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Nutrient removal from municipal wastewater using mixture of two algae, *Scenedesmus obliquus* and *Chlorella vulgaris*

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Abstract: Algae are the main components of primary production in sea and refinery wastewater. Microalgae and cyanobacteria are the most-used groups. In the present study, refined wastewater was collected from drainage of a refinery in Gorgan to evaluate efficiency of two algae mixture, *Scenedesmus obliquus* and *Chlorella vulgaris*, to remove water nitrate, phosphate, BOD and COD during 14 days. An equal number of either of the algae (24000 cells) was added to each treatment at the experiment initiation. The algae number were daily counted; whereas, the media nitrate, phosphate, ammonia, chlorophyll-a, biomass, BOD and COD were measured every other day. At the end of study, phosphate was completely depleted in M50 treatment and the highest phosphate level (1 mg/l) was observed in M0 treatment ($p < 0.05$). Water nitrate was completely depleted in M50 treatment. The highest biomass levels (0.4 g/l) were observed in M0 and M50 treatments ($p < 0.05$). The highest chlorophyll-a was observed in M0 treatment (2.83 mg/l). BOD and COD had similar pattern among the treatments. The lowest COD was related to M50 treatment (5 mg/l); whereas the lowest BOD level was observed in M0 treatment (0.05 mg/l). Overall, the algae mixture was efficient to uptake nutrients and produce biomass and chlorophyll.

Keywords: nutrient removal, algae mixture, *Scenedesmus obliquus*, *Chlorella vulgaris*

Introduction

Anthropogenic human wastewaters are the main source of environmental pollution (Oswald and Gotass, 1957). Biological refinement is an important part of municipal and industrial wastewater refinement, because of low cost and high efficiency (Chuang and Mitch, 2017). Municipal wastewaters are rich source of nutrient and organic materials thus stimulating the microalgae growth. Ammonium, nitrate and phosphate are of these materials which participate in eutrophication. Microalgae grow well in nitrogen- and phosphorous-rich wastewater with strong potential to remove these compounds (Pittmanm *et al.*, 2010). In the traditional refinement processes, organic carbon compounds are degraded to carbon dioxide with sludge formation; however, in wastewater culture, algae are capable to Photosynthesis using carbon dioxide and sun light to convert nutrient rich water to cellular structures such as lipids and carbohydrates (Wang *et al.*, 2010).

Wastewaters are forage for degraded microorganisms. Using green algae such as *Chlamydomonas* and *Chlorella* in drainage of large-shallow chambers (wastewater oxidation) is the cheapest and fastest method to convert dangerous materials into valuable fertilizer with catalyzer effects and no smell (Maity *et al.*, 2014). Algae are important in wastewater channels (Aziz and Ng, 1992). These algae use nitrate and phosphate for their metabolic processes and release oxygen through photosynthesis; oxygen help aerobic bacteria to degrade crude materials of wastewater. Industrial and municipal wastewaters contain high amount of organic and mineral compounds. Refinement of these wastewaters is mainly considered an oxygen generating process (Wang *et al.*, 2010). Oxygen generation using algae is common and some algae such as *Scenedesmus, Euglena, Chlorella* and *Chlamydomonas* are effective ones. Oxygen elevation is necessary for wastewater deodorization. Thus, algae are important in wastewater treatment and this occurs naturally in some cases (Gisi and Notarnicola, 2017). *Ch. vulgaris* is an algae belonging to family *Chlorellaceae*, order *Chlorellales*, and class *Trebouxiphyceae*; while, *Sc. obliquus* belongs to family *Scendesmaceae*, order *Chlorococcales*, and class *Chlorophyceae* (Saison *et al.*, 2010). Algae like *S. obliquus* are able to uptake nutrients at low concentrations due to high resistance to changes in temperature and pH; also their production technology

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is easy and cheap (Yalcin *et al*., 2006; Voltolina *et al*., 2004; Saison *et al*., 2010). *Sc. obliquus* is a non-motile algae belonging to green algae with worldwide distribution (Li *et al*., 2010; Lee and Lee, 2001). Li *et al.* (2010) reported that *Scenedesmus* is able to remove wastewater nitrate and that the algae is capable to live and uptake nutrients in culture systems. *Ch. vulgaris* is single-cell green algae with worldwide distribution (Li *et al*., 2010; Lee and Lee, 2001).

Therefore, the aim of the present study was to investigate growth performance and efficacy of two algae mixture, *Sc. obliquus* and *Ch. vulgaris*, to remove nutrients from different concentration of municipal wastewater.

Materials and methods

Algae isolation and purification

In the present study the algae was first identified under microscopy using Lavens and Sorgeloos (1996) and then purified on agar medium. After confirmation of pure algae culture, they were inoculated into 2-L flasks containing Zinder (Z_{8+n}) medium to obtain initial algae stocks (Miller *et al*., 1978; Komarek, 1973).

Experiment protocol

In this study, 500-ml bottles containing 250 ml culture medium were used under sterile conditions at 23±2°C. Municipal wastewater was collected after the last stage of refinery; then, filtered and autoclaved to ensure no microbial load. Three treatments consisting of the mixture of the two algae and three wastewater concentrations (M0, M50 and M100) were assigned for this experiment. To prepare the wastewater concentrations, 1 part of distilled water was mixed with 2 parts of wastewater (50%), or 1 part of distilled water was mixed with 1 part of wastewater (100%), or no dilution (0%). This study was conducted with 3 replications as a completely randomized design. The initial nutrients concentrations are presented in Table 1.

Tab. 1: Nutrient concentration (mg/L)in medium with different dilutions.

Compounds	0%	50%	100%
Nitrite	58.2	38.9	32.2
Phosphate	52.2	24	21.3
Nitrate	116.4	107	69.15
Ammonia	2.05	1.55	0.95

Each 500-L flask was filled with 250 ml of wastewater. The experiment period was 14 days, during which algae cells were counted daily and chlorophyll-a, dry biomass, nitrate and phosphate were determined every other day. Algae count was performed following Martinez *et al.* (2000). Dry biomass was determined according to Lavens and Sorgeloos (1996) by weighing a certain volume of the counted cells. Chlorophyll-a levels were determined according to Parsons *et al.* (1984). Nitrate and phosphate levels were determined using Wagtech kits, after the cell biomass removal by centrifugation. Specific growth rate (SGR) was calculated using formula $SGR= (LnN_2-LnN_1)/t$; where, N_2 was final algae cell number, and t was the experiment period. Algae population doubling time was calculated using formula $DT=log^2{}_e/SGR$ (Omori and Ikeda, 1984).

Statistical analysis

The data were analyzed as a completely randomized design using software SAS9.4. Also, correlation test was conducted for the data. Software Excel 2013 was used to draw graphs. Mean comparison was performed using Duncan test at P=0.05.

Results

Phosphate and nitrate are the main nutrients in wastewaters and in the present study, the algae efficiency to uptake these compounds was investigated. Phosphate uptake was affected by the wastewater concentration, time and their interaction (Tab. 2). As shown in the Table 2, wastewater concentration had a significant effect on the medium phosphate removal, however, no significant interact-tion was found on day 8.

Tab. 2: effects of algae, wastewater concentration and their interaction on phosphate uptake.

Time	Algae	Con.	Algae × Con.
2	417.29**	177.74**	81.7*
4	915.41**	231.65**	598.7**
6	201.17**	213.67**	206.78**
8	171.38**	174.57**	9.43
10	120.58**	93.32**	89.3*
12	74.17**	55.59**	139.58**
14	49.34**	40.61**	121.1**

Con. = Concentration
** P<0.01; * P<0.05

Phosphate residuals in the medium of different treatments are shown in Table 3. Accordingly, the highest phosphate removal was observed in the M50 treatment with 100% phosphate uptake after 14 days. The M100 treatment had low amount of phosphate (0.04 mg/l) after 14 days, which was not significantly different compared to the M50 treatment.

Tab. 3: phosphate concentration (mg/l) in different treatments during the experiment (mean±SD). Lowercase letters show significant difference in each column and uppercase letters show significant difference in each row.

Day	Treatment		
	M0	M50	M100
0	50.4±4.65^{aA}	24±4.65^{bA}	21.3±4.65^{cA}
2	13.2±0.91^{aB}	10.5±0.91^{bB}	7.2±0.91^{cB}
4	10.8±0.93^{aC}	6.5±0.93^{bC}	4.8±0.93^{bC}
6	4.2±0.45^{aD}	5.5±0.45^{aC}	3.1±0.45^{bD}
8	2.5±0.3^{aE}	2.9±0.3^{aD}	2.4±0.3^{aD}
10	2.2±0.32^{aE}	1±0.32^{aE}	2.4±0.32^{aD}
12	1.8±0.32^{aE}	0.8±0.32^{bE}	0.8±0.32^{bE}
14	1±0.16^{aE}	0±0.16^{bE}	0.04±0.16^{bE}

According to Table 4, nitrate uptake was affected by the wastewater concentration, time and their interaction. Similar to phosphate, nitrate uptake was mainly affected by wastewater concentration, however, there was no significant effects of waste-water concentration and their interaction on days 10 and 14.

Tab. 4: effects of algae, wastewater concentration and their interaction on nitrate uptake.

Time	Algae	Con.	Alga × Con.
2	354.5**	200.41**	130.91**
4	152.33**	161.98**	91.23**
6	320.89**	355.85**	145.76**
8	103.96**	89.87**	85.88**
10	92.69**	103.98**	73.18
12	95.49**	90.44**	75.95**
12	95.49**	90.44**	75.95**
14	76.87**	87.4**	34.21

Con. = Concentration

Nitrate residuals in the medium of different treatments are shown in Table 5. Accordingly, the highest nitrate removal was observed in the M50 treatment with 100% nitrate uptake after 14 days. The M0 treatment had the highest amount of nitrate after 14 days (0.36 mg/l).

Figure 1 shows phosphate removal patterns using the algae mixture during the experiment. Figure 2 shows nitrate removal patterns using the algae mixture during the experiment. According to the Figures 1 and 2, nitrate removal rate, particularly in M0 treatment compared to the other treatments, was higher than those of the phosphate suggesting that increase in wastewater concentration resulted in decrease in nitrate removal rate.

Overall, the results indicate that mixture of *Chlorella* and *Scenedesmus* grow well in different treatments and suppressed nitrate and phosphate concentration.

Tab. 5: nitrate concentration (mg/l) in different treatments during the experiment (mean±SD). Lowercase letters show significant difference in each column and uppercase letters show significant difference in each row.

Day	Treatment		
	M0	M50	M100
0	116.4±7.22^{aA}	107±7.22^{aA}	69.15±7.22^{aA}
2	48.98±5.62^{aB}	33.45±5.62^{bB}	11.16±5.62^{cB}
4	46.5±5.73^{aC}	14.85±5.73^{bC}	9.96±5.73^{cBC}
6	34.08±3.89^{aD}	12.85±3.89^{bD}	9.18±3.89^{cC}
8	13.02±0.91^{aE}	8.85±0.91^{bE}	7.2±0.91^{bD}
10	4.7±0.64^{aE}	1.06±0.64^{bF}	4.65±0.64^{aE}
12	1.94±0.31^{aE}	0.18±0.31^{bF}	1.42±0.31^{aF}
14	0.36±0.05^{aE}	0±0.05^{bF}	0.27±0.05^{aF}

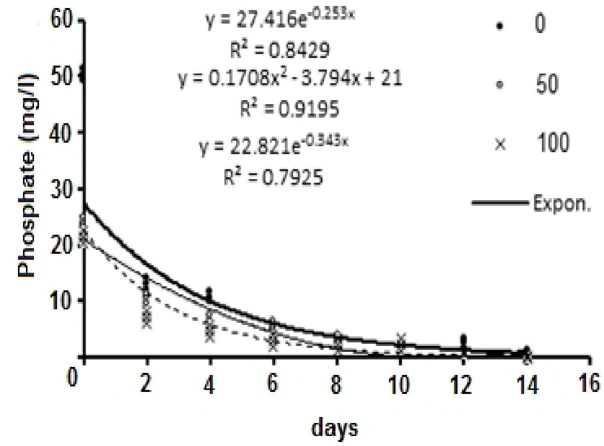

Fig. 1: Phosphate removal patterns using the algae mixture.

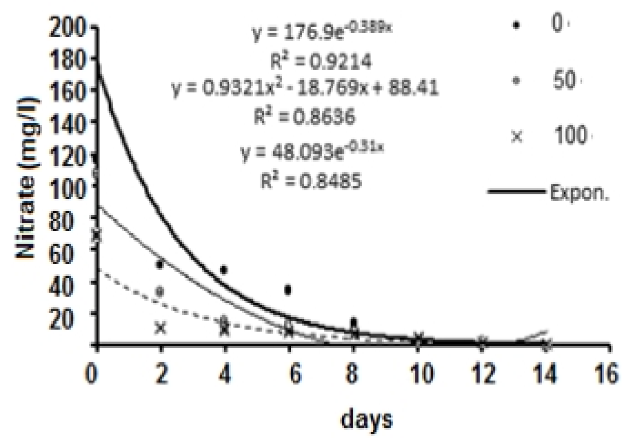

Fig. 2: Nitrate removal patterns using the algae mixture.

Algae biomasses during the experiment were positively correlated to nutrient concentration (Tab. 6). The highest biomass was observed in the M0 and M50 treatments (0.4 g/l) and the lowest biomass was observed in the M100 treatment (0.3 g/l). In the M50 treatment, the highest biomass was observed on day 10 (0.4 g/l) (Tab. 6).

Tab. 6: Algae biomass (g/L) in different treatments during the experiment. Lowercase letters show significant difference in each column and uppercase letters show significant difference in each row (α=0.05).

	Treatment		
Day	**M0**	**M50**	**M100**
2	0.1±0^{aD}	0.1±0^{aC}	0.1±0^{aB}
4	0.1±0^{aD}	0.1±0.01^{aC}	0.1±0.011^{aB}
6	0.2±0.01^{bC}	0.3±0.01^{aB}	0.3±0.01^{aA}
8	0.2±0.01^{bC}	0.3±0.01^{aB}	0.3±0.01^{aA}
10	0.2±0.01^{bB}	0.4±0.01^{aA}	0.3±0.01^{bA}
12	0.4±0.01^{aA}	0.4±0.01^{aA}	0.3±0.01^{bA}
14	0.4±0.01^{aA}	0.4±0.01^{aA}	0.3±0.01^{bA}

The highest cell number in the middle of the experiment (the day 10) was related to the M50 and M100 treatments. The highest cell number at the end of the experiment was related to the M100 treatment (2.17). The highest cell number during the experiment was related to the M50 treatment and on day 8 (Tab. 7).

Tab. 7: Algae cell number (×0.00001) in different treatments during the experiment (mean±SD). Lowercase letters show significant difference in each column and uppercase letters show significant difference in each row.

	Treatment		
Day	**M0**	**M50**	**M100**
1	0.24±0^{aK}	0.24±0^{aM}	0.98±0.021^{cEF}
2	1.12±0.02^{aJ}	1.04±0.02^{bL}	3.37±0.06^{aA}
3	3.19±0.06^{bH}	2.96±0.06^{cI}	3.37±0.06^{aA}
4	6.68±0.64^{aF}	3.86±0.64^{bH}	2.29±0.64^{cB}
5	6.6±0.91^{aF}	6.47±0.91^{bG}	1.04±0.91^{cEF}
6	7.72±1.37^{bE}	10.49±1.37^{aE}	1.22±1.37^{cDE}
7	17.74±3.81^{bA}	2.77±3.81^{aB}	1.57±3.81^{cC}
8	14.49±4.52^{bB}	32.74±4.52^{aA}	1.57±4.52^{cC}
9	14.49±3.44^{bD}	25.35±3.44^{aD}	1.64±3.44^{cC}
10	12.66±3.69^{bC}	27.10±3.69^{aC}	1.59±3.69^{cC}
11	4.88±1.02^{bG}	8.54±1.02^{aF}	1.43±1.02^{cCD}
12	2.18±0.22^{bI}	2.72±0.22^{aIJ}	1.21±0.22^{cDEF}
13	1.72±0.25^{cIJ}	2.34±0.25^{bJK}	3.34±0.25^{aA}
14	1.57±0.08^{cIJ}	2±0.08^{bK}	2.17±0.08^{aB}

Chlorophyll contents in different treatments during the experiment are presented in Table 8. The highest chlorophyll-a content was observed on day 10 in the treatment M100 (4.97 mg/l) (Tab. 8). The highest chlorophyll-a content in all treatments was observed on day 10. At the end of the experiment, the highest and the lowest chlorophyll-a contents were observed in the M0 (2.83 mg/l) and M50 (2.23 mg/l) treatments, respectively. There was a correlation between chlorophyll-a content and algae cell number in the M50 treatment with the highest cell number on day 8. Increase in algae cell number resulted in increase in biomass in the M50 treatment.

Tab. 8: Chlorophyll contents (mg/l) in different treatments during the experiment (mean±SD). Lowercase letters show significant difference in each column and uppercase letters show significant difference in each row.

	Treatment		
day	**M0**	**M50**	**M100**
2	1.7±0.71^{bD}	1.88±0.74^{aC}	1.77±0.72^{bC}
4	4.25±0.65^{aA}	3.94±0.61^{bA}	4.85±0.72^{aA}
6	3.57±0.76^{aB}	3.02±0.65^{cB}	3.27±0.72^{bB}
8	3.64±0.76^{bB}	3.71±0.78^{aA}	3.68±0.77^{bB}
10	3.43±0.67^{cB}	4±0.88^{bA}	4.97±0.91^{aA}
12	3.42±0.78^{aB}	2.98±0.57^{bB}	3.53±0.81^{aB}
14	2.83±0.59^{aC}	2.23±0.48^{bB}	2.45±0.51^{bB}

In Table 9, BOD5 showed decreasing pattern during the experiment with the highest (1.4 mg/l) and lowest (1 mg/l) in the M0 and M50 treatments, respectively. COD showed a trend similar to that of the BOD5 during the experiment with the highest (7 mg/l) and lowest (5 mg/l) in the M0 and M50 treatments, respectively (Tab.10). According to the results of Table 11, the highest SGR (0.18 per day) and lowest DT (3.71 days) were related to the treatment M100.

Tab. 9: BOD5 (mg/l) values in different treatments during the experiment (mean±SD). Lowercase letters show significant difference in each column and uppercase letters show significant difference in each row.

	Treatment		
Day	**M0**	**M50**	**M100**
2	3.72±0.85^{aA}	2.26±0.43^{aB}	2.56±0.57^{aB}
4	2.9±0.89^{bA}	2±0.71^{bB}	2.2±0.79^{bB}
6	2.06±0.97^{cA}	1.5±0.7^{cB}	1.75±0.78^{cB}
8	2.32±0.96^{cA}	1.6±0.7^{cB}	1.96±0.88^{cB}
10	2±0.97^{dA}	1.4±0.67^{dB}	1.82±0.96^{cB}
12	2±0.84^{dA}	1.2±0.5^{eC}	1.62±0.68^{dB}
14	1.4±0.61^{eA}	1±0.31^{fB}	1.36±0.54^{dA}

Tab. 10: COD (mg/l) values in different treatments during the experiment (mean±SD). Lowercase letters show significant difference in each column and uppercase letters show significant difference in each row.

	Treatment		
Day	**M0**	**M50**	**M100**
2	18.6±1.37^{aA}	11.3±0.98^{aB}	12.5±1^{aB}
4	14.5±1.58^{bA}	10±1.23^{aB}	11±1.32^{aB}
6	12.4±1.67^{cA}	9±1.1^{bB}	10.5±1.23^{aB}
8	11.6±1.76^{cA}	8±1.11^{cC}	9.8±1.28^{bB}
10	10±1.25^{cA}	7±1.03^{dB}	9.1±1.17^{bA}
12	10±1.34^{cA}	6±1.08^{eC}	8.1±1.22^{cB}
14	7±1.27^{dA}	5±1.1^{fB}	6.8±1.25^{dA}

Tab. 11: SGR and DT in different treatments during the experiment (mean±SD). Different letters show significant difference during the experiment

	Treatment		
	M0	M50	M100
SGR	0.33 ± 0.1^{B}	4.72 ± 0.23^{A}	0.18 ± 0.09^{B}
DT	2.09 ± 0.76^{B}	1.75 ± 0.54^{C}	3.71 ± 0.79^{A}

Discussion

Biological method is one of the methods to reduce nitrogen and phosphorous in aqueous medium. Microalgae have high potential to refine wastewater (De la Noüe *et al.*, 1988). Wastewater treatment by microalgae has several advantages such as nutrient recycling, valuable biomass production and no secondary pollution (De la Noüe *et al.*, 1988; Voltolina *et al.*, 2004). Yalcin *et al.* (2006) stated that microalgae are efficient to remove water nutrients.

The present results showed that the algae mixture grew well in nutrient rich medium, and considering the high survival and suitable growth rate, the mixture was highly capable to biologically refine wastewater. The results showed that phosphate uptake in the first days of the experiment was higher than the last days. The medium phosphate and nitrate completely depleted in the M50 treatment. In this case, there was no significant difference between the M50 and M100 treatments suggesting that high concentration of nutrients may sometimes inhibit algae growth at the first stage. Kong *et al.* (2010) showed that high nutrient concentrations adversely affected growth of *Chlamydomonas reinhardtii*.

The algae mixture was capable to remove 99.8% of the medium nitrate within 12 days. Heydari *et al.* (1390) showed that *Scenedesmus quadricauda* can remove 68 and 73% of water ammonia and nitrite within 21 days, with initial concentrations of 0.108 and 0.623 mg/l, respectively. Also, they reported that there was a correlation between the wastewater nitrogen uptake and algae cell number and dry weight, which is in line with the present study's results. Wang *et al.* (2010) found that *Chlorella* sp growth in a wastewater after an initial precipitation removed 90.6% of phosphate. Mallick *et al.* (2002) reported that *Phormidium. Bonheri* is able to completely remove phosphate and nitrate with initial concentrations of 30 and 42 mg/l. Similar to the present study, Martinez *et al.* (2000) found that *Sc. obliquus* can remove 97% of medium nitrogen and phosphorous within 14 days, with initial concentrations of 27.4 and 11.8 mg/l, respectively. Sreesai and Pakpain (2007) reported that *Ch. vulgaris* removed 55% of total medium phosphorous. Li *et al.* (2010) showed that *Scenedesmus* sp. can efficiently uptake low concentrations of phosphate and found positive relationship between the nutrient concentration and nutrient uptake rate.

The results of the present study suggest that there is a correlation between nitrogen removal rate and the algae cell number and dry weight as phosphate and nitrate removal percentage increased along with increase in the algae cell number and dry weight. Sing and Dhar. (2007) found a significant correlation between nitrogen removal rate and the cell number and dry weight of *Nostoc moscorom* and *Anabaena variabilis*.

The highest biomass (0.4 g/l) was observed in the M0 and M50 treatments; whereas, the lowest value (0.3 mg/l) was related to the M100 treatment, suggesting that nitrogen was more important than phosphorous in algae biomass production. Algae cell number and SGR had positive correlation with biomass and thus the highest biomass, algae cell number and SGR of *Sc. obliquus* was observed in the M50 treatment. It was expected that biomass decrease in the middle of the experiment due to increase in dead cell number but this dead cells may increase biomass. Sing and Dhar. (2007) found a significant correlation between nitrogen removal efficiency and dry weight of *N. moscorom* and *A. variabilis*. They reported that dry weight after 12 days was not significantly different between the algae being 0.25 and 0.3 g/l for *N. moscorom* and *A. variabilis*, respectively. Órpez *et al.* (2009) found that *Botryococcus braunii* can uptake 81% (180 mg/l) of nitrate from agricultural wastewater at the second stage of refinery. Also the algae dry weight was 0.4 g/l suggesting that this species grows well in agricultural wastewater.

The highest chlorophyll-a value was related to the M50 treatment. This treatment had the highest cell number at the day 8th with a correlation between cell number and chlorophyll-a content. According to the Table 7, the algae mixture (M100) had the highest chlorophyll-a compared to the other treatments, which was due to higher cell number at the end of the experiment. Ruiz-Marin *et al.* (2010) found that growth rate of *Ch. vulgaris* (8 h) was higher than that of the *Sc. obliquus* (20 h) in wastewater. Also, they reported that *Sc. obliquus* was more resistant to environmental conditions and had higher chlorophyll-a (0.22 vs. 0.54 mg/l) compared to *Ch. vulgaris*. Ruiz-Marin *et al.* (2010) also reported that *Sc. obliquus* was more resistant to environmental conditions and had higher chlorophyll-a (0.31 vs. 0.56 mg/l) compared to *Ch. vulgaris*. Martinez *et al.* (2000) reported that bacteria and protozoa in wastewater may affect algae cell growth and number. Increase in algae cell density results in shading condition, suppressing growth and reproduction and affects chlorophyll-a content, as chlorophyll-a content on day 14 was 22% lower than that of the day 10th. The results of the present study were in accordance with the other studies (De la Noüe and Javier, 1985).

BOD and COD showed a decreasing pattern during the experiment. The highest BOD and COD was observed in the M0 treatment (1.4 and 7 mg/l) and the lowest was observed in the M50 treatment (1 and 5 mg/g). Maity *et al.* (2014) used *Leptolyngbya* sp. to produce biofuel and refine wastewater and found that COD decreased from 255 to 112 mg/l. Liu *et al.* (2017) used a mixture of a bacterium *Acinetobacter* sp. and an alga to remove nutrients from wastewater. They reported that the mixture reduced the wastewater COD and phosphorous by 93.01 and 98.78 %, respectively. Li *et al.* (2010) found that *Scenedesmus* sp. can remove 83-99% of nitrogen and 99% of phosphorous within 250 h when N:P ratio was 5.1 and 12.1. The initial concentration of nitrogen and phosphorous was 189 and 73.1 mg/l, respectively. Also, BOD showed 82% decrease at the end of the experiment.

Conclusion

The aim of this study was to investigate the efficacy of two algae mixture to remove nutrients. The results showed that the complete nitrate and phosphate removal was only observed in the M50 treatment. The mixture was also effective to decrease BOD5 and COD with the highest efficiency in the M50 treatment. The highest biomass was observed in the M50 and M0 treatments with no significant difference. The highest biomass was observed in the day 10th in the M50 treatment. The highest chlorophyll-a content was related to the M0 treatment at the end of the experiment, however, the highest chlorophyll content was related to the M100 treatment, which was significantly higher than the other treatments. The highest cell count was related to the M100 treatment at the end of the experiment. Also, the highest cell count was observed in the day 8th in the M50 treatment, which was significantly higher than the other treatments.

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