mgL^{-1}) among the trial groups. Similarly, it has been reported in a previous study that a high N/P ratio caused phosphorus deprivation for microalgae growth [78].

Table 4. Nutrient removal performance of *C. turgidus* compared to previous studies. "–": not measured. NR: no removal occurred in wastewater P-MWW: primary MWW, S-MWW: secondary MWW, E-MWW: Effluent of discharge.

Growth Medium	Species	Removal (%)							Biomass Efficiency (gL^{-1} d^{-1})	Time (d)	Ref.
		NH_4-N	NO_2-N	NO_3-N	TN	$P0_4$-P	BOD_5	COD			
P-MWW	*Chroococcus turgidus*	83.01 ± 0.01	NR	59.68 ± 4.11	71.58 ± 1.99	95.29 ± 0.12	76.20 ± 1.2	82.56 ± 3.9	0.10 ± 0.02	7	This study
S-MWW		82.78 ± 0.06	22.22 ± 2.22	67.99 ± 1.57	74.68 ± 0.56	92.41 ± 3.35	72.23 ± 1.43	58.02 ± 4.9	0.09 ± 0.02		
E-MWW		74.62 ± 1.59	63.64 ± 1.57	71.14 ± 2.17	72.16 ± 0.84	89.00 ± 5.00	50.00 ± 1.05	73.21 ± 2.2	0.07 ± 0.02		
BG-11		79.07 ± 1.03	NR	15.97 ± 10.77	19.96 ± 0.84	90.42 ± 0.07	70.00 ± 1.32	77.23 ± 5.5	0.02 ± 0.00		
Tannery WW	*Chroococcus turgidus*	70.0	-	-	70.0	95.0	50.0		-	10	[32]
Pharm WW		30.0	-	-	70.0	95.0	50.0	50.0	-		
Oil drill WW (lab)	*Chlorococcum humicola*	-	-	78.04	-	17.54	96.17	52.19	-	10	[31]
Oil drill WW (pilot tank)		-	-		-	-	70.27	-	-		
Oil drill WW (scaled up tank)		-	-	7.31	-	-	93.20	15.04	-		
Sewage and Industrial WW	*N. muscorum*, *A. subcylindric*	20.9–96	-	19.6–80	-	20.8–95	-	20–57.1	-	10	[79]
P-MWW	*Nostoc muscorum*	-	-	-	72.0	88.2	-	85.7	5.901 mg L^{-1} d^{-1}	7	[4]
MWW	*Chlorella*, *Cryptomonas*, *Scenedesmus*	80.0	-	-	75.0	95.0	92.0	-	3.5 to 22.7 $gm^{-2}d^{-1}$	7	[80]
	Consorsium diatom	-	-	-	95.1	88.9	51.0	91	0.9 gL^{-1}	10	[55]
	C. humicola	-	-	58.84	-	73.20	92.6	-	-	16	[57]
	Oscillatoria sp.	-	-	50.55	-	69.98	89.77	-	-		
	Oscillatoria sp.	98.125	100	-	-	84.718	-	-	-	5	[58]
	Scenedesmus obliquus	81.9	-	100	-	94	-	71.2	0.88 g L^{-1}	16	[59]
Aquaculture WW	*Chlorella sorokiniana*	98.2	81.8	75.8	-	100	-	88	269 mg L^{-1} d^{-1}	14	[62]
		75.6	96.4	84.5	-	74	-	71.88	498.14 $mgL^{-1}d^{-1}$	7	[63]
Swine WW	*Spirulina platensis*	92.0	-	49.0	-	67.0	45.0	67	-	16	[81]
Agriculture runoff WW	*Cyanobacteria*	-	-	-	95	96	-	-	24 mg TSS·L^{-1}·d^{-1}	15	[65]

In this study, *C. turgidus* showed very high P removal efficiency and the remediation ratios were also reflected in the specific growth rate obtained for each WW. In terms of P removal performances, *C. turgidus* provided 95.29%, 92.41%, 89%, and 90.42% removal efficiency for PE, SE, E, and C, respectively. On the other hand, when the pH of the medium rises above 9, the pH of the medium is an important indicator for removal by microalgae, as some of the P in the medium will be removed by precipitation [82]. As of the fourth day, the pH increased above 9 in all experimental groups, so that some P precipitation could have occurred. However, until the fourth day, pH remained below 9 in all WW samples. In the first three days, 92.6%, 90.76%, 88.6%, and 82.69% PO_4-P removal had already been occurred in the PE, SE, E, and C groups. Considering that the uptake of P by microalgae continues even after the fourth day, it can be said that almost all of the P% removal was removed by *C. turgidus*. According to the results obtained, *C. turgidus* showed similar results compared with the PO4-P removal efficiencies of different cyanobacteria species in similar wastewater (Table 4), depending on C:N:P and initial concentration differences [4,32,57,58,79].

This study also investigated the COD and BOD5 removal performance of the species. COD and BOD5 were calculated on each trial group's first and last day (Table 4.). BOD de-

pletes the dissolved oxygen of the surface waters, leading to the death of aquatic organisms and the creation of anaerobic conditions. BOD is a general indicator of the pollution load in the WW, so its removal is the primary goal of WWT. It is also an important parameter for determining both the biodegradability capacity and the performance of a treatment plant. For microalgae to reduce COD and BOD in WW, the species must first be able to use organic carbon as well as inorganic N and P, and light and CO_2 play an important role in this process [83]. Moreover, the photoperiod can play an important role in this process; COD and BOD removal ratios may vary according to varying photoperiods. In a previous study, it was reported that there was a 38% increase in the COD removal efficiency of *Chlorella sorokiniana* under continuous light conditions (24:0 L:D) in a batch culture system [84]. The amounts of BOD_5 reduced for the PE, SE, E, and C trials were determined as 152.16 ± 0.4, 105.75 ± 0.1, 24 ± 0.85 and 84 ± 0.9 mgL^{-1}, respectively. In this study, the highest COD removal rate of 82.56% was obtained from the PE trial, while the lowest COD removal was obtained from the SE trial with a rate of 58.02% in the trial systems of *C. turgidus* species, which were carried out in continuous light and batch culture systems. COD removal efficiencies of *C. turgidus* gave comparable results to previous studies, with ratios of 82.56%, 58.02%, 73.21%, and 77.23% in PE, SE, E, and C groups, respectively (Table 4.). The amounts of removed COD for the PE, SE, E wastewater, and C medium were determined as 284.01 ± 0.01, 168.26 ± 0.1, 135.86 ± 1, and 310.89 ± 0.06 mg L^{-1}, respectively. On the other hand, the amounts of removed COD were significantly different in each trial and were ordered as BG11 > PE > SE > E from the highest amounts of removed COD to the lowest. The degradability of organic matter depends on the type of organic form as well as on the species-specific features of the microalgae species used [85]. In the comparison table given in Table 4, the COD removal performances of microalgae were highly variable (15–91%) in previous studies on similar WW types [6,86]. Although some previous research has shown that the rate of COD reduction in both heterotrophic and mixotrophic modes is higher than in the autotrophic mode [87,88], *Chlorella sorokiniana* in autotrophic mode (24:0) has been reported to show the highest COD removal compared to mixotrophic modes (12:12 and 16:8 L: D). The 7-day COD removal efficiency of the strain under autotrophic conditions (24:0 L: D) in this study was higher than the previous study conducted in pharmaceutical wastewater under mixotrophic conditions. However, considering the initial microalgae cell density, origin of the species, wastewater type, and composition of both studies, further studies under the same conditions will be needed to make a more objective comparison.

3.3. Biochemical Performance

The characteristics of the WW to be used for wastewater-based microalgae cultivation may greatly affect the amount and content of biomass to be obtained. MWW is seen as a very rich source for obtaining microalgae biomass [36–38]. For this purpose, MWWs with different characteristics (PE, SE, E) were used to evaluate cyanobacteria *C. turgidus* biomass.

Microalgal organisms in WWT can be used for many different purposes, including reduction of COD and BOD, removal of N and P, inhibition of coliforms, removal of heavy metals, and balancing of pH [33,37]. The high concentration of N and P in most WWs provides an advantage in providing an inexpensive nutrient source for microalgae biomass production [36,38]. However, the biochemical composition of the obtained microalgae biomass is as essential as the microalgae treatment performance. The lipid content of almost all microalgal organisms ranges from 10 to 30% without any additional treatment [89], but the variation of this content is determined by microalgae ability and environmental conditions. Table 2 shows the biochemical compositions of the biomass of different microalgae species obtained in different WWs. In one of these previous studies, carbohydrate, protein, and lipid ratios obtained from *C. turgidus* culture in CFTRI medium were reported as 78.89 ± 0.26%, 17.80 ± 0.41%, and 3.28 ± 0.39%, respectively [52]. In this study, *C. turgidus* showed different biochemical compositions in different WW characterizations. However, the biochemical content of the species is determined by the characteristics of the WW as well as the specific features of the species used. Indeed, in a previous study, it was shown

that different microalgae species exhibit different protein and carbohydrate profiles in the same WW [90].

As a result of the trials with *C. turgidus*, the lowest lipid content was obtained from BG11 at 10.03%, and the highest lipid content was obtained from the SE experimental groups at 28.05% (Figure 5). In terms of lipid content, the experimental groups were ordered from high to low as SE > E> PE > BG11. A recent study conducted in BG11 medium reported that *C. turgidus* has the same 10% total lipid percentage, although *Haematococcus pluvialis* and *Scenedesmus obliquus* are not in the same group of algae [68]. The similarity of lipid content obtained in this and the previous study in BG11 medium was indicative when evaluating the strain's performance in WW. It is a general trend that microalgae limit protein synthesis while increasing carbohydrate and lipid accumulation under N-deficient conditions [91]. Similarly, when *C. turgidus* was examined in terms of N concentrations in the experimental groups, it was seen that the microalgae lipid and carbohydrate accumulation increased as the N in the medium decreased. *C. turgidus* increased the carbohydrate content inversely with increasing N concentration. The highest carbohydrate accumulation, with similar values, was obtained from the E and SE trials at 43.93% and 43.05%, respectively, followed by the PE trial group at 39%, and the lowest carbohydrate content of 17.8% was obtained in the BG11 medium (Figure 5). On the other hand, according to the results obtained, the opposite is the case for protein synthesis; protein synthesis increased due to increasing N concentration. Indeed, nitrogen enrichment of the medium is expected to result in high protein synthesis [11]. In other words, although protein production varies for each species under the influence of different factors, nitrogen deficiency generally results in a decrease in protein synthesis [92]. At the end of the experiment, the harvested microalgae % protein ratios were obtained as SE < BG11 < E < PE. While the highest protein ratio was obtained from the PE experiment with 35.25% among the trials, the lowest protein ratio was obtained from the SE trial group, with 21.18%. It was found that lipid percentages were lower than protein ratios in all WW groups except the SE trial. In a previous study conducted for 10 days in the BG11 medium, it was reported that *C. turgidus* obtained 17.80% protein content, which is considerably lower than the ratios obtained from all WW and the BG11 mediums in this study. These different results obtained for *C. turgidus* species are likely to be caused by many factors such as adaptation time, differences in strains used, and culture conditions [93].

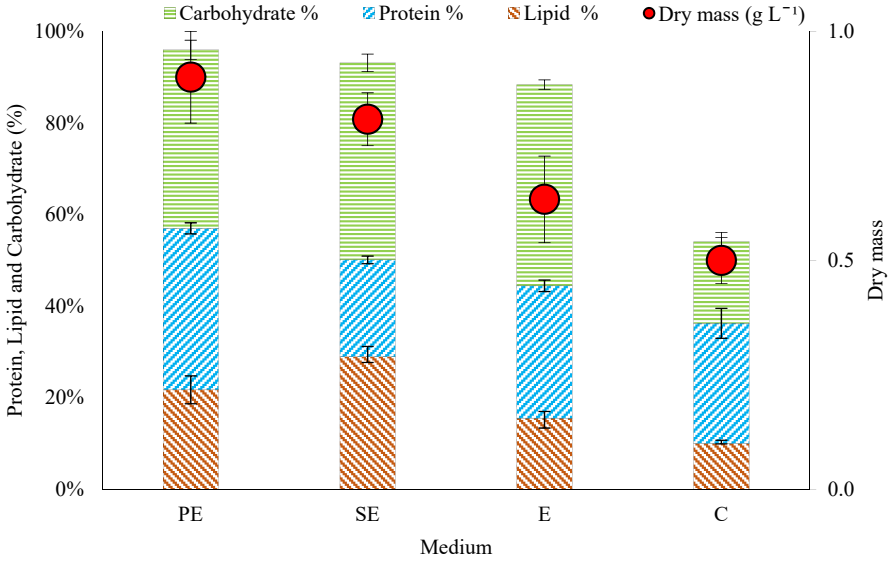


Figure 5. *C. turgidus* carbohydrate, protein, lipid percentage content, and dry mass values.

4. Conclusions

In this study, the growth ability, biomass yield, and content and treatment capacity of the blue-green alga *Chroococcus turgidus* were investigated through the major nutrients (NH_4^-, NO_2^-, NO_3^-, and PO_4^{3-}), COD, and BOD_5 in different stages of municipal wastewater. The results of this study suggested that *C. turgidus* may a new candidate for bioremediating the pollution load of municipal wastewater, and its biomass may have the potential to offer options for valuable bio-product production. Today, where the basic principles of sustainability gain a perspective toward zero waste, the implementation of such an integrated microalgae treatment system can also facilitate the operation of the ecosystem service cycle.

Supplementary Materials: The information in this part should be: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su142417021/s1, Table S1: The composition of enrichment medium BG11; Table S2: Lowry reagent for total protein analysis.

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