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Nutrients removal from raw municipal wastewater using *Chlorella vulgaris* microalgae

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ABSTRACT

This study evaluated the ability of *Chlorella vulgaris*, a freshwater microalgae species, to remove nutrients from raw municipal wastewater. The wastewater was collected from the initial sedimentation-stage discharge of the treatment plant and used to cultivate the microalgae in both a shaker-incubator and a photobioreactor. The results showed that the microalgae effectively reduced the nitrate, nitrite, phosphate, and ammonium ion concentrations in the wastewater by over 90%. Phosphate removal was particularly efficient in the photobioreactor, with a removal rate of 91%, while the shaker- incubator had a removal rate of 44%. In addition to removing nutrients, the microalgae were also able to significantly reduce the wastewater's chemical oxygen demand (COD), with a reduction of over 90% from 264 to 23.1 mg/l. The microalgae also had a symbiotic effect on the bacterial colonies present in the wastewater, reducing their numbers by 99% while allowing the microalgae to thrive. The final biomass concentration in the photobioreactor was 2.03 g/l, a higher value compared to similar studies. These results demonstrate the potential of *Chlorella vulgaris* and other microalgae species for use in wastewater treatment systems.

1. Introduction

In recent decades, urbanization and industrialization have increased the production and release of wastewater into rivers and lakes without proper treatment, causing many environmental problems [1]. For example, the eutrophication phenomenon occurs due to the high concentration of organic nitrogen and phosphorus in wastewater when wastewater is discharged to surface waters [2-4]. During this process, excessive accumulation of soluble nutrients such as phosphorus and nitrogen naturally leads to an increase in algal biomass in stagnant water. The latter phenomenon ultimately reduces the oxygen content and the quality of surface water. Some countries set strict regulations for treating municipal wastewater, whether for reuse or discharge to water resources

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and the environment. Despite the improvement of conventional wastewater treatment technologies, most of the existing treatment plants still face the challenge of removing mostly nitrogen and phosphorus nutrients and achieving the required standards [5]. Common methods of wastewater treatment are based on aerobic and anaerobic digestion by activated sludge, coagulation, and sedimentation operations [3,6], which consume a great deal of energy and chemicals [7,8]. Although these methods can significantly reduce COD, they are not very effective in removing nitrogen and phosphorus. Several methods have been proposed to remove these nutrients, including the use of microalgae. In recent years, wastewater treatment with microalgae has been considered an alternative to conventional methods. The potential of microalgae in nutrient removal from different types of wastewater to reach higher productivity has been shown in several studies [9-12]. In this regard, microalgae can be used in combination with activated sludge due to their high potential to remove contaminants to a negligible extent [4,13]. Microalgae can grow in wastewater effluents by utilizing carbon, nitrogen, and phosphorus as the main source of growth [14] to produce a valuable biomass product (i.e., the source of sustainable biofuels). Consequently, microalgae can also reduce energy consumption in comparison to conventional wastewater treatment methods [6,8]. Many researchers have used microalgae in wastewater treatment as one of the complementary treatment stages, and its high efficiency in the removal of nutrients has been proven. In many of these studies, effluent from the secondary treatment stage [13,15,16], as well as in the stages of centrate [17] and synthesis [2,4,6,18,19], have been used. The proper selection of an efficient microalgae species with considerable cell growth and good tolerance to wastewater is crucial to promote the advantages of microalgae [20]. Applying the microalgae-bacterial consortium in one step to eliminate maximum nutrients and reduce the cost of wastewater treatment has also been considered in recent years [6,7,21-24]. The microalgae-bacterial consortium is primarily suitable for wastewater treatment with high levels of nutrients and low organic matter [25,26]. In some studies, the microalgae-bacterial composition has been used to remove nutrients in both raw and synthetic wastewater [7,21,27,28]. Raw wastewater can naturally be an ideal substrate for microalgae growth, and in wastewater treatment, since the main load of treatment is on microalgae, routine secondary treatments can be eliminated [6,29,30]. Nevertheless, a significant number of studies on microalgae have focused on the treatment of synthetic wastewater or the effluent of the secondary treatment (activated sludge), and a small number of studies have dealt with raw wastewater. However, wastewater treatment with *C. vulgaris* has been studied after the secondary treatment, leading to the high efficiency of nutrient removal [4,6,8,21,31]. Even so, the studies that have employed only *C. vulgaris* in the treatment of raw wastewater without any additional operations are scarce. In the present study, the effect of using *C. vulgaris* native to Iran in the removal of contaminants in raw municipal wastewater was investigated. The main objectives were to only apply *C. vulgaris* in the removal of nutrients and to study the microalgae cultivation along with bacteria that naturally exist in wastewater. Samples of raw municipal wastewater were prepared from a treatment plant in the western part of Tehran after initial sedimentation. The experiments were performed in two processes, including semi-batch photobioreactors with aeration and a closed shaker incubator, where the amount of nutrient removal and the growth of microalgae in the wastewater were compared. The total amount of bacteria and the biomass in the culture medium were also evaluated.

2. Materials and methods

2.1. Microalgae strain and wastewater sample

The Iranian freshwater microalgae *Chlorella vulgaris* (PTCC 6033, Persian Type Culture Collection), isolated from mangrove forests, was used for wastewater treatment and nutrient removal. The carbon sources for the growth and reproduction of *Chlorella vulgaris* microalgae were CO_2 in the air and organic carbon in the wastewater; the light was provided by purple light lamps. For culturing the microalgae, synthetic culture medium, BBM, containing $NaNO_3K_2HPO_4$, KH_2PO_4, $MgSO_4$, $CaCl_2$, NaCl, $MnCl_2$, MoO_3, H_3BO_3,

$ZnsO_4$, $FeSO_4$, $CusO_4$, $(Co(NO_3)_2$, Na_2EDTA, KOH, and HCl was initially used [1]. All materials were laboratory-grade and made by the Merck Company. Meanwhile, real municipal wastewater (WW) was used as a raw material for microalgae growth in the photobioreactor. The raw municipal wastewater after the initial settling stage was freshly collected for each experiment from the Shahrak-Gharb WW treatment plant in the west of Tehran. The compositions of the raw and treated wastewater are given in Table 1. The concentration of components in the influent and effluent of the plant is related to the raw wastewater before the waste collection stage and the treated wastewater after aerobic and anaerobic treatment in the treatment plant.

Table 1. Characteristics of wastewater from Shahrak-Gharb treatment plant.

Components	Influent concentration (mg/l)	Effluent concentration (mg/l)
NH4-N	57	6.65
COD	446	19.4
Organic-N	10.9	2.28
Total P	6.21	2.88
NO_3^-	-	4.52

2.2. Preparation of C. vulgaris inoculum and culturing conditions in the shaking incubator

In preparing the inoculum, a synthetic BBM culture medium was used after sterilization in an autoclave for 2 hours at 110 °C. Then its pH was adjusted to 6.8 by the addition of 0.1 N NaOH and HCl solutions. A refrigerated stock suspension (5% vol.) of C. *vulgaris* was used to inoculate the microalgae into a 100 cm^3 culture medium. The percentage of microalgae inoculation was then increased to 10% in the subsequent 500 and 1000 cm^3 culture mediums to increase the volume of microalgae. At each stage, the media were placed in a shaker-incubator (Noor Sanat Ferdows Co.) at 24 °C and a shaking rate of 150 rpm. Cultivation time continued at each stage until reaching the logarithmic phase. Three factors of light intensity, dark-to-light period, and wavelength were considered to create suitable light for microalgae growth.

2.3. Microalgae culture in column photobioreactor

While the microalgae reached the logarithmic phase stage in the synthetic culture medium in the shaker- incubator, it was inoculated to the culture medium in a column photobioreactor with a cell concentration of 2×10^7 cell/ml. Figure 1 shows an image and schematic sketch of the laboratory photobioreactor used in this research. The photobioreactor consisted of a 145 cm height open bubble column with inner and outer diameters of 5 cm and 10 cm, respectively. The volume of the photobioreactor was six liters, with 43% filled by culture media. The column was placed inside a white wooden cabin to provide better light reflection. At the bottom of the column, a sparger was installed to distribute the air in the form of small bubbles. Air entered the reactor through an air pump in which the flow rate was adjusted at 2.5 l/min using a rotameter. The exterior of the bioreactor column was illuminated by six 90 cm of 30-watt fluorescent (flora) lamps. Also, the distance from the lamp to the column is 1.5 cm. The light intensity of the lamps in the middle of the reactor was 6300 (lux), and the light and dark periods were set to 16 and 8 hours, respectively. The experiments were carried out at ambient temperature. Each photobioreactor experiment lasted 12 days, and concentrations of biomass, ammonia (NH_4^+ -N), nitrate (NO_3^- -N), nitrite (NO_2^- -N), phosphate (PO_4^{3-} -P), COD, total bacterial culture, and chlorophyll were measured at regular intervals.

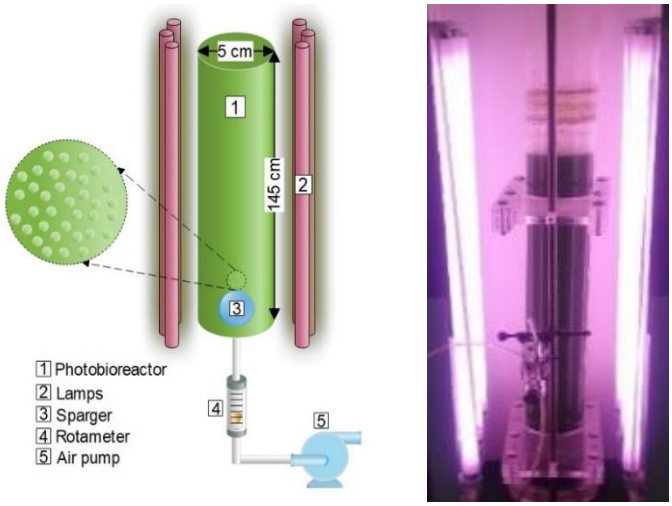

Fig. 1. Image and schematic sketch of the photobioreactor set-up used for the cultivation of microalgae.

2.4. Methods of analyses

In investigating the growth rate of microalgae in the culture medium, the cell density (turbidity) of the medium at 680 nm was measured using a DR3900 Hach spectrophotometer set. Also, viable cell count was performed using a 16-square neo bar slide positioned in a microscope at a magnification of 40. The average number of cells (number of cells per cubic centimeter, N) was calculated by Equation (1):

$$\text{Cell No} = \frac{\text{N}}{16} \times 4 \times 10^6 \quad (1)$$

The light intensity was measured using a light meter, model TES1332, and was kept at 6300 (lux). The COD of the samples was measured according to the standard of the APH5220-D method. The digester solution (mercury sulfate) and acidic indicator (combination of silver sulfate and concentrated sulfuric acid) were prepared and added to the glass vials along with some of the samples and placed in the reactor at 148°C for two hours for digestion. When the vials reached ambient temperature, their value was read using a Hach DR3900 spectrophotometer. Nitrate, nitrite, and ammonia concentrations were measured using spectroscopic methods [32]. The phosphate concentration was analyzed by the Semi-Automated Calorimetry method [33]. To measure the dry weight of the microalgae biomass, a 50 cm^3 microalgae culture taken from the photobioreactor was filtered using a filter paper and weighed after drying at 100 °C for 24 hours. The total count of the bacteria (colony-forming unit, CFU) was measured by culturing the sample without any filtration on sterile BHI agar medium plates. The plates were incubated at 28-30 °C, and bacterial growth was counted after 24 and 48 hours. The pH of the media was measured by a Hach pH meter.

3. Results and discussion

3.1. Microalgae growth in synthetic culture medium

The growth of *C. vulgaris* was initially performed in a synthetic culture medium (BBM) using both a shaker- incubator and a photobioreactor. Microalgae cells were cultured in the shaker-incubator in volumes of 500 and 1000 cm^3 and then inoculated into the photobioreactor. Figure 2 shows the variation of cell growth (cell density, cell count) and pH factors versus the microalgae cultivation time for the volumes of 500 and 1000 cm^3 and in the photobioreactor. The changes in cell density and cell count shown in the graphs indicate that the growth of the microalgae in the Erlenmeyer's with 500 and 1000 cm^3 had an increasing trend until the eighth day and then reached a stationary phase. In the photobioreactor, the microalgae entered the logarithmic growth phase after a one-day delay due to the larger volume, and the cells' growth continued until the tenth day. In addition, the growth rate of microalgae increased significantly from the fifth day. Changes in ambient pH in the shaker- incubator increased in the range of 7 to 10, but in the case of a photobioreactor, this increase was limited to the range of 8 to 9. The microalgae-bacterial activity decreased with increasing pH in the photobioreactor (open system) and was controlled due to the presence of carbon dioxide (was at 8-9). But in closed conditions that lacked carbon dioxide, there was no pH control, and the pH increased to 11 [34].

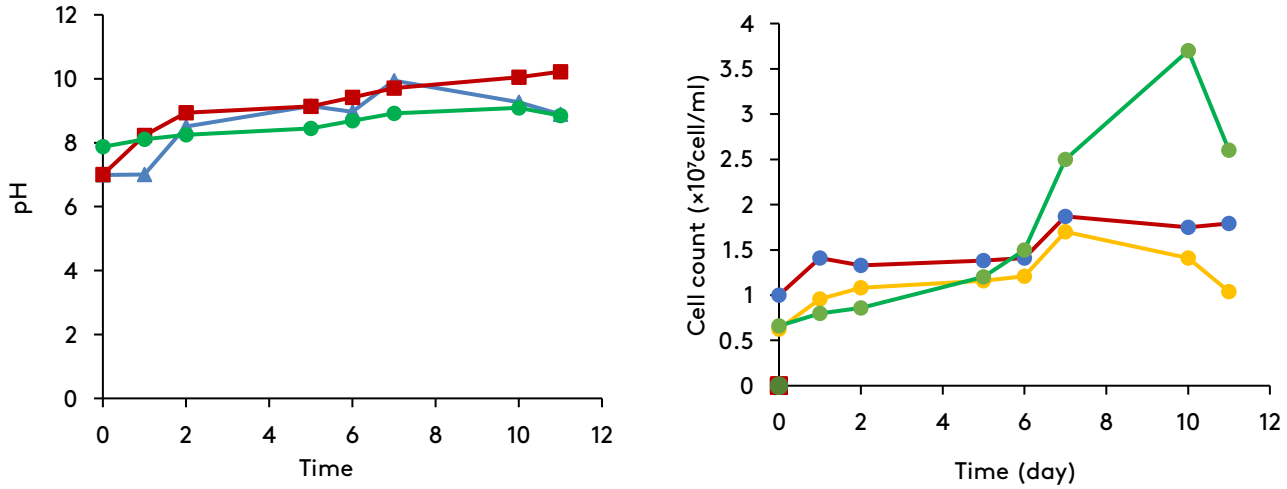

Fig. 2. Changes in pH (A) and cell count (B) of *C. vulgaris* culture in 500 cm^3 (▲) and 1000 cm^3 (■) in the shaker-incubator and in the photobioreactor (●) using synthetic culture medium.

By measuring the dry weight of the microalgae on the tenth day, the biomass concentration of the microalgae in the photobioreactor reached 1.00 g/l.

3.2. Comparison of microalgae growth in municipal raw wastewater and synthetic culturing medium

After performing microalgae experiments in a synthetic culture medium, microalgae culture studies were carried out in the municipal raw WW in two systems: shaker- incubator and photobioreactor. Figure 3 shows the results of the growth rates of *C. vulgaris* in the shaker- incubator and photobioreactor column, as well as microalgae growth in the synthetic culture medium and shaker- incubator as a control. Comparing the curves, it is clear that the mixture of microalgae-WW in the photobioreactor quickly entered its exponential phase and, after seven days, reached an optical density (OD_{680}) of 2.5 and a maximum cell count of 2.61 cells/ml on day 11. However, both the microalgae-WW and the control sample in the shaker- incubator reached a maximum OD of 1.49 and 2.06, respectively, on day 14. The different operating conditions in the photobioreactor, including proper aeration, led to such an improvement. Compared to the growth of microalgae in the synthetic culture medium, the growth of microalgae in the municipal WW was much higher and had the highest growth rate after eleven days due to its richness of nutrients [5,6]. It should be noted that in this study, the microalgae growth results were obtained by sampling the raw wastewater without performing side operations such as filtration. This study showed a higher maximum growth rate for *C. vulgaris* in the photobioreactor compared to other studies [22]. Wang et al. [15] obtained the highest growth rate of *Chlorella sp.,* less than 1.0 cells/ml, from its cultivation in municipal wastewater. On the other hand, Otondo et al. cultivated microalgae in synthetic and real wastewater and reached a maximum biomass of 0.05-0.125 cells/l [6].

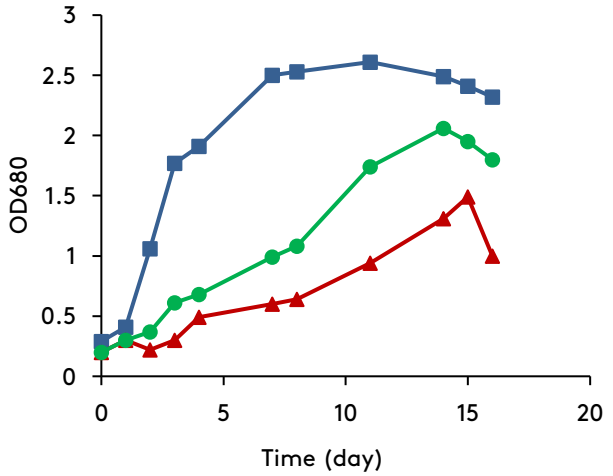

Fig. 3. Comparative diagrams of *C. vulgaris* growth in raw municipal wastewater in the shaker- incubator (▲) and photobioreactor (■). The control sample of growth in the synthetic culture medium in the shaker- incubator (●) is also shown.

3.3. Nutrients removal by C. vulgaris in the shaker-incubator and photobioreactor

The removal of nutrients from raw municipal WW was studied in the shaker-incubator and photobioreactor. The control experiments were performed only in the presence of raw WW in the incubator.

3.3.1. Removal of nitrate, nitrite, ammonia, and phosphate

The changes in the nitrate, nitrite, and ammonia concentrations during the cultivation of *C. vulgaris* in raw municipal WW in the shaker-incubator and photobioreactor are compared in Figures 4 and 5. The microalgae-WW results showed that the ammonia and nitrate in both the incubator and column had a decreasing trend and reached their lowest levels on the 11^{th} day (Figures 4A and 4B). In other words, more than 90% of the ammonia and nitrates were removed (Figure 6). The results obtained in this study were better than other works, in which 58% of nitrate was eliminated from the secondary activated sludge stage employing microalgae as the tertiary wastewater treatment [35]. It has been reported as 87-89% and nearly 97% for nitrogen and ammonia nitrogen removal by *Chlorella sp.*, respectively when treated swine wastewater was used [14]. In this regard, W. M. Lopez Ponte et al. [36] stated that about 95% of nitrate was removed by culturing *Chlorella sp.* in tertiary treatment of wastewater. The data shown in Figure 4 indicates that nitrite concentrations remained stable over time due to their low initial concentration in the feed stream. In contrast, the concentration of nitrate in raw wastewater alone (in the shaker-incubator) increased to a maximum on the third day, while the concentrations of ammonia and phosphate decreased over time. This increase in nitrate is likely due to the nitrification process occurring in the closed environment of the shaker-incubator, where the decrease in ammonia concentration (influenced by the bacteria present in the raw wastewater) is accompanied by an increase in nitrate. When microalgae (*C. vulgaris*) are present in the wastewater, the removal of ammonia and nitrate becomes more pronounced, while in its absence, the conversion of ammonia to nitrate by bacteria plays a more significant role. The data also shows that microalgae are more effective at removing nutrients from wastewater compared to bacteria alone, as seen by comparing Figure 4A and Figure 4C. Figure 5 shows the changes in phosphate concentration in the raw municipal WW along with *C. vulgaris* microalgae in the photobioreactor and shaker-incubator, as well as the raw WW without microalgae in the incubator. As seen, the effect of microalgae on reducing the phosphate concentration is quite evident. The microalgae significantly removed phosphate in municipal wastewater in the photobioreactor (~ 91%), while in the closed incubator conditions, the elimination was much lower (~ 44%) (Figure 6). In other recent studies, Z. Chen et al. [14] reported a phosphate removal close to 92-93% by applying *Chlorella* sp. s in swine wastewater treatment, while W. M. Lopez Ponte et al. [36] achieved around 69% removal using *Chlorella* sp. in tertiary wastewater treatment. In recent years, researchers have also been able to remove some of the nutrients using synthetic [31] and UV-treated wastewater [30]. Most research has focused on using microalgae to treat the effluent of the secondary- or tertiary-stage of activated sludge treatment [37]; however, this study demonstrates the efficient nutrient removal of raw wastewater using *C. vulgaris*.

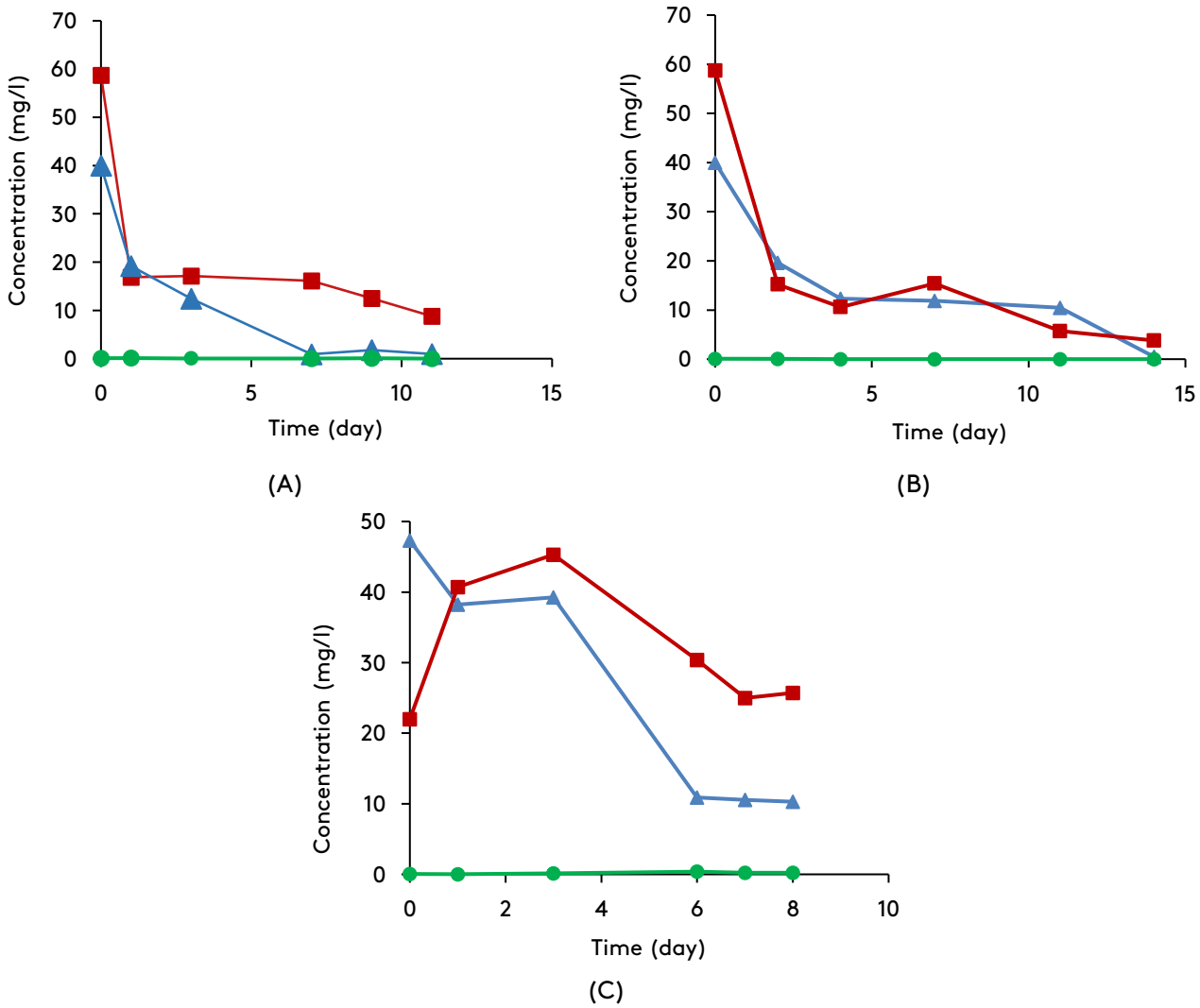

Fig. 4. Changes in nitrate (■), nitrite (●), and ammonia (▲) concentrations during cultivation of C. *vulgaris*-WW in the shaker- incubator (A), C. *vulgaris*-WW in the photobioreactor (B), and only WW in the shaker- incubator (C).

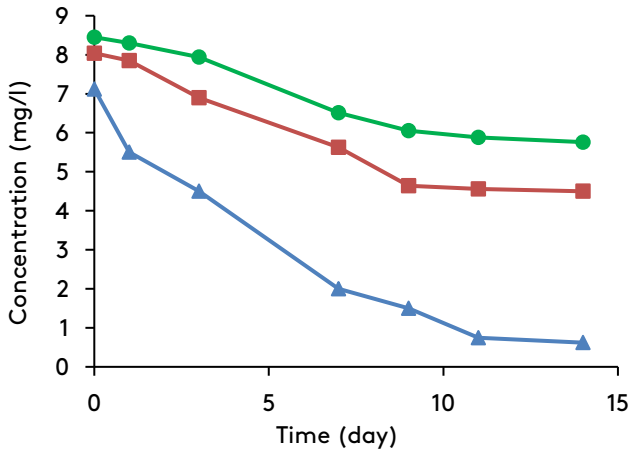

Fig. 5. Changes in phosphate concentration during cultivation of C. *vulgaris*-WW in the shaker- incubator (■), C. *vulgaris*-WW in the photobioreactor (▲), and only WW in the shaker- incubator (●).

Figure 6 demonstrates the removal efficiencies (%) of different nutrients during the cultivation of C. *vulgaris*-WW in the shaker- incubator and photobioreactor.

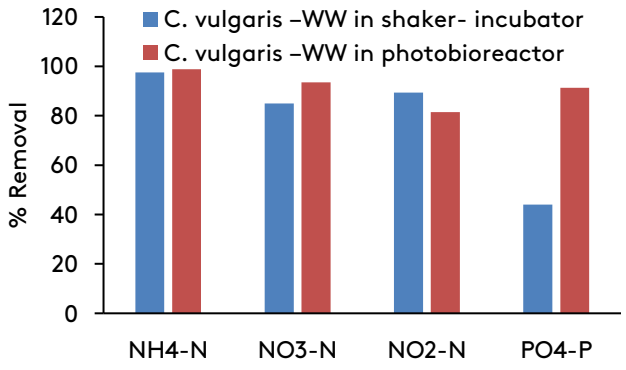

Fig. 6. Comparison between nutrient removal efficiency in the shaker- incubator and photobioreactor during cultivation of C. *vulgaris* –WW.

3.3.2. COD Changes in the photobioreactor

The rate of COD changes in the raw microalgae-municipal WW in the photobioreactor was only compared to the raw WW in the shaker- incubator in Table 2.

Table 2. COD changes during treatment of microalgae-raw WW in the photobioreactor compared to the COD changes in WW only in the shaker- incubator.

Day	COD (mg/l)	
	Raw WW in the shaker- incubator	WW-microalgae in the photobioreactor
0	264	264
2	256	143.2
4	160.5	35.3
7	129.3	30.4
11	144.8	22.8
14	121.6	23.1

As can be seen from the table, the COD in the raw WW by microalgae decreased from 264 to 23.1 mg/l during 14 days. Comparing the latter result with the raw WW alone, as a control, (264 to 121.6 mg/l) confirmed the removal of more than 90% of the COD in WW by C. *vulgaris,* proving the effectiveness of adding microalgae to WW for removing pollutants. In another study, the growth of microalgae in synthetic wastewater could reach this amount [18]. According to various studies, the maximum removal rate of COD has been reported to be between 40%-60% [8]. In recent works, the COD removal efficiency was reported to be up to 70% by changing culturing conditions when *Chlorella* sp. was used for the tertiary treatment of municipal wastewater [37]. Carbon is a major source of microalgae growth. When there is a source of organic carbon and light, algae growth is considered mixotrophic growth, which allows carbon dioxide and organic carbon to be absorbed simultaneously, indicating the fastest way to grow algal biomass. If so, the COD decreases during the process because the microalgae use carbon for photosynthesis [6,7,17].

3.4. Bacterial load variations of raw WW in the photobioreactor

Table 3 shows the results of counting total bacterial cells in the raw WW and C. *vulgaris*-WW culture inside the photobioreactor on different days. Figure 7 shows images of bacterial cultures at the beginning of the experiment and on the seventh day in the photobioreactor in the presence of a mixture of microalgae and raw wastewater.

Table 3. Total bacterial counts in the raw WW and C. *vulgaris*-WW culture inside the photobioreactor on different days.

DAY	WW (CFU)	Microalgae-WW (CFU)
1	2×10^{8}	1.4×10^{7}
9	-	5×10^{6}
13	-	1.4×10^{6}
17	-	6×10^{4}

As seen in Figure 7, the bacterial culture in the microalgae-WW medium greatly decreased, during microalgae cultivation (i.e., after 17 days). The results were confirmed by previous microalgae growth diagrams and the removal of nutrients in the mixture of wastewater and algae in which the presence of bacteria did not inhibit the growth of microalgae. In addition to the nutrients in the wastewater, microalgae use bacteria for their growth. In other studies, the microalgae-bacterial combination has been evaluated to remove harmful substances in wastewater [2-24]. Bacteria have also been used as a growth factor [17].

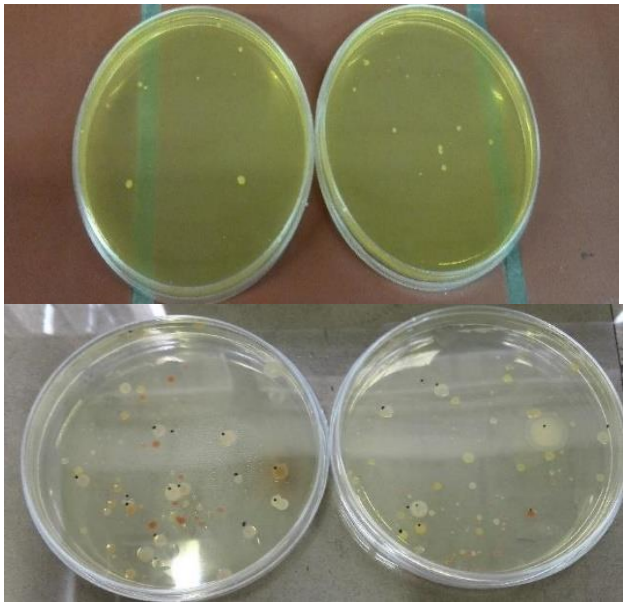

Fig. 7. Bacteria total count experiment at the beginning of treatment (down) and on day 17th (up).

3.5. C. vulgaris microalgae biomass in the photobioreactor

The dry weight changes of the microalgae biomass in the photobioreactor and shaker- incubator during cultivation of *C. vulgaris*-WW are shown in Figure 8. The highest dry biomass weight was obtained on the fourth day of cultivation in the photobioreactor, equivalent to 2.03 g/l, representing a significant value compared to other studies. It was clear that the dry weight changes did not exist significantly through the *C. vulgaris* culture in the shaker- incubator when WW was available.

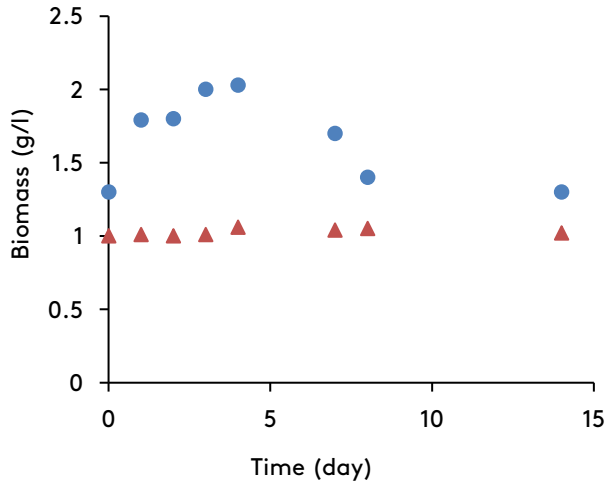

Fig. 8. Changes in the dry biomass of microalgae during cultivation of *C. vulgaris* –WW in the photobioreactor (●) and in the shaker- incubator (▲).

4. Conclusions

This research evaluated the ability of *Chlorella vulgaris* microalgae to remove contaminants such as nitrate, nitrite, phosphate, COD, and ammonium ions from raw municipal wastewater. The results showed that raw municipal wastewater was a suitable medium for the growth of microalgae due to its high nutrient content. While previous research has primarily focused on using microalgae to treat synthetic wastewater or the output of the secondary stage of activated sludge treatment, this study demonstrated the potential of using *Chlorella vulgaris* as a standalone treatment for raw municipal wastewater. The microalgae were able to remove over 90% of contaminants without the need for additional bacteria or filtration, making it a potentially effective alternative for the biological treatment of raw municipal wastewater. In addition to removing contaminants, the microalgae were also able to produce biomass with a concentration of 2.03 g/l and significantly reduce the COD and bacterial content of the wastewater.

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