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Influence of CO_2 concentration and N:P ratio on *Chlorella vulgaris*-assisted nutrient bioremediation, CO_2 biofixation and biomass production in a lagoon treatment plant

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ABSTRACT

Microalgae are among green trends for wastewater nutrient bioremediation, valuable biomass production, and CO_2 biofixation. Currently, limited information is available regarding combined effects of nitrogen:phosphorus (N:P) ratio and CO_2 concentration on growth characteristics and nutrient removal capacity of *Chlorella vulgaris* cultivated in lagoon systems. The current work sought to address simple effects and interaction effects of various N:P ratios and CO_2 concentrations on growth kinetics of microalgae, using samples taken from effluents of a domestic settling lagoon. The findings revealed that the medium supplemented with 16% CO_2 and N:P ratio of 10 was the most productive culture, generating maximum biomass concentration, specific growth rate, biomass productivity, and CO_2 biofixation rate of 0.7900 g L^{-1}, 0.4170 d^{-1}, 0.08500 g L^{-1} d^{-1} and 0.1430 gCO_2 L^{-1} d^{-1}, respectively. Moreover, *C. vulgaris* adapted and grew well even under CO_2 levels as high as 24% in the wastewater. The microalga also demonstrated to uptake both nitrogen and phosphorous in the range of 70.00–95.00%. These observations support the possibility of CO_2 bioremediation along with removal of nitrogen and phosphorous to below the most European restrictive limits for effluent discharges, while the increase in COD concentration caused by microalgae should be taken into account.

1. Introduction

Microalgal biomass has gained a great attention from researchers by virtue of its advantages in accumulating various biologically active macromolecules such as lipid, protein, carbohydrates, and pigments, being used in both industry and biomedical research [1]. The potential of microalgae for macromolecule production has been attributed to their high photosynthetic rate and ability to store huge amounts of bioactive compounds [2]. However, commercial production of microalgae is often more costly than conventional crop production, as it requires significant quantities of water and various nutrients. Accordingly, sources like effluents from conventional wastewater treatment plants provide a low cost medium for microalgae growth, where no additional chemicals are required.

The use of wastewater for cultivation of microalgae could be considered multi-faceted approaches to manage important environmental challenges. Nowadays, the rapid increase of human population and urbanization has resulted in production of large amounts of wastewater in many countries, where uncontrolled discharge of such effluents most likely results in serious health and environmental problems [3]. Although several unit processes have been developed for nutrient removal [4], environmental and economical challenges with these processes reflect the urgent need for ongoing research to find superior and sustainable solutions. Moreover, consumption of fossil fuels has led carbon dioxide (CO_2) emission, which has triggered irreversible climate changes. As a green solution, microlagal photosynthesis may be applied for CO_2 fixation and nutrients removal from wastewater concurrently. Microalgae-assisted wastewater treatment may also provide additional benefits, including an increase in dissolved oxygen, a reduction in pathogenic bacteria population, and the removal of heavy metals [5].

Thus far, a number of studies have reported successful cultivation of several species of microalgae using wastewaters

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[5,6]. Significant achievements have been also made regarding the capability of microalgae for coupling nutrient reduction from wastewater with CO_2 biofixation [7,8,9]. However, it remains unclear that how nitrogen:phosphorus ratio and CO_2 concentration interact with each other in a microalgae-assisted wastewater treatment. Moreover, limited knowledge is presently available regarding the suitable step of a lagoon treatment plant at which N:P ratio is optimal for microalgae growth. Lagoon systems are frequently applied as conventional treatment processes in developing countries, warranting research to find a point in a process flow of treatment plant which would be suitable for employing microalgae. In this context, this study sought to analyze effects of different levels of N:P ratios and CO_2 concentrations on the growth characteristics of microalga *Chlorella vulgaris*. Consequently, the applicability of *C. vulgaris* cultivated in secondary treated effluent from a lagoon treatment plant for coupling biomass production, CO_2 biocapturing, and green tertiary treatment was evaluated.

2. Materials and methods

2.1. Microalga strain

Chlorella vulgaris (ATCC ® 30,821™) was inoculated in 1-L Erlenmeyer flasks containing BG-11 medium under sterile condition at 25 ± 1 °C with 48 μmolm^{-2} s^{-1} cool-white fluorescent light illumination and a 12:12 light/dark cycle. The media was eliminated by centrifugation for 20 min at 4500 rpm and residual microalgal biomass was added to the bioreactors for inoculation.

2.2. Experimental procedure

Real samples were taken from the effluent discharge of settling lagoons of a municipal wastewater treatment plant, located in the north east of Iran where the wastewater was subjected to screening and biological treatment (aerated lagoons and settling lagoons) followed by disinfection. The measured quality of effluent was as follows: $NH^{+1}{}_4$-N = 64.84 mg L^{-1}, $NO^{-1}{}_3$-N = 4.210 mg L^{-1}, $PO^{-3}{}_4$-P = 3.780 mg L^{-1}, COD = 82.00 mg O_2 L$^{-1,}$ pH = 8.520 and alkalinity = 91.80 $mg_{CaCO_3}L^{-1.}$ The samples were autoclaved at 121 °C for 15 min. Two different sets of growth media were used: without changing the N:P ratio (18:1) and the N:P ratio of 10:1 (by the addition of KH_2PO_4 to the effluent). The N:P ratios were selected based on the fact that the optimal inorganic N P^{-1} ratio for freshwater algae growth is 6.8–10 [6]. Accordingly, in this study, N:P ratio of 10:1 was imposed to the medium to compare microalgal growth with that of N:P ratio of 18:1 available in the secondary effluent of the lagoon. Microalga was cultured in the batch mode, using a 5-L flat-plate photobioreactors with 8 × 28 × 30 cm dimensions. The cultures were continuously fed with an air stream containing CO_2-enriched air (8%, 16% and 24% *v/v*) at 0.4 vvm, using a rotameter. The CO_2 range in this study is in the line with the typical content of fossil fuel (5–20%) combusted and emitted into atmosphere [10]. Little information is available regarding the influence of CO_2 levels higher than 20% on the growth, CO_2 biofixation rate, nutrient removal, lipid and protein production of *C. vulgaris* grown under elevated CO_2.

The cultures remained for 9 days under 25 ± 1 °C with a light supplied by two cool white fluorescent lamps with an irradiance of 160 μmolm^{-2} s^{-1} and a 12:12 light/dark photoperiods. At the beginning of the study, all reactors were inoculated with a calculated volume of microalga to obtain a similar initial concentration of biomass in all assays (initial optical density of 0.1800 at 760 nm). Two reactors containing culture media without the microalga was used as negative controls.

2.3. Analytical methods

Microalga biomass were measured every other day by optical density measurement at 760 nm [11], using a UV–vis spectrophotometer (Agilent, USA). dry weight (X) was calculated using linear regression analysis of a standard curve:

$$X\left(\mathrm{g\,L^{-1}}\right) = (0.1940 \times OD_{760}) - 0.001\left(R^2 = 0.9960\right) \quad (1)$$

Liquid samples for nutrient analysis were taken every other day during the 9 day test period. The samples were centrifuged at 4500 rpm for 20 min and the supernatants were collected. Nitrate, ammonium, phosphate, and COD were analyzed according to the standard methods [12]. Because effluent from settling lagoons was used as cultural media, ammonium and nitrate were considered as total nitrogen. Alkalinity was measured by titration of 10 mL of the cell-free medium with 0.1 N HCl [12]. pH of the medium was also monitored with a pH meter (Metrohm- Swiss made 827).

Nutrient concentrations within the cultivation time were calculated to determine nutrients removal efficiencies (%R), using the following equation:

$$\%R = (Si - S_f)/Si \quad (2)$$

Where S_i and S_f correspond to nutrients concentration (mg L^{-1}) at the beginning and end of cultivation time, respectively [8].

Total nitrogen content of microalga was measured by an elemental analyzer (Costech ECS 4010), estimating crude proteins. using the following equation [13].

$$\text{Crude protein} = 6.25(\%\,\mathrm{N}) \quad (3)$$

Total lipid content was extracted from freeze-dried algal biomass using Bligh and Dyer method [14].

2.4. Determination of growth kinetic parameters

The specific growth rate (d^{-1}) was determined using the exponential logarithmic phase [15]:

$$\mu = (\ln X_1 - \ln X_0)/t_t - t_0 \quad (4)$$

Where X_1 and X_0 are the biomass concentration (g L^{-1}) on days t_1 and t_0 (the end and beginning of the exponential growth phase, respectively).

Biomass productivity (g L^{-1}d^{-1}) was also calculated:

$$P = (X_t - X_0)/t_t - t_0 \quad (5)$$

Where X_0 is the initial biomass concentration (g L^{-1}) at time t_0 and X_t is the biomass concentration in (g L^{-1}) at any time t subsequent to t_0 [16].

2.5. Measurement of carbon dioxide biofixation rate

The carbon dioxide biofixation rate (P_{CO_2}) was calculated according to De Morais and Costa [16] equation:

$$P_{CO_2} = C_c P(M_{CO_2}/M_C) \quad (6)$$

Where C_c is the carbon content in dried biomass obtained by elemental analysis; P is the maximum biomass productivity (g L^{-1}d^{-1}); and M_{CO_2} and M_C are 44 and 14 (the molecular weight of CO_2 and C respectively).

2.6. Statistical analysis

An experiment array based on the 2 × 3 multilevel categoric design was used to evaluate the effects of different CO_2 concentrations (8%, 16% and 24%) and two N:P ratios (10 and18) on the *Chlorella* vulgaris-assisted nutrient bioremediation, CO_2 biofixation

Table 1
Effects of N:P ratio and CO_2 concentration on growth characteristics of *C. vulgaris* (means ± SD).

Independent variables		Quantitative parameters		
CO_2 (%,v/v)	N:P ratio	X_{max} (g L^{-1})	P_{max} (g L^{-1} d^{-1})	μ (d^{-1})
8	**10:1**	0.5500 ± 0.04000^{BCD}	$0.07600 \pm 0.008000^{AB}$	0.3930 ± 0.01100^{AB}
16	**10:1**	0.7900 ± 0.02000^{A}	0.08500 ± 0.003000^{A}	0.4170 ± 0.01000^{A}
24	**10:1**	0.6500 ± 0.03000^{B}	$0.06800 \pm 0.003000^{AB}$	0.3860 ± 0.006000^{BC}
8	**18:1**	0.5100 ± 0.08000^{CD}	0.06800 ± 0.01100^{AB}	0.3760 ± 0.01500^{BC}
16	**18:1**	0.6400 ± 0.07000^{BC}	0.06700 ± 0.008000^{B}	0.3950 ± 0.006000^{AB}
24	**18:1**	0.4700 ± 0.04000^{D}	0.04800 ± 0.004000^{C}	0.3590 ± 0.01700^{C}

Means in each column without a common superscript uppercase letter are different ($p < 0.05$).

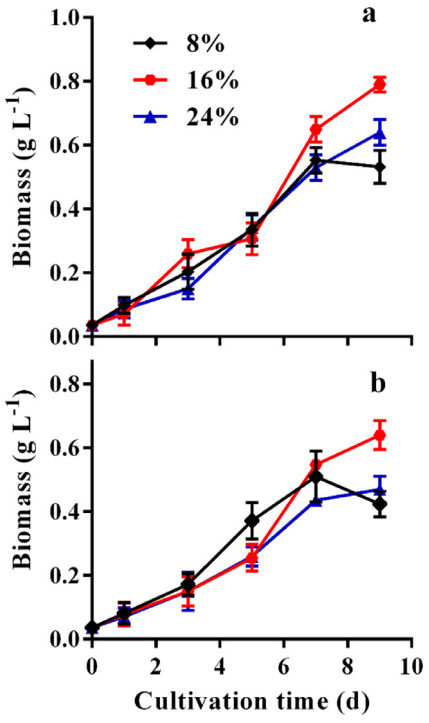

Fig. 1. Growth curves of *C. vulgaris* under different levels of CO_2 at (a) N:P = 10:1 and (b) N:P = 18:1.

and biomass production . The experiments were independently repeated at least two times, analyzed by SPSS 19.0 (SPSS Inc., USA). The statistical significance was evaluated using one-way ANOVA. A $p < 0.05$ value was considered statistically significant. In addition, the numerical method provided by Design-Expert 11 was employed to run an optimization in order to determine the best factor levels that would maximize the system's responses under specific user-defined criteria. The last day (ninth day) was considered as the end-point time. The detailed method is presented in Supplementary material (Method S1).

3. Results and disscution

3.1. Microalgal growth kinetics

The *C. vulgaris* growth kinetics showed no obvious lag phase under any of the treatments so that the algal growth began exponentially from the beginning (Fig. 1).

The lack of lag phase in the *C. vulgaris* growth is in agreement with previous reports [6,8], indicating that *Chlorella* spp. survives high CO_2 levels and quickly adapt to wastewater media. Under different N:P ratios and CO_2 concentrations, growth kinetic parameters of *C. vulgaris* were also determined, including specific growth rate (μ), maximum biomass concentration (X_{max}), and maximum biomass productivity (P_{Max}) (Table 1). For both N:P ratios, μ increased as the CO_2 concentration of the air flow raised from 8% to 16%. The maximum μ was attained under 16% CO_2 and N:P ratio of 10:1. However, the continuing increase in the CO_2 content (up to 24%) caused a decline in μ values so that the lowest value was observed for 24% CO_2 and N:P of 18:1 ($p < 0.05$). The pattern of changes in μ caused by different CO_2 concentrations is supported by other studies, although different CO_2 values have been reported [8,16,17].

The changes in X_{max} showed a pattern similar to that obtained with μ. The condition of 16% CO_2, N:P of 10:1 generated a X_{max} of 0.7900 g L^{-1}, while the value was 0.4700 g L^{-1} under 24% CO_2, N:P of 18:1. Besides, N:P ratio of 10:1 caused higher values of X_{max} compared to N:P ratio of 18:1 regardless of CO_2 concentration. This issue could be attributed to high phosphorous content in lower N:P ratios, as validated by Xin et al. [18].

P_{max} was also noticed to be a function of the CO_2 levels and N:P ratio. An increase in CO_2 from 8% to 16% led to higher values of P_{max}, followed by an apparent decrease where CO_2 reached 24% (Table 1). Accordingly, the highest calculated P_{max} was 0.08500 g $L^{-1}d^{-1}$ in N:P ratio of 10:1 grown under 16% CO_2, while the lowest value (0.04800 g $L^{-1}d^{-1}$) was obtained at N:P ratio of 18:1 with 24% CO_2 ($p < 0.05$) (Supplementary materials: Table S1 and Fig. S2)

These findings indicate that an increase in CO_2 concentration up to 16% does not lead to a significant decline in the algal biomass. This issue contradicts with some reports [17,19,20], while it is in agreement with another study indicating algal biomass of *C. pyrenoidosa* was not reduced by the CO_2 concentrations up to 30% [21]. Although there are variations in the reported optimum CO_2 concentration for achieving maximum algal biomass, our findings suggest that *C. vulgaris* grow well under concentrations of CO_2 up to 24%. However, the decline in growth parameters under 24% CO_2 (as compared to 16% CO_2) can be associated to a decrease in carbonic anhydrase (CA) and Rubisco enzyme activity which have direct effects on algal growth [22].

Comparison between growth parameters of *C. vulgaris* in N:P ratio of 18:1 and N:P ratio of 10:1 indicated that phosphorous can be considered a limiting nutrient for *C. vulgaris* grown in the effluents of settling lagoon. Phosphorylation made phosphorus to be incorporated into algal organic compounds which leads to adenosine triphosphate (ATP) production (as an energy input) from adenosine diphosphate (ADP). Therefore, an increase in the phosphorous concentration of the media results in improvement of algal growth parameters [23].

3.2. Effect of CO_2 and N:P ratios on carbon content and CO_2 biofixation rate (P_{CO_2})

The carbon content of *C.vulgaris* was not significantly affected by N:P ratio or CO_2 concentrations (Table 2), being at an average of 46.46% (w/w). These findings support other studies in that CO_2 concentrations or N:P ratios do not make significant changes in

Table 2
Effects of N:P ratio and CO_2 concentration on carbon content (C_c), CO_2 biofixation (P_{CO_2}) and yield coefficient of *C. vulgaris* (means ± SD).

Independent variables		Quantitative parameters		
CO_2 (%,v/v)	N:P ratio	C_c (% W^{-1})	P_{CO_2} ($gco_2 L^{-1} d^{-1}$)	Y_{CO_2}(gCO_2/gSS)
8	**10:1**	45.71 ± 0.5600^{A}	0.1240± 0.01100^{AB}	1.630^{A}
16	**10:1**	46.16 ± 1.130^{A}	0.1430 ± 0.008000^{A}	1.680^{A}
24	**10:1**	48.11 ± 0.03000^{A}	0.1200 ± 0.003000^{AB}	1.760^{A}
8	**18:1**	44.30 ± 1.030^{A}	0.1100 ± 0.01700^{AB}	1.620^{A}
16	**18:1**	47.51 ± 1.4100^{A}	0.1170 ± 0.01000^{B}	1.740^{A}
24	**18:1**	46.71 ± 1.230^{A}	0.08200 ± 0.01000^{C}	1.710^{A}

Means in each column without a common superscript uppercase letter are different ($p < 0.05$).
$Y_{CO_2} = P_{CO_2}/P_{MAX}$

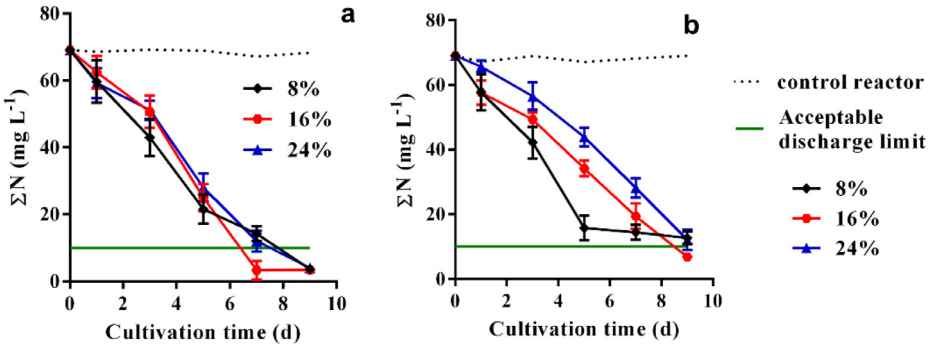

Fig. 2. Evolution of total nitrogen as ΣN: $NH^{+1}{}_4$-N + $NO^{-1}{}_3$-N concentration at different CO_2 levels in reactors. (a) N:P = 10:1, (b) N:P = 18:1.

carbon content of algal cells [8,21,24]. P_{CO2} showed a minimum and maximum level of 0.08200 and 0.1400 gCO_2 L^{-1} d^{-1} at N:P ratio of 18:1, 24% CO_2, and N:P ratio of 10:1, 16% CO_2, respectively ($p < 0.05$). The differences in P_{CO_2} could be attributed to the differences in biomass productivity under various treatment conditions.

Yield coefficients (Y_{CO_2}) of the tested microalga were also found to range from 1.620 to 1.760 mg CO_2 per mg of biomass (Table 2). Y_{CO_2} values reported here are compatible with other studies where Y_{CO_2} is approximately similar to the theoretical value of 1.880, being calculated using the molecular formula of $CO_{0.48}H_{1.83}N_{0.11}P_{0.01}$ for microalgal biomass [25] (Supplementary materials: Table S1 and Fig. S3).

3.3. Wastewater nutrient removal by C. vulgaris

According to European Union (EU) legislation [26], total nitrogen and phosphorous in the effluent from treatment plants must not exceed 10 and 1 mg L^{-1}, respectively. Thus, the capability of *C. vulgaris* to uptake nutrients present in the effluent of settling lagoon was compared with target values defined by EU legislation. In addition, no nitrogen or phosphorous removal in the control reactors occurred as the pH was maintained at low levels by the addition of CO_2; therefore, no ammonia stripping or phosphate precipitation were observed; ensuring only biological processes are involved in nutrient removal [27].

The trend of changes in total dissolved nitrogen (ΣN: NH_4^+-N + $NO^-{}_3$-N) and phosphorous concentrations is depicted in Fig. 2a,b, and Fig. 3a,b, respectively. A decrease in the both was observed under all the tested conditions. The findings also revealed that the total nitrogen and phosphorous removal percentages were higher than 80.00% and 70.00%, respectively, for all CO_2 and N:P ratio levels at the end of the period. Maximum total nitrogen removal was 95.20% under 16% CO_2 and N:P of 10:1, while the least efficiency was 81.60% for 8% CO_2 and N:P of 18:1 ($p < 0.05$). The maximum efficiency for phosphorus removal was 96.50% under 8% CO_2 and N: P of 18:1, while the minimum uptake was 72.88% with 8% CO_2 and N:P 10:1 ($p < 0.05$) (Table 3).

The higher levels of total nitrogen removals at N:P ratio of 10:1 compared to N:P of 18:1 ($p < 0.05$) can be explained by the fact that N:P of 10:1 is close to the ratio of 7(−8):1 g^{-1} reported for typical composition of algae [28]. When *C. vulgaris* grew in an environment with N:P ratio of 18:1, the microalgae had a continuous phosphorous limitation, resulting in a high internal nitrogen pool, also indicated by Kapdan and Aslan [29]. Unlike nitrogen, the microalga removed phosphorous at great levels in N:P of 18:1 as compared to N:P of 10:1, because phosphorous uptake can be limited by nitrogen in N:P of 10:1 (Supplementary materials: Table S2 and Fig. S4).

The time required to reach the nitrogen and phosphorus discharge limits of 10 mg L^{-1} ($T_{10\,(N)}$) and 1 mg L^{-1} ($T_{1\,(p)}$), respectively, were determined and compared across all the treatments (Table 3). Regardless of CO_2 condition, $T_{10\,(N)}$ values were lower at N:P ratio of 10:1 than those obtained at N:P ratio of 18:1 ($p < 0.05$). Minimum $T_{10\,(N)}$ was 153.2 h under 16% CO_2, N:P ratio of 10:1, while no $T_{10\,(N)}$ was achieved under 8% and 24% CO_2, N:P of 18:1. Similarly, total residual nitrogen at N:P of 10:1 ranged between 3.370 and 4.000 mg L^{-1} which was below the limits defined by EU legislation. Contrary to $T_{10\,(N)}$, $T_{1\,(p)}$ was lower at N:P ratio of 18:1 compared to N:P ratio of 10:1. The lowest $T_{1\,(p)}$ of 120.0 hours occurred at N:P ratio of 18:1 and 8% CO_2. Expectedly, residual phosphorous at N:P of 18:1 was below the limits defined by EU legislation (ranged from 0.1300 to 0.3500 mg L^{-1}).

Despite differences in nutrient removal efficiency among N:P ratios and CO_2 concentrations, the discharge concentrations of nitrogen and phosphorus reached their limits under 16% CO_2 and N:P ratio of 18:1. These findings propose the possibility of simultaneous removal of nitrogen and phosphorus to below the most restrictive limits. However, the time needed to reach these limits were longer than those reported by others [7,23], creating an obstacle in widespread application of this method for tertiary wastewater treatment compared with the other treatment process.

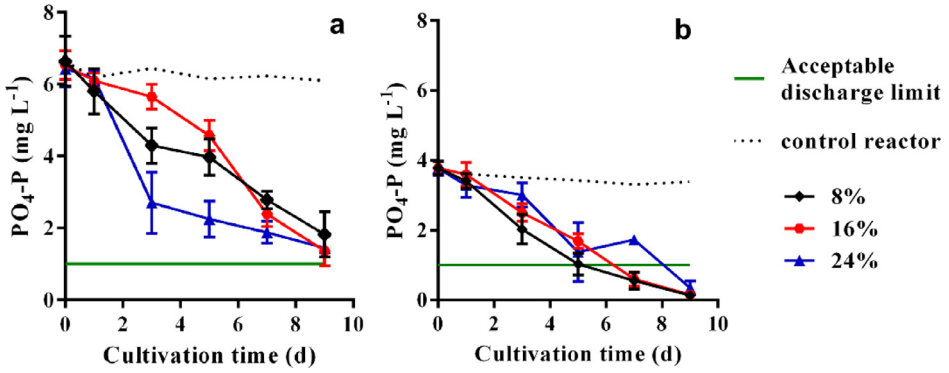

Fig. 3. Evolution of PO^{-3}_4 concentration at different CO_2 levels in reactors. (a) N:P = 10:1, (b) N:P = 18:1.

Table 3
Nutrient removal by *C. vulgaris* under different N:P ratios and CO_2 concentrations (means ± SD).

Independent variables		Quantitative parameters					
CO_2 (%,v/v)	N:P ratio	ΣN removal (%)	T_{10N} (h)[1]	Residual ΣN (mg L^{-1})	P removal (%)	T_{1P} (h)[1]	Residual p (mg L^{-1})
8	**10:1**	94.80 ±0.01000^{A}	187.2	3.590	72.88 ±0.07000^{B}	*	1.820
16	**10:1**	95.12 ± 0.01000^{A}	153.2	3.370	78.98 ±0.05000^{B}	*	1.360
24	**10:1**	94.20 ±0.01000^{A}	180.1	4.000	77.60 ±0.000^{B}	*	1.440
8	**18:1**	81.60 ±0.02000^{C}	*	12.70	96.50 ±0.03000^{A}	120.0	0.1300
16	**18:1**	90.10 ±0.01000^{B}	203.9	6.830	95.81 ±0.01000^{A}	150.4	0.1600
24	**18:1**	82.44 ±0.01000^{C}	*	12.12	90.46 ±0.06000^{A}	193.8	0.3500

Means in each column without a common superscript uppercase letter are different ($p < 0.05$)
[1] Times needed to reach 10 mg N L^{-1} (t_{10N}) and 1 mg P L^{-1} (t_{1P}).
* Discharge limit not achieved.

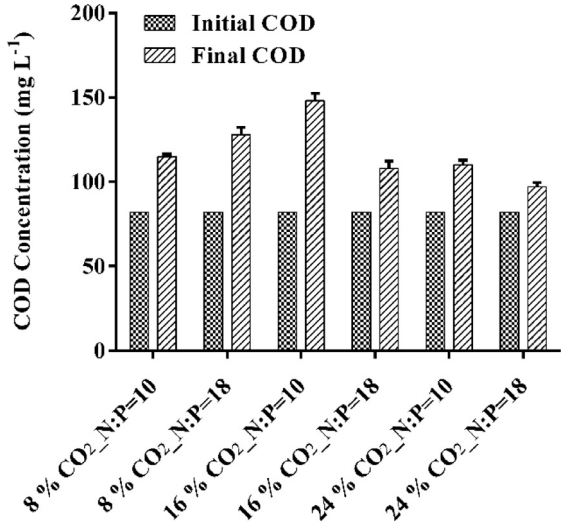

Fig. 4. Initial and final chemical oxygen demand (COD) concentrations.

3.4. Change in COD concentration

Chemical Oxygen Demand (COD) is widely used as an indicator of organic loads in wastewater treatment plants. Therefore, COD was measured at the beginning and at the end of experimental period (Fig. 4).The increased COD at the end of period indicated producing considerable amounts of organics. Because carbon matters present in the settling effluent are poor biodegradable and cannot easily be utilized by microalgae, microalgae grow as an autotroph and uses CO_2 as the carbon source. Under these autotrophic conditions, exocytosis by *C. vulgaris* cause biopolymers and volatile organic compounds to be released into the media, which in turn increase COD. Besides, during the microalgae cell growth, aliquots of the polysaccharidic material of both capsules and slimes may be released into the surrounding medium, causing a progressive increase of media viscosity and COD [6,30] (Supplementary materials: Table S1 and Fig. S4)

Table 4
Effects of N:P ratio and CO_2 concentration on protein and lipid contents of algal biomass (means ± SD).

Independent variables		Quantitative parameters	
CO_2 (%,v/v)	N:P Ratio	Lipids (% dw)	Crude protein (% dw)
8	**10:1**	10.41 ± 0.9200^{A}	61.31 ± 2.350^{A}
16	**10:1**	9.910 ± 1.470^{A}	49.37 ± 1.450^{BC}
24	**10:1**	10.09 ± 1.250^{A}	43.06 ± 2.590^{D}
8	**18:1**	11.46 ± 1.670^{A}	53.62 ± 2.000^{B}
16	**18:1**	8.100 ± 0.5600^{A}	47.81 ± 1.710^{CD}
24	**18:1**	11.16 ± 1.570^{A}	46.37 ± 2.220^{CD}

Means in each column without a common superscript uppercase letter are different ($p < 0.05$).

3.5. Lipid and crude protein contents

The total lipid content of *C. vulgaris* ranged from 8.100% to 11.46% dw (Table 4). Athough the total lipid contents were slightly affected by nutrient and CO_2 concentrations, the differences were not significant ($p \geq 0.05$). Elevated CO_2 concentrations did not lead to lipid accumulation in the microalgal biomass, most likely due to the relatively poor solubility of CO_2 in water. These results are in accordance with several recently reported studies where different concentrations of CO_2 caused only 1–6% increment in lipid content in microalgae cells [19,31]. However, the higher productivity of algal biomass at N:P ratio of 10:1, 16% CO_2 (Table 1) may lead to an increase in lipid productivity. The protein measurements also showed high amounts of crude proteins in the microalgal mass for all the treatments (Table 4) where the highest crude protein content (61.31%) was observed at 8% CO_2 concentration, N:P of 10:1. The total protein contents of *C. vulgaris* grown in the real samples of wastewater evaluated in this study are higher than those

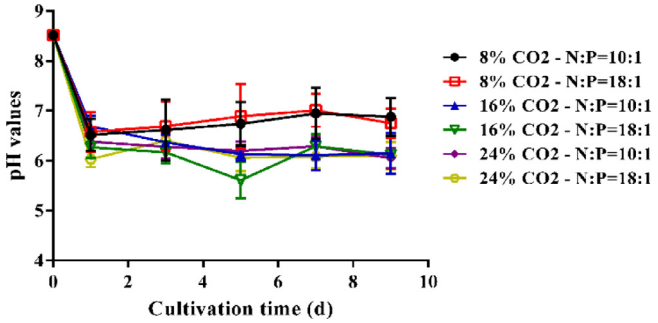

Fig. 5. The relationship between pH and cultivation time.

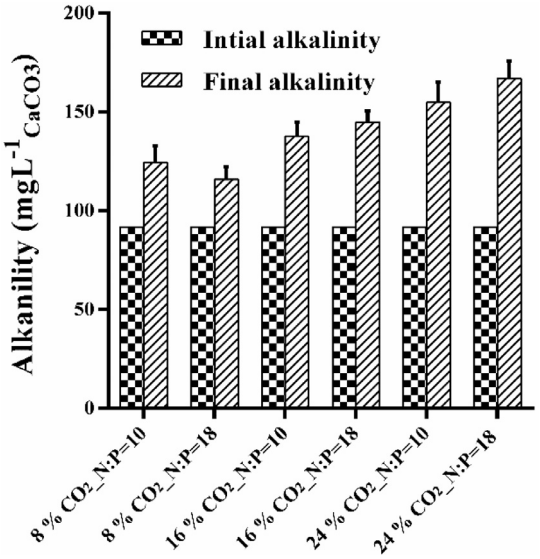

Fig. 6. Initial and final alkalinity concentrations.

cultivated in synthetic wastewater. Moreover, the total protein content of *C. vulgaris* was higher than that of vegetables such as soybean and peanut. Thus, the findings of the present study indicate that a nutrient-rich domestic wastewater is suitable for synthesis of macromolecules such as lipid and protein in *C. vulgaris* [32]. (Supplementary materials Table S1 and Fig. S5)

3.6. Changes in pH and alkalinity

At the beginning of the experimental period, all bioreactors showed a drop in pH due to the CO_2 injection, followed by a slight increase through the remaining time period. This increase is thought to be a result of flowing CO_2 out of bioreactors and CO_2 consumption during the algal photosynthetic as well (Fig. 5). During photosynthesis, bicarbonate ions are converted to CO_2 which in turn are fixed by RuBisCO enzyme. Based on the chemical equilibrium of $H^+ + HCO_3^{-1} \leftrightarrow CO_2 + H_2O$, as the H^+in the cell is consumed, the OH^- concentration increases. In order to neutralize the cell, H+ must be uptaken from the growth media, resulting in pH increment [33]. Providing high levels of CO_2 into culture media prevents elevation of pH during culture period.

The alkalinity of media before the *C. vulgaris* cultivationwas measured and compared with those of after the cultivation (Fig. 6). The data showed an increase in the alkalinity at the end of the period. Because pH ranged from 6 to 7 throughout the period, bicarbonates were most likely dominating forms of CO_2 which increased with elevated influent CO_2 concentration. These results are consistent with other reports indicating that CO_2 was mostly transformed to bicarbonates which in turn would increase the total alkalinity [34].

4. Conclusions

This study for the first time demonstrated that the growth characteristics of *C. vulgaris* under N:P ratio of 18:1 present in the secondary effluent of lagoon may not be optimum compared with that under N:P ratio of 10:1 imposed to the medium. Thus, in a lagoon plant, stream from units with lower N:P ratios might provide a better medium for microalgae. The findings enabled us to conclude that the condition of 16% CO_2 and N:P ratio of 10:1 can generate the highest amounts of algal biomass. The higher productivity of biomass at N:P ratio of 10:1 also increased lipid productivity, which could have implications in the production of biofuel. The microalga utilized in this study adapted to grow well under CO_2 levels as high as 24%, indicating its suitability for bioremediation of CO_2 from a simulated flue gas. Nitrogen and phosphorous were also found to be significantly removed by *C. vulgaris* in the range of 70.00–96.00% under all the CO_2 concentrations and N:P ratio conditions. However, the increase in COD concentration caused by microalgae should be taken into account.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jtice.2019.01.005.

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