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# Removal of high concentration of nitrate and phosphate from aqueous mixotrophic solution by *Chlorella vulgaris*

**Tuğba Şentürk, Şükran Yıldız**

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Manisa Celal Bayar University Faculty of Science and Art Department of Biology Manisa, Turkey

**ORCID IDs of the author(s):**

T.Ş. 0000-0002-9882-0079

Ş.Y. 0000-0003-3195-2269

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**Correspondence:**

**Tuğba ŞENTÜRK**

**E-mail:** tugba_sen34@hotmail.com



## ABSTRACT

Microalgae exhibit large potential as an alternative to advanced biological nutrient removal in wastewater or simulated wastewater at laboratory conditions. Therefore, it is necessary to determine the optimum conditions for nutrient removal. This study investigated the total carbohydrate, chlorophyll-a, -b, carotenoid and lipid production and nutrient removal of mixotrophic microalgae (*C. vulgaris*) cultured in different nitrate/phosphate rich modified BG-11 medium (0-200 mg $L^{-1}$) at longer growth periods (10 days). The mean removal efficiency of $NO_3$-N (in nitrate source), and $PO_4$-P (in phosphate source) (88.29 ±0.12 and 31.06 ±0.22%, respectively) was reached in the mixotrophic culture. Under the optimum conditions (200 µmol photon $m^{-2}s^{-1}$ 16 h photoperiod and 28% inoculum size), 63.61-99.05% of $NO_3^-$ and 13.97-63.77% of $PO_4^{3-}$were successfully removed. The lipid and carbohydrate productivities were 27.95 and 29.53 g $L^{-1}d^{-1}$, 0.2869 and 0.2435 g $L^{-1}$ $d^{-1}$ respectively, which were approximately 9-12 times higher than those in photoautotrophic condition. The BG-11 growth media containing 10 g $L^{-1}$ glucose and excessive amount of nutrient effect results indicate that the Chl-a, -b and carotenoid contents of *C. vulgaris* is higher at 100 mg $L^{-1}$ N and 50 mg $L^{-1}$ P growth media composition compared to 100% growth media composition. Thereby, the findings of this study provided an insight into the role of algal uptake of nutrients under the nutrient rich mixotrophic medium for the future algae-based treatment application.

**Keywords:** Bioremediation, *Chlorella*, Mixotrophic solution, Nutrient removal, Algal removal

## Introduction

The application of microalgae for wastewater treatment has gained much attention due to the potential of microalgae to simultaneously remove nutrients and produce valuable biomass. Their great potential in producing biodiesel, which is a renewable energy source, can reduce the greenhouse gas emissions (Abe et al., 2008; Khan and Yoshida, 2008; Bruce, 2008; Groom et al., 2008; Azianabiha et al., 2019).

The production of biofuels from microalgae is associated with high demands of nutrients required for growth (Barbera et al., 2016). Their lipid productivity/biomass (dry weight) is about 15–300 times that of conventional crops (Chisti, 2008). Therefore, microalgae are considered as a promising substitute for fossil fuels in the future (Li et al., 2010).

Phosphorus is one of the most important nutrient in domestic waste-water. It is difficult to remove and hence along with nitrogen is responsible for eutrophication of water bodies, especially where untreated sewage is discharged. Nutrient removal is becoming a regular approach for wastewater treatment plant, since excess nitrogen and phosphorus in discharged wastewater can lead to downstream eutrophication and ecosystem damage (Swati et al., 2017).

Based on these considerations, it is clear that the only way to obtain an economically and environmentally sustainable microalgal biofuels production is to recycle the nutrients, the majority of which is not included in the lipid fraction destined to biofuels,and remains in the residuals. This possibility is clearly highly connected with the method employed for biomass treatment after harvesting (Sialve et al., 2009; Heilmann et al., 2011; Biller et al., 2012; Rösch et al., 2012; Garcia Alba et al., 2013; Levine et al., 2013; López Barreiro et al., 2013; Zhang et al., 2014; Ward et al., 2014; Barbera, 2016).

Microalgae growth is possible under heterotrophic or mixotrophic conditions as well as autotrophic conditions depending on specific characteristics of the species (Andrade and Costa, 2007) and some microalgae species like *Chlorella vulgaris* (Mitra et al., 2012), *Haematococcus pluvialis* (Kobayashi et al., 1992), *Spirulina platensis* (Marquez et al., 1993), *C. sorokiniana* (Wang et al., 2012), *Botryococcus braunii* (Zhang et al., 2011), and *C. zofingiensis* (Liu et al., 2011) have been observed under autotrophy, heterotrophy, and mixotrophy conditions. Mixotrophic cultivation of microalgae provides higher biomass and lipid productivities than cultivation under photoautotrophic conditions, the cost of the organic carbon substrate is estimated to be about 80% of the total cost of the cultivation medium (Bhatnagar et al., 2011).

The objective of this study was to quantify some biochemical changes (lipids, chlorophyll-a and -b, carotenoids and total carbohydrate and removal of nutrients) in mixotrophic condition (glucose substrate) of *Chlorella vulgaris* grown in nitrate-phosphate rich conditions. Nitrate and phosphate concentrations were measured on the initial and final days of cultivation to evaluate nutrient removal rates. Therefore, the aim of the present study was to determine nutrient uptake performance and efficiency of *Chlorella* cells under the nutrient rich mixotrophic medium for the future algae-based wastewater treatment application.

## Material and Methods

### *Algal Growth Medium and Experimental Design*

*C. vulgaris* was obtained from the Culture Collection of Microalgae at the University of Ege, Izmir, Turkey. The modified and non-modified BG-11 medium were used as the growth medium in the experiments. The growth and nutrient uptake experiments were conducted at four different nutrient levels as presented in Table 1. $NO_3$-N ($NaNO_3$) and $PO_4$-P ($K_2HPO_4$) were used as the nitrogen and phosphorus sources, respectively. A standard initial inoculum of the algae was inoculated to culture flasks (200 mL each) that contained BG-11 medium and incubated at 28 ± 1ºC under 14 h light (20 E $m^{-2}$ $s^{-1}$ ±20 %), with magnetic stirring (100 rpm). For mixotrophic cultures, glucose was added to the culture broth in concentration of 10 g $L^{-1}$ maintaining the same L/D photoperiod of 14:10 h. BG11 medium and BG11 medium containing glucose were used for autotrophic culture and mixotrophic culture of *Chlorella* cells, respectively. 10 g $L^{-1}$ glucose has been proved to be an ideal organic matter source for the mixotrophic cultivation of microalgae in some previous studies (Liang et al., 2009; Cheirsilp & Torpee, 2012). To examine the removal effect of nitrogen and phosphorus from modified medium by using *C. vulgaris* cells, the selected microalgae were triplicate cultured in medium with 0, 50, 100, 200 mg $L^{-1}$ concentration of nitrate and phosphate for 10 days. The initial pH was adjusted to 7 using 10% HCl and the contents of chlorophyll-a, chlorophyll-b, lipid and carotenoids in the supernatant were determined by UV-VIS spectroscopy.

**Table 1.** Initial nutrient levels for batch experiments with *C. vulgaris.*

| Experiment | $NO_3$-N (mg/L) | $PO_4$-P (mg/L) | Glucose (g/L) |
|---|---|---|---|
| **Control* (n-mm Bg-11 )** | 0.06 ±0.003 | 0.001 ±0.001 | No glucose |
| **mm BG-11** | 0 | 0 | 10 ±0.01 |
| | 50 ±0.24 | 50 ±0.77 | 10 ±0.04 |
| | 100 ±0.58 | 100 ±0.96 | 10 ±0.05 |
| | 200 ±1.33 | 200 ±1.46 | 10 ±0.01 |

*Key to subscripts: n-mm: non modified medium, mm: modified medium.

### *Determination of Chlorophyll and Total Carotenoids Concentration*

Chlorophylls and carotenoids in *C. vulgaris* were extracted with methanol and spectrophotometrically determined as described by Dere et al. (1998). Total pigment content was obtained by summing chlorophylls and carotenoids contents

### *Lipid Analysis*

Lipid contents of the microalgae were directly measured by sulpho-phospho-vanillin (SPV) colorimetric method (Mishra et al., 2014). At the end of the cultivation, algal biomass was harvested to measure lipid content. The relationship between the lipid content of the 100 µL microalgae suspensions and the absorbency at 530 nm was acquired from a previous study (Tao et al., 2017; Eq. 1):

$$\text{Lipid (mg)} = 0.123 \text{ X } OD_{530} + 0.003 \ (R^2 = 0.999) \quad (1)$$

### *Dry Weight and Nutrient Removal Analysis*

The dry weight of algal biomass was determined using the method of suspended solid (SS) measurement. For the measurement of water quality, the algal culture was centrifuged (10.000 rpm X 10 min at 4°C) and filtered through a 0.45 µm filter. After that, the weight of *C. vulgaris* was calculated from the calibration curve that obtained from the dry cell weight method (Eaton, 2005). The filtered supernatant was then used for the determination of nitrate and phosphate concentrations. To determine nutrient removal rates, $NH_3^+$-N and $PO_4^{3-}$-P were measured on initial and final days of the experimental period. The samples were filtered with a 0.2-µm pore-size membrane filter prior to the measurement to exclude suspended materials. Nutrient removal rate (R, %; Eq. 2) and removal capacities (q, mg/L day, Eq. 3) were calculated as (Babaei et al., 2013):

$$R = 100 \times (C_i - C_f) / C_i, \quad (2)$$

$$q \text{ (mg/L day)} = (C_i - C_f) \text{ x } V/m \quad (3)$$

V: Solution volume (mL)

m: Dry weight of the adsorbent (g)

$C_i$ and $C_f$: initial and final nutrient concentrations of $NH_3^+$-N or $PO_4^{3-}$-P on initial and final days of the experimental period, respectively.

All experiments were performed in 3 replicates. The data are presented as the mean±standard deviation of the mean (SDM).

## Results and Discussion

### *Chlorophyll-a and b and Carotenoid Contents*

In this work, the effects of mixotrophic medium, which is contain high concentration of nitrate and phosphate, were systematically investigated on *C. vulgaris*, regarding the nutrient uptake, the lipid productivity, the chlorophyll, carotenoid and carbohydrate content.

Chl-a and b and carotenoid levels for the control group were measured 0.6565, 0.9883 and 0.0985 µg/L, respectively under mixotrophic cultivation. At the end of the experiment, the highest chlorophyll-a and -b and carotenoid contents were observed in the 50 mg $L^{-1}$ (1.33 µg $L^{-1}$) and 50 mg $L^{-1}$ (2.24 µg $L^{-1}$) and 100 mg $L^{-1}$ (3.57 µg $L^{-1}$), respectively. Measurements for the Chl-a and b and carotenoid content, for the 100 mg $L^{-1}$ and 50 mg $L^{-1}$ concentration of nitrate and phosphate solution, showed that the high concentration of $NO_3^-$ and $PO_4^{3-}$ treatment causing an increase in Chl-a and b and carotenoid, respectively (Figure 1). Chlorophyll content results showed that 100 mg $L^{-1}$ nitrate treatment caused an increase in Chl-a and b and carotenoid levels, while 50 mg $L^{-1}$ phosphate treatment decreased.

Chlorophyll is one of the cellular compounds on the basis of which microalgal biomass in the culture is estimated and it can be used to measure cell growth (Kong et al., 2013). According to a previous report, the utilization of an external organic carbon source may affect the photoautotrophic growth processes, such as photosynthesis and respiration (Kong et al., 2013). As shown in Figure 1, the effect of glucose and 100 mg $L^{-1}$ and 50 mg $L^{-1}$ concentration of nitrate and phosphate solution on the photosynthetic pigment content and

productivity of mixotrophic *C. vulgaris* was significant. Our results showed that the mixotrophic cultures experience an increase in photosynthetic pigment productivity that was dependent on the increase of high concentration of nutrient in the medium content (Kong et al., 2013).

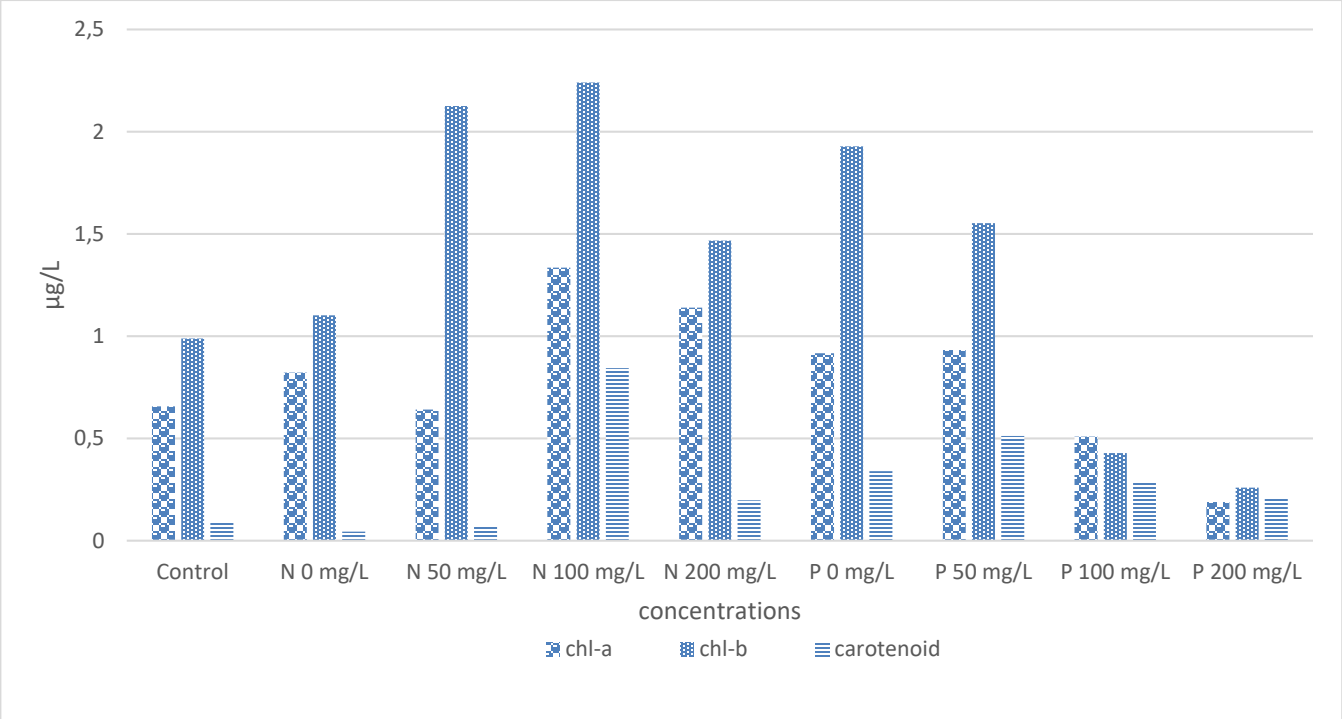


**Figure 1.** Chl-a and b and carotenoid changes in µg/L

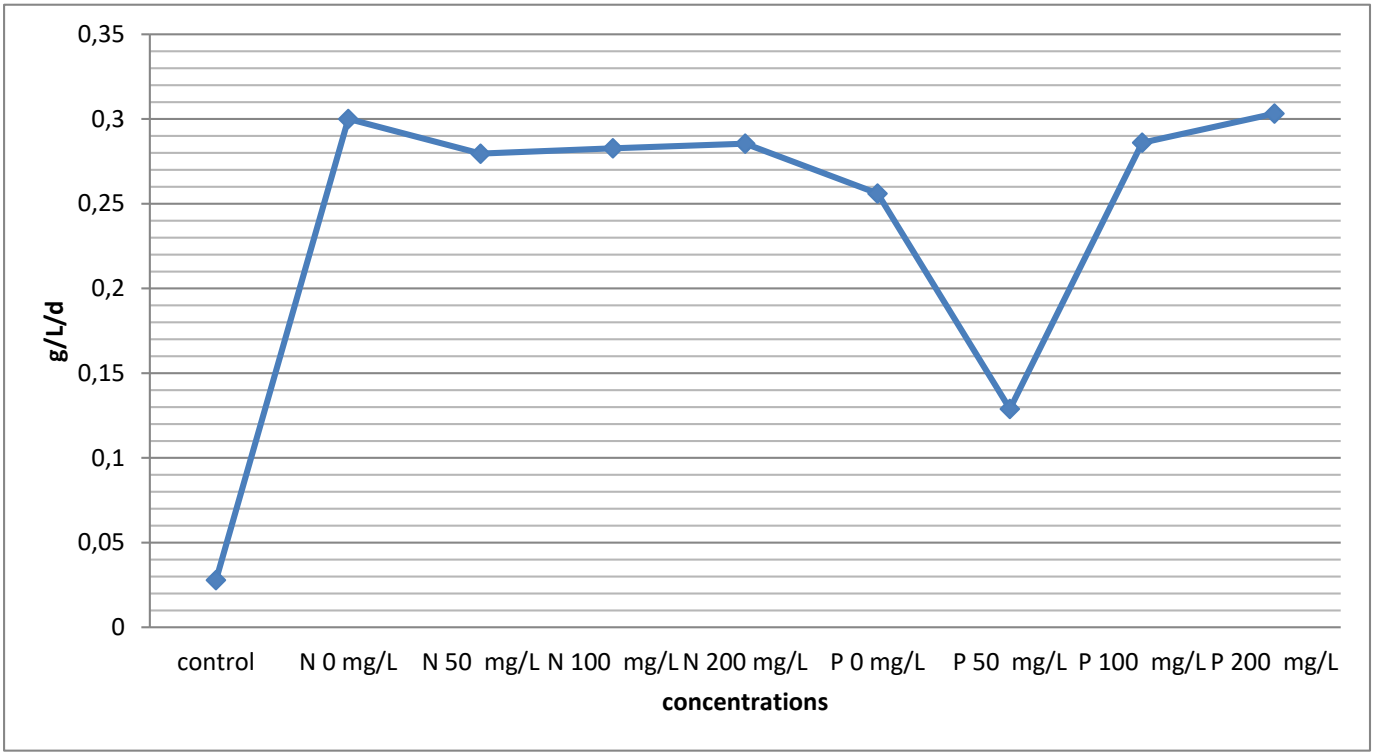


**Figure 2.** Carbohydrate content changes in g/L after the nutrient treatment

### *Total Carbohydrate Contents*

The effects of high concentrations of nutrient on the carbohydrate content and productivity of *C. vulgaris* under mixotrophic cultivation can be seen in Figure 2. Carbohydrate content for the control group were measured 0.0278 g $L^{-1}$ under mixotrophic culture conditions. Measurements for the carbohydrate content, for the 200 mg $L^{-1}$ concentration of nitrate and phosphate solution, showed that the high concentration of $NO_3^-$ and $PO_4^{3-}$ in the culture media causing an increase in carbohydrate, respectively. The average carbohydrate content for nitrate and phosphate treatment measured as 0.2869 and 0.2435 g $L^{-1}$, showing that these nutrients cause an increase on the increasing concentrations.

Carbohydrates are found as the intermediary reserves in some algae, due to the fact that they are required when the nitrogen becomes limited in the lipid synthesis (Kong et al., 2013). In the present study, when chlorophyll content in *C. vulgaris* increased, both lipid and carbohydrate content increased by nitrogen depletion. A common trend can be since, in which the carbohydrate content increased rapidly after the nitrogen source concentration decreased to the lowest level, which is consistent with previous findings showing that carbohydrate accumulation in microalgae is often triggered by nitrogen depletion (Orus et al., 1991; Kong et al., 2013). These results suggested that changes in the cellular biochemical composition were influenced by the trophic conditions and nutrient concentration in the medium.

### *Result of Lipid Analysis*

The measurements for the lipid content for the different nutrient concentration treatment showed that $NO_3^-$ and $PO_4^{3-}$ treatment causing an increase in lipid levels. The max. lipid content was 27.95 and 29.53 mg $L^{-1}$ under nitrate and phosphate treatment medium, respectively (Figure 3). Woertz et al. (2009) studied the lipid productivity and nutrient removal by green algae including *Scenedesmus, Chlorella* and *Glolenkinia* species grown during the wastewater treatment in batch cultures and reported that the maximum lipid content range was 14-29% and volumetric productivity of lipid was 17 mg/L/d. The highest lipid content (30.74 and 39.88 mg $L^{-1}$) occurred in mixotrophic cultivation when the culture was loaded with a high concentration of nitrate and phosphate (100-200 mg $L^{-1}$), higher than under autotrophic cultivation.

The lipid productivity obtained in the present work was not necessarily superior or inferior to those reported elsewhere using different strains of microalgae. For instance, Converti et al. (2009) and Woertz et al. (2009) reported that *C. vulgaris* growing in Bold's basal medium had somewhat higher production rates ranging from 8 to 20 mg/d/L and 17 to 24 mg/d/L, respectively. This suggests that in laboratory culture mode the lipid productivity in wastewater or simulated wastewater might be improved by continuous supplementation of nutrients such as nitrate or phosphate (Wang, 2012).

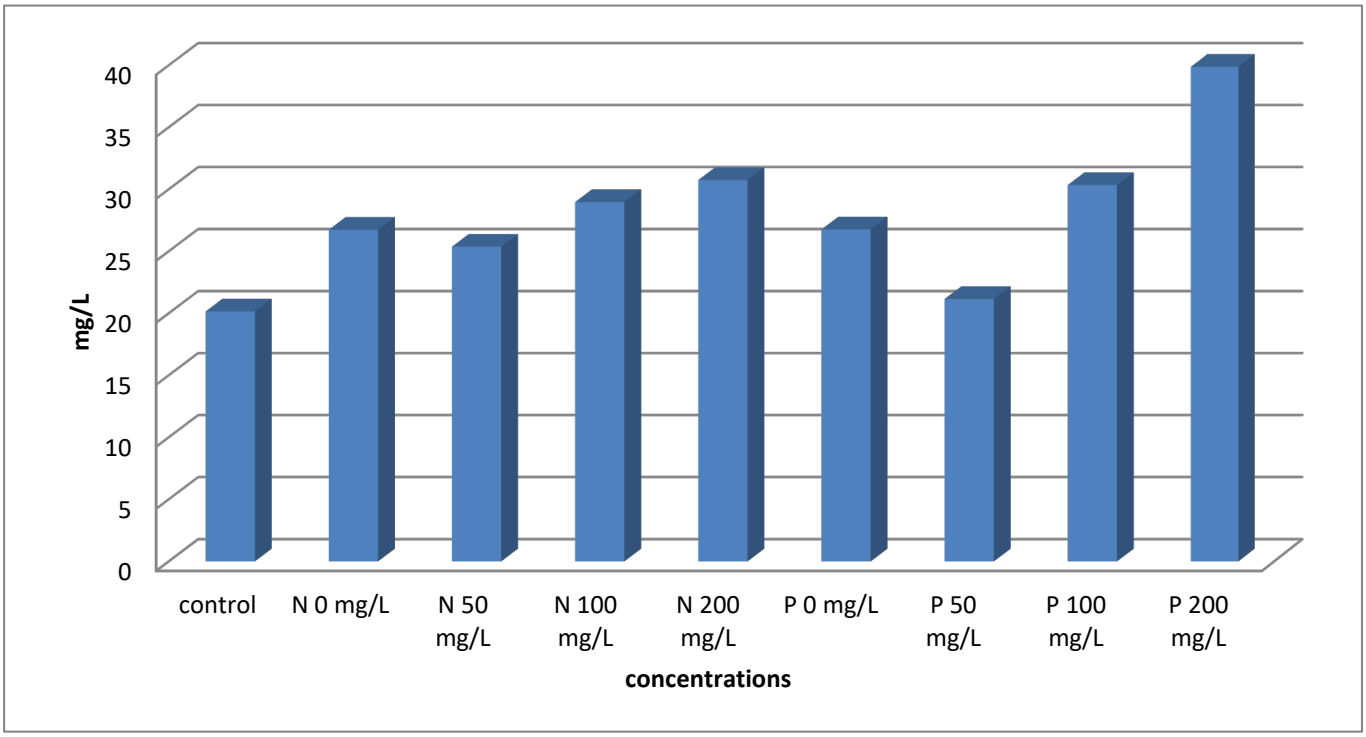


**Figure 3.** Lipid content changes in mg/L after the nutrient treatment

### *Nutrient Removal Efficiencies*

The removal amounts and removal efficiency of total nitrogen and phosphorus depending on the four different concentration of culture medium are presented in Figures 4 and 5. The min. and max. nitrate removal amounts and efficiency were 0.2302- 0.3584 mg $L^{-1}$ and 63.61- 99.05% under mixotrophic conditions, respectively. The results showed that the mixotrophic cultures experience an increase in nitrate uptake that was dependent on the increase of high concentration of nutrient in the medium content. The $NO_3^-$ uptake capacities was average 88.29%. It means that mixotrophic microalgae approximately consumed about 89% of the initial nitrate after 10 days to produce biomass.

Max. phosphate removal amount and efficiency were also high, as great as 0.2226 mg $L^{-1}$ and 63.77% in mixotrophic conditions compared to the other concentration of culture medium at 50 mg $L^{-1}$ nitrate concentration of nutrient in the medium content. Lowest phosphate removal capacity was observed in 100 and 200 mg $L^{-1}$ concentration of treatment. This might be to the fact that the organic carbon concentrations in this experiment were low compared to those in the reviewed literature de-Bashan et al. 2011.

Under the mixotrophic and optimum conditions (200 µmol photon $m^{-2}s^{-1}$ 16 h photoperiod and 28% inoculum size), 63.61-99.05% of $NO_3^-$ and 13.97-63.77% of $PO_4^{3-}$were successfully removed (Tab 2).

Mixotrophic cell cultivation utilizing both light and organic carbon source has been considered the most efficient process for the production of microalgal biomass (Lee et al., 1996). When the light energy used for $CO_2$ fixation is decreased in mixotrophic cultures, most of the energy is used for carbon assimilation. Therefore, since the amount of energy dissipated is minimal, mixotrophy provides higher energetic efficiency than other cultivation modes (Lalucat et al., 1984). On the other hand, Shi et al. (2000) reported that glucose can be considered the best organic C-substrate for the growth of *Chlorella.*

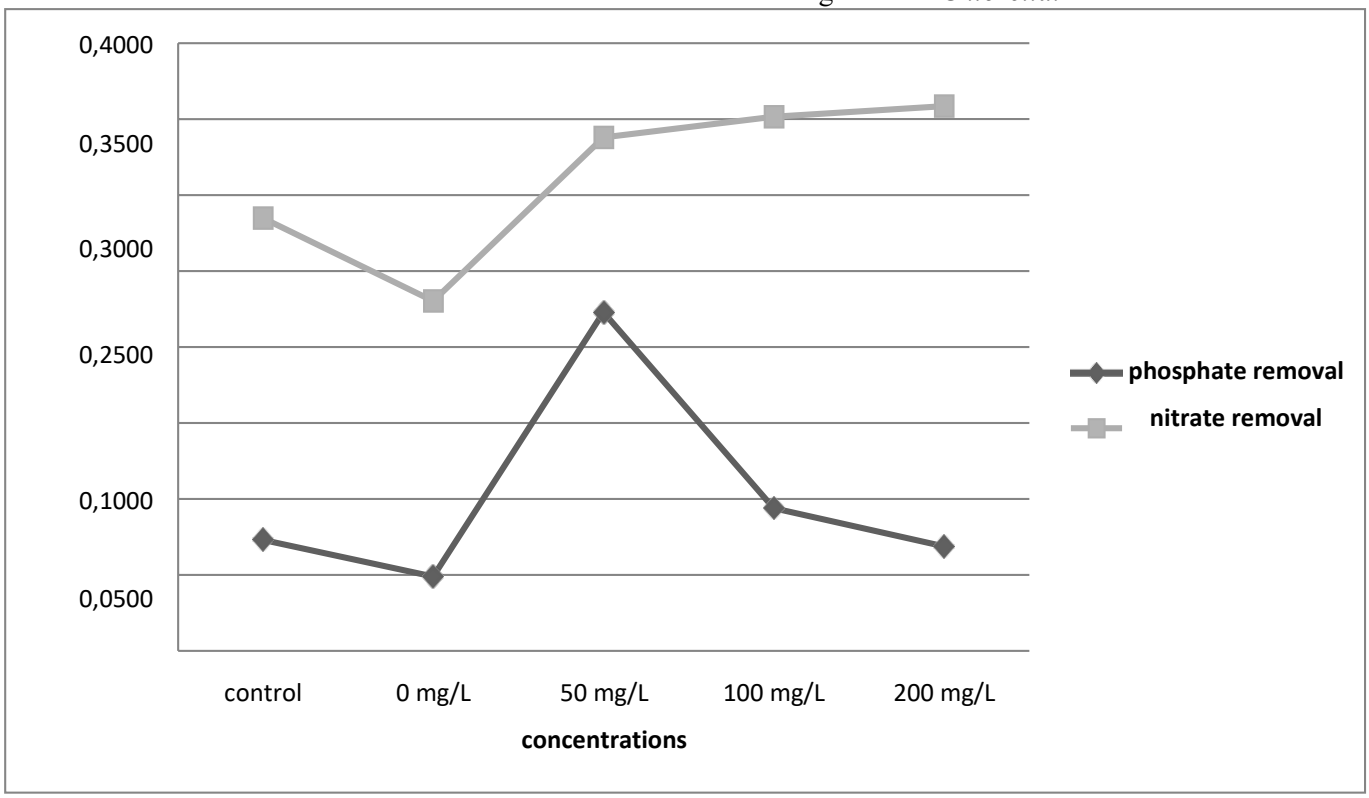


**Figure 4.** Nutrient removal levels measured for 0; 50; 100; 200 mg $L^{-1}$ and control values.

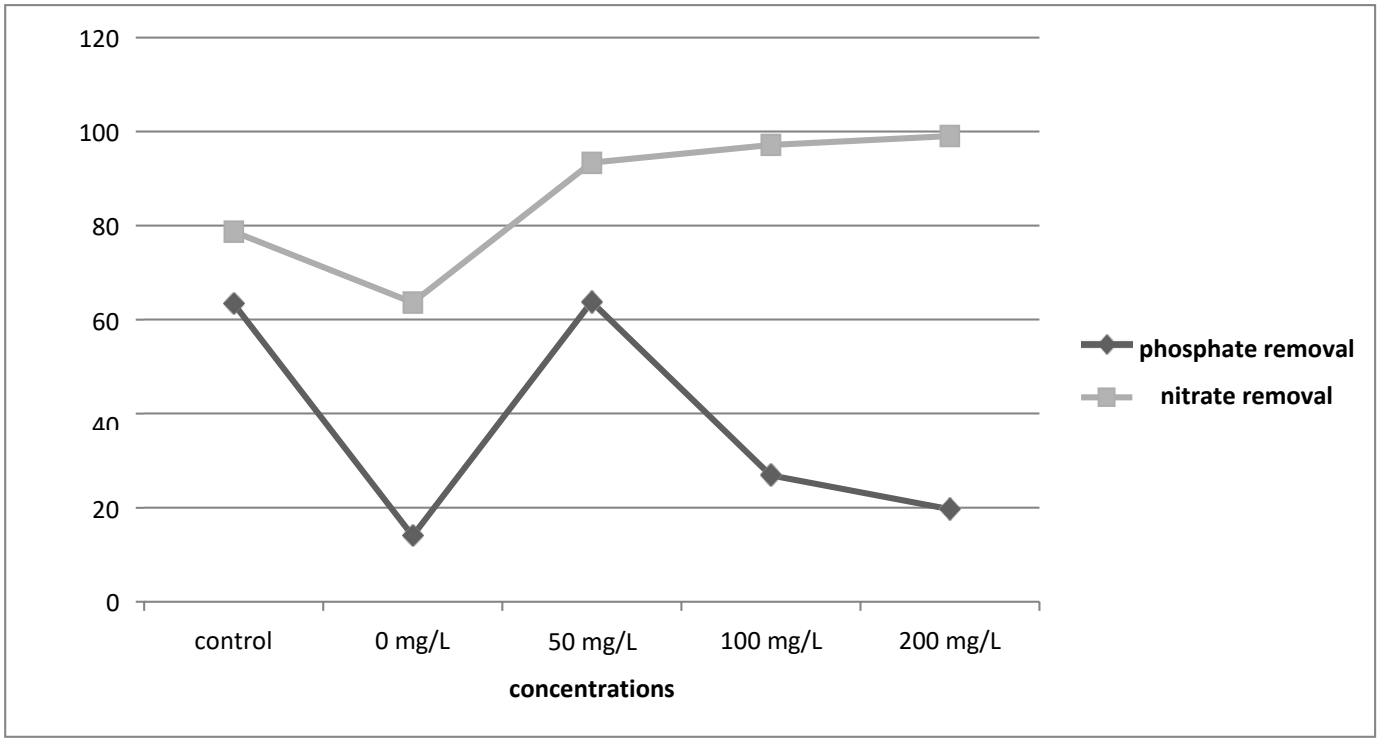


**Figure 5.** Nutrient removal efficiency of *C. vulgaris* under the mixotrophic conditions

Under the mixotrophic and optimum conditions (200 µmol photon $m^{-2}s^{-1}$ 16 h photoperiod and 28% inoculum size), 63.61-99.05% of $NO_3^-$ and 13.97-63.77% of $PO_4^{3-}$were successfully removed (Tab 2).

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## Conclusion

To conclude, this study describes the nutrient removal efficiency of *C. vulgaris* under the mixotrophic conditions while illustrating the effect of high concentration of nitrate and phosphate solution on carbohydrate, chlorophyll and carotenoid content as well as its relation between lipid synthesis levels. The findings from the study show that the uptake of nutrient with *C. vulgaris* green microalgae for excess nitrogen and phosphorus removal is effective. Generally, *C. vulgaris* removed more nutrients from mixotrophic medium than the control medium. Uptake of nitrate by the culture was the highest under mixotrophic conditions than the autotrophic conditions (control medium) at 0, 50, 100 or 200 mg $L^{-1}$ nutrient concentrations. Uptake of phosphate was higher under autotrophic conditions at 50 mg $L^{-1}$ nutrient concentrations. It was concluded that the mixotrophic regime, using glucose, is superior to autotrophic regime for the uptake of nitrate. The activity of *C. vulgaris* microalgae on practical aqueous solution nutrient removal will reduce drastically the concentration of excess nitrogen that will be discharged into the various compartments of the environment, and can even find use in agricultural farms as irrigation water.

**Table 2.** Standardized conditions (control) and under high concentration of nitrate and phosphate treatment from the 10th day of mixotrophic culture condition. Values are expressed as amount of substances in relation to the dry matter. Each value represents the mean of three replicates ± standard deviation.

| | Chl-a (µg/L) | Chl-b (µg/L) | Carotenoid (µg/L) | Carbohydrate (g/L) | Lipid (mg/L) | Adsorption capacities (mg/L) | Uptake efficiency (%) |
|---|---|---|---|---|---|---|---|
| **Control** | 0.6565±0.006 | 0.9883±0.005 | 0.0945±0.004 | 0.027±0.004 | 20.15±0.3 | 0.1788±0.001 | 71.04±0.1 |
| **N 0 mg/L** | 0.82181±0.005 | 1.1019±0.007 | 0.0444±0.004 | 0.3000±0.004 | 26.73±0.9 | 0.2302±0.002 | 63.61±0.4 |
| **N 50 mg/L** | 0.6408±0.002 | 2.1255±0.006 | 0.0689±0.006 | 0.2795±0.005 | 25.36±0.2 | 0.3379±0.005 | 93.35±0.1 |
| **N 100 mg/L** | 1.3348±0.005 | 2.2404±0.004 | 0.8437±0.006 | 0.2827±0.006 | 28.96±0.9 | 0.3515±0.005 | 97.13±0.3 |
| **N 200 mg/L** | 1.1393±0.004 | 1.4674±0.005 | 0.1974±0.004 | 0.2854±0.002 | 30.74±0.7 | 0.3585±0.002 | 99.05±0.3 |
| **P 0 mg/L** | 0.9163±0.006 | 1.9277±0.006 | 0.3396±0.001 | 0.001±0.001 | 26.75±0.9 | 0.0488±0.003 | 13.97±0.5 |
| **P 50 mg/L** | 0.9317±0.004 | 1.5525±0.002 | 0.5120±0.001 | 0.1288±0.002 | 21.15±0.9 | 0.2227±0.004 | 63.76±0.3 |
| **P 100 mg/L** | 0.5087±0.004 | 0.4285±0.003 | 0.2891±0.006 | 0.2860±0.001 | 30.34±0.8 | 0.0938±0.004 | 26.85±0.6 |
| **P 200 mg/L** | 0.1890±0.005 | 0.2589±0.003 | 0.2096±0.005 | 0.3032±0.004 | 39.88±0.4 | 0.0686±0.003 | 19.65±0.1 |

**Compliance with Ethical Standard**

**Conflict of interests:** The authors declare that for this article they have no actual, potential or perceived conflict of interests.

**Ethics committee approval:** There is no need ethics committee approval.

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