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Research Article

Combined effect of nitrogen and phosphorus on growth and biochemical composition of Tetradesmus obliquus (Turpin) M.J. Wynne

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Abstract: Microalgae have many biotechnological applications in various industries including food and feed, fertilizer, biofuel, cosmetics, pharmaceutics, and wastewater treatment. Since hey produce secondary metabolites under stress conditions such as pigments, carotenoids, hydrocarbons, and vitamins, investigating the effects of stress factors on growth parameters and biochemical composition of microalgal biomass is needed in producing bioproducts.

In this paper, the combined effects of nitrogen and phosphorus on growth and the protein/amino acid and Lipid-FAMEs profiles of microalgae *Tetradesmus obliquus* (MAKUMACC-037) were investigated.

Nitrogen and phosphorus deficiency reduced the algal growth. Biochemical composition was changed in a nitrogen and phosphorus dependent manner.

High concentration of protein and lipid were associated with increased nitrogen and phosphorus concentration However, the FAMEs profiles were changed depending on only the nitrogen concentration.

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1. INTRODUCTION

Microalgae are unicellular aquatic organisms, ranging in size from 1 µm to 2 mm. They are photoautotrophic microorganisms that produce their own food utilizing light, CO_2, and H_2O and thus primary producers in the aquatic system (Sirakov *et al.*, 2015; Maizatul *et al.*, 2017). Microalgae can be cultivated and can produce sufficient biomass for use in different biotechnological applications under optimum conditions. These microorganisms can survive in a diverse set of environmental habitats such as salt water, inland water, and wastewater. Their growth rates, biomass productivity, and biochemical compositions are affected by different cultivation conditions (Yaakob *et al.*, 2021).

Microalgal biomass is commonly used in food, feed, cosmetic, bioenergy, pharmaceutical, biofertilizer, and wastewater treatment sectors. Microalgae contain 28–70% of protein (dry biomass), 10–20% of lipids, and 10–50% of carbohydrates (Amaro *et al.*, 2011). Because of

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their high macromolecule rates, they are quite suitable for fish, shrimp, and livestock feed. Moreover, biomass can be used for human consumption with ease. FAO's report on potential food supplies and agricultural products emphasizes importance of finding alternative food sources (FAO, 2017). The high oil content of these organisms also makes them important for use in biofuel production. Also, they produce many other metabolites including pigments and secondary metabolites which are valuable in producing cosmetic and pharmaceutical components. (Zachleder *et al.*, 2014; Fernandes *et al.*, 2013).

Regarding the biochemical capacities of microalgae mentioned above, the following research question has been raised, "In which culture medium and conditions do they produce these valuable metabolites the most?" In order to answer this question, maximum biomass efficiency studies have been carried out by changing growth parameters such as nutrients, temperature, light intensity, and quantity (Zarrinmehr *et al.*, 2013). Since the most important ones among those are the nitrogen (N) and the phosphorus (P) concentrations of the culture media, studies mainly focus on their impact on growth (Xin *et al.*, 2010).

Nitrogen is the basic element for constructing cell structure and producing physiological molecules such as enzymes and hormones. On the other hand, phosphorus has importance in terms of the structure of membranes, RNA, DNA, and ATP. Additionally, the amounts and types of N and P have found to promote microalgal growth, and biomass productivity. Changing the N and the P amounts of the culture media stresses the algal cells and causes a modification in the biochemical composition (Beuckels *et al.*, 2015).

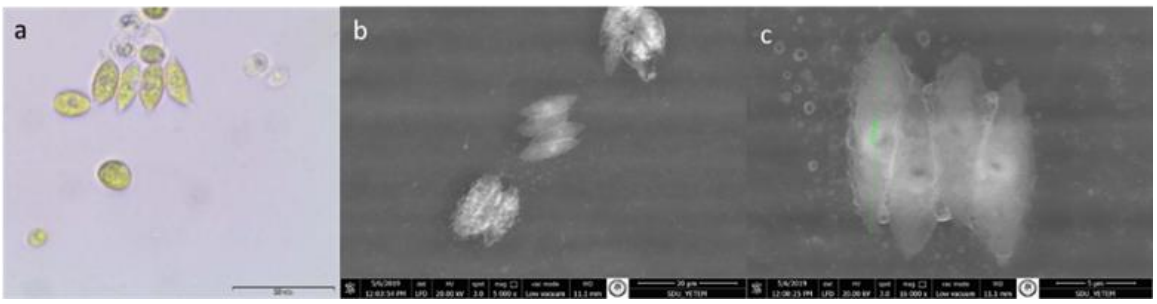
This study primarily focused on the combined effects of N and P on growth and biomass productivity of *Tetradesmus obliquus.* Secondly, the coupling effects of N and P on biochemical composition such as total protein, amino acid, lipid, and FAMEs (Fatty Acids Methyl Esters) of *T. obliquus* were addressed.

2. MATERIAL and METHODS

2.1. Algal Strain and Culture Conditions

In this study, *Tetradesmus obliquus* (MAKUMACC-037), which was isolated from the Ergene River Basin (Turkey) and deposited in the Mehmet Akif Ersoy University Microalgae Culture Collection (MAKUMACC) was used. It was characterized according to the ITS gene region as *Acutodesmus obliquus* (NCBI-KF470790) in 2014. Then this microalga was reclassified as *Tetradesmus obliquus* (Turpin) M. J. Wynne, so the current name is used in this paper (Wynne *et al.*, 2015). Figure 1 shows the light and scanning electron micrographs of the study material, *T. obliquus.*

Figure 1. Light and scanning electron micrographs of *T. obliquus*; Light micrograph (a), scale bar: 20 µm; general view (b), scale bar: 20 µm; length of a cell (c), scale bar: 5 µm.



To obtain the stock culture, BG11 medium (Rippka *et al.*, 1979) was used for culturing until the exponential phase under 16:8 h light: dark photoperiod (70 µmol $m^{-2}s^{-1}$) at 25±2°C and pH 7.2 as a first step. The culture was semi-continuously aerated (1 L/minute). The BG 11 medium was consisting of (in mgL^{-1} H_2O) $NaNO_3$, 1500; K_2HPO_4, 40; $MgSO_4.7H_2O$, 75; $CaCl_2.2H_2O$, 36; citric acid, 6; ferric ammonium citrate, 6; Na_2EDTA, 1; and Na_2CO_3, 20. 1 mL trace solution was added, its composition (in gL^{-1}) is H_3BO_3, 2.86; $MnCl_2$, 1.81; $ZnSO_4.7H_2O$, 0.222; $Na_2MoO_4.2H_2O$, 0.39; $CuSO_4.5H_2O$, 0.079; and $Co(NO_3)2.6H_2O$, 0.0494.

The second step of the study is harvesting and transferring microalgae into 3 different media that have been modified according to N and P concentrations as follows: BG11 (Control Medium); %50N+%50P+ (Medium I); %50N-%50P- (Medium II). [N+P+: contains more N and P than BG11; N-P-: contains less N and P than BG11]. The starter OD (Optical Density) value of all cultures is 0.09 and the cell number value is $3.6x10^5$ cells/mL. The cultures were grown under the same conditions as stock cultures.

2.2. Determinations of Culture Growth Parameters

Cell number (CN), optical density (OD), and dry weight (DW) values were used as culture growth parameters. The R^2 correlation values of these data were detected with Microsoft Office Excel 2007 (Microsoft, USA). The CN (cell mL^{-1}) was determined every other day by counting three replicate samples in a Thoma haemocytometer under a light microscope. To detect cell density, the OD at 680 nm was measured every other day by a UV/visible spectrophotometer (Shimadzu UV-1650) (Santos-Ballardo *et al.*, 2015). DW was determined according to Boussiba *et al.* (1992) where 10 mL of culture was filtered through a filter (Whatman GF/C, 1.2 lm, UK) and dried at 105°C for two hours and weighed.

All culture growth measurements were conducted to detect the stationary phase. After reaching the stationary phase the cultures were harvested to obtain dry biomass.

The specific growth rate (µ), Doubling Time (DT); Biomass productivity ($P_{Biomass}$) values were estimated according to these formulas.

$$\text{Specific growth rate } (\mu): \mu = \ln (x_2-x_1)/(t_2-t_1).$$

Where x_2 and x_1 are the cell density at t_2 and t_1, respectively (Chia *et al.*, 2013).

$$\text{Doubling Time (DT): } DT = 0.6931/\mu.$$ (Godoy-Hernández *et al.*, 2006).

Biomass productivity ($P_{Biomass}$): the dry biomass produced per day ($gL^{-1}day^{-1}$). It was calculated using $P_{Biomass}$ ($gL^{-1}day^{-1}$) = (X_2-X_1) x $(t_2-t_1)^{-1}$.

where X_1 and X_2 were the biomass concentrations (gL^{-1}) on days t_1 (starting point of cultivation) and t_2 (endpoint of cultivation), respectively (Hempel *et al.*, 2012). The results were converted to mg $L^{-1}day^{-1}$.

2.3. Determining the Protein-Amino acid and Lipid-FAMEs Profiles

To obtain the dried biomass, all the cultures were centrifuged after the stationary phase. Then the cultures were washed with distilled water, and dried in an oven at 40°C for 48 hours. Protein content was detected by using a CHNS elemental analyzer (Perkin- Elmer Model 2 400, USA) with the total N content. The crude protein contents of all groups were estimated following the equation below (Lopez *et al.*, 2010).

$$\text{Protein content} = \text{Nitrogen content} \times 6.25.$$

The results were expressed as a percent of dry weight. Amino acid compositions were detected by using the HPLC method (Köse *et al.*, 2011). The dried samples were hydrolyzed at 110°C for 24 hours with 6.0 M hydrochloric acid. Hydrolysates were filtered through a 0.20 µm PTFE syringe filter and then all the hydrochloric acid was evaporated. The levels of amino

acids were measured using EZ: fast kits (EZ: fast GC/FID Protein Hydrolysate Amino Acid Kit) by gas chromatography.

To detect the lipid amount, at least 100 mg of dry biomass was used (Bligh & Dyer (1959)). Profiles of FAMEs were analyzed with the method of Metcalf *et al.* (1966) through gas chromatography (Agilent 5975 C, Agilent 7890 A GC) with column DB WAX (50*0.20 mm, 0.20 μm).

2.4. Statistical analysis

All experiments were conducted with three replicates (n=3). The values were reported as the mean ± standard deviation (SD) of three replicates. Data were analyzed with ANOVA (one-way analysis of variance) in Minitab Statistical Software 2016 2 (Microsoft, USA). A difference at a significant level is at $p < 0.001$.

3. RESULTS

3.1. Combined Effect of Nitrogen and Phosphorus on Culture Growth of *T. obliquus*

For determining the culture growth of *T. obliquus*, cell number (CN), optical density (OD) and dry weight (DW) values were detected every other day under stable cultivation. A robust linear correlation was obtained between CN and OD measurements and OD and DW (Table 1).

Table 1. R^2 correlation values between CN-OD and OD-DW.

Media	CN-OD	OD-DW
BG 11 (Control Medium)	0.949	0.972
%50 N+ %50P+ (Medium I)	0.940	0.966
%50 N-%50P- (Medium II)	0.949	0.932

In the control medium (BG11) the highest CN ($2.4x10^7$ cell/mL); OD (1.05) and DW (0.041 mg/L) values are detected on the 24th, 26th, 28th days of culture, respectively (Figure 2, Figure 3, Figure4). At the same culture days; Medium II has the lowest CN ($0.8x10^7$ cell/mL); OD (0.73) and DW (0.029 mg/L) (Figure 2, Figure 3, Figure 4).

Figure 2. Cell numbers (CN) of *T. obliquus* in 3 media. The SD of three replicates for each media were shown with error bars ($n = 3$).

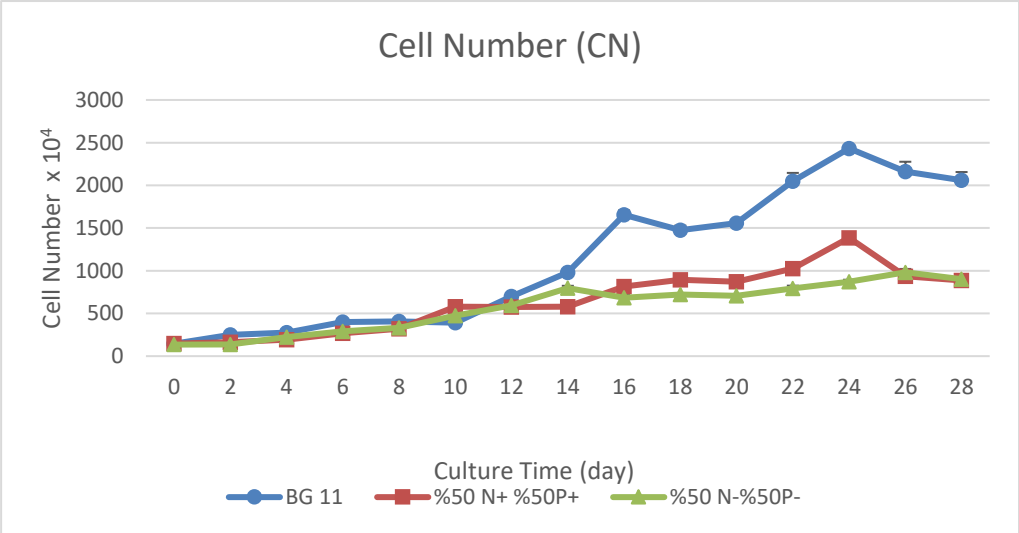


Figure 3. Optical Density (OD) of *T. obliquus* in 3 media. The SD of three replicates for each media were shown with error bars (n = 3).

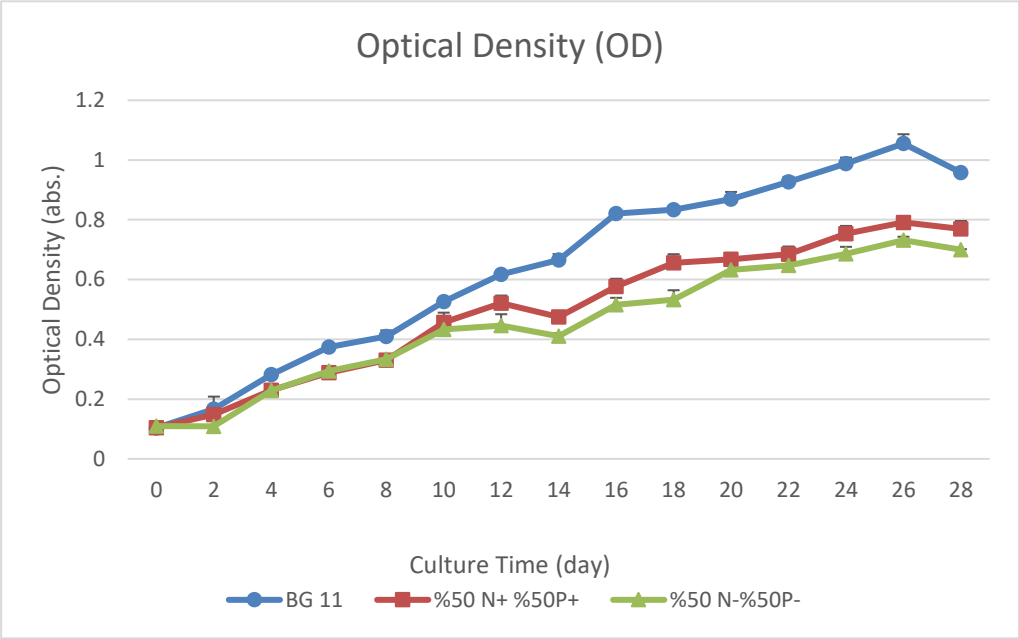
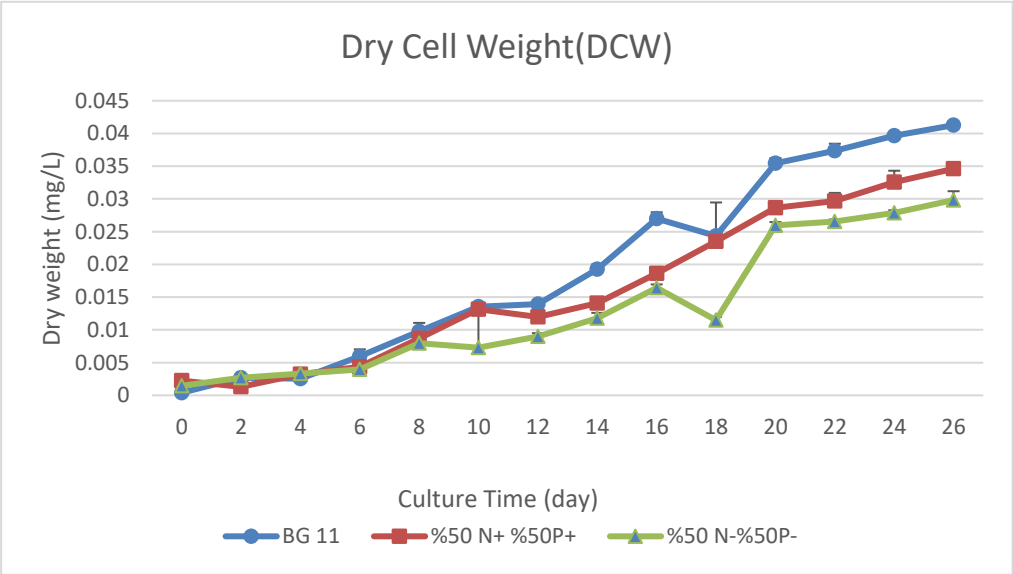


Figure 4. Dry cell weight (DCW) of *T. obliquus* in 3 media. The SD of three replicates for each media were shown with error bars (n = 3).



If Medium I and Medium II are compared between themselves; it is shown that Medium I has higher values than Medium II in all parameters. Same results can be seen in Table 2 which shows the Specific Growth Rate (SGR), Doubling Time (DT) and Biomass Productivity ($P_{Biomass}$) values. The highest SGR and $P_{Biomass}$ values are in the Control medium, Medium I and Medium II, respectively. Of course, there was an inverse order in the DT values, as expected.

Table 2. Culture growth parameters of *T. obliquus* in different media.

Media	SGR (μ)	Doubling Time (DT)	Biomass Productivity ($P_{Biomass}$)
BG 11 (Control Medium)	0.214±0.013	3.246±0.012	0.0008±0.008
%50 N+ %50P+(Medium I)	0.197±0.011	3.509±0.017	0.0007±0.005
%50 N-%50P-(Medium II)	0.183±0.010	3.781±0.015	0.0006±0.004

When all the results are evaluated together, it is seen that N or P deficiencies cause a decrease in culture growth. Many studies have stated that depletion of N in culture medium causes a decrease in growth (Vooren et al., 2012; Zhu et al., 2014; Ji et al., 2011). Anand & Arumugam researched the effects of N concentrations on the growth of *Scenedesmus quadricauda* and they declared that N enrichment causes high growth and N deficiency causes the opposite. So, it can be detected previously, N concentration of culture media is the principal factor in culture growth (Zhu et al., 2014; Breuer et al., 2012; Delgado et al., 2020; Procházková et al., 2013).

Also, P is another essential nutrient for microalgae growth, it can be used for the the synthesizing of cellular components such as components of the cell membrane, DNA, RNA and ATP (Atiku et al., 2016). The papers aim to research the effects of P concentrations on algal growth show that P deficiency causes a decrease in algal growth (Xin et al., 2010; Procházková et al., 2013; Lin et al., 2016).

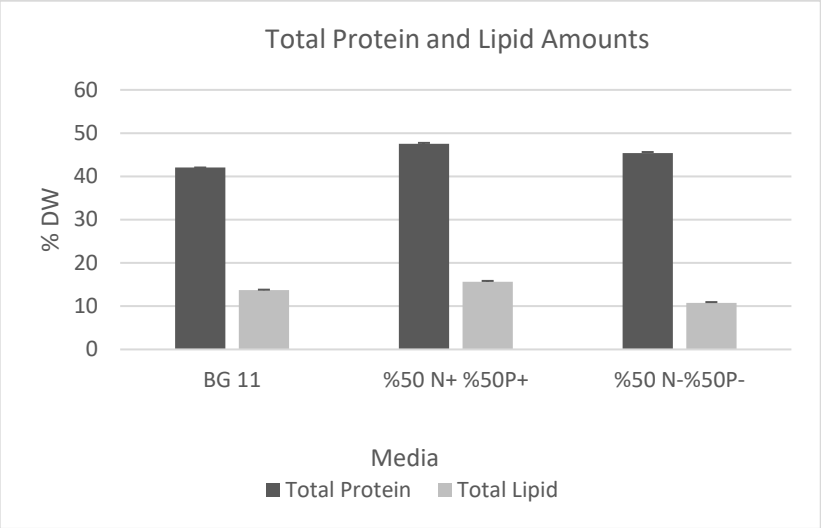
In addition, investigating the effects of N and P on culture growth separately, there are many studies investigating the combined effects of N and P (Singh et al., 2015; Zhuang et al., 2018). Huang et al., (2021) stated that the N-P coupling effect was significant on algal nutrient uptake, dry mass and pigments. Bongiovani et al. (2020) indicated that in their study about the effects of Nitrate or Phosphate Deprivation on *Nannochloropsis oceanica.*

3.2. Combined Effects of Nitrogen and Phosphorus on Protein-Aminoacids and Lipid-FAMEs Profiles of *T. obliquus*

N is the most important macronutrient for microalgal growth and an essential building molecule of proteins, carbohydrates and lipids (Yodsuwan et al., 2017; Zarrinmehr et al., 2019). N concentration affects the culture growth parameters as well as the biochemical composition of microalgal biomass (Vooren et al., 2012). Beside this, the biochemical composition of microalgae depends on the nutrient usability, type and concentrations of nutrients (Gao et al.,2018; Rani et al., 2020)

The biomass obtained from Medium I has the highest protein content (47.53 %DW) followed by Medium II (45.42 %DW), Control medium (42.03 %DW), respectively (Fig. 5). These results show the N and P are very important for synthesizing protein. So high proteins content was obtained in the medium that has high N and P concentrations. On the other hand, Medium II has higher protein content than Control Medium because of low growth rate of Medium II. Studies have shown that N deficiency generally pauses protein synthesis and shifts photosynthesis reactions towards lipid synthesis (Chu et al., 2014; Ho et al., 2014). However, our results contradict this. But these effects are very different than the mutual effect of N and P (Ji et al., 2013; Cao et al., 2014). So, the recent studies have focused on mutual effects of nutrients like ours.

Figure 5. Total protein and lipid amount of *T. obliquus* biomass harvested from 3 different media (p<0.001).



Total amino acid contents do not change according the modification of media, but amino acid composition and amount of amino acid types change (Table 3). In the Medium I the most abundant amino acid is Arginine (40.65 %DW) and least abundant amino acid is Phenylalanine (0.18 %DW). Whereas the control medium has different concentrations of Arginine (15.09 %DW) and Phenyl alanine (8.41 %DW). On the other hand, the highest amount of amino acid is Leucine (25.63 %DW) and lowest amount of amino acid is Lysine (0.1 %DW) in the Medium II. It can be understanding due to these results that N and P concentrations change the compositions and amounts of amino acids strongly. Total aa amounts of *T. obliquus* are the highest in control medium and lowest in Medium II. These results are not surprised because of N starvation causes to reduce the amino acid contents in the microalgae cells (Salbitani et al., 2020).

Table 3. Amino acids composition and amounts (mgg^{-1} DW) of *T. obliquus* biomass harvested from 3 different media.

	BG 11 (Control Medium)	%50 N+ %50P+ (Medium I)	%50 N-%50P- (Medium II)
Arginine	15.09±0.11	40.65±0.11	6.859±0.10
Serine	2.048±0.09	0.759±0.05	1.367±0.09
Glycine	1.057±0.08	1.435±0.06	0.537±0.07
Alanine	1.082±0.04	0.454±0.04	0.724±0.04
Proline	2.435±0.09	0.638±0.09	1.315±0.03
Valine	8.982±0.08	2.159±0.10	1.534±0.05
Threonine	2.209±0.07	0.997±0.09	4.128±0.01
Methionine	4.214±0.05	0.975±0.04	1.893±0.06
Isoleucine	1.646±0.06	0.788±0.03	1.551±0.04
Leucine	2.611±0.08	0.628±0.04	25.63±0.02
Phenylalanine	8.415±0.12	0.18±0.05	3.795±0.03
Tyrosine	3.301±0.11	0.198±0.07	4.909±0.10
Aspartic acid	3.61±0.13	4.331±0.08	0.725±0.11
Glutamic acid	3.799±0.14	1.929±0.09	0.674±0.12
Histidine	1.009±0.06	1.785±0.10	0.148±0.13
Lysine	1.005±0.06	4.24±0.02	0.162±0.11

Data were expressed as mean ± *SD*. n=3. p<0.001.

Also, some researchers stated that the types of N sources are affecting the assimilation of N by microalgae cells and synthesizing of amino acids (Imamura et al., 2010). Since this situation directly affects the synthesis of photosynthetic pigments and enzymes, changes occur in the metabolic pathway (Vona et.al.,1999; Mark et al., 2007; Hockin et al., 2012).

Our lipid analysis results (Fig. 5) show that Medium I (oversupplied both N and P) have the highest lipid content (15.6 %DW), Control medium (13.71 %DW), Medium II (10.72 %DW); respectively. Synthesizing the lipids and depositing the FAME are regarding to supplying of both N and P, directly. Many studies declared that when the culture medium has the sufficient N amount, carbohydrate accumulation shifts to depositing of lipid and protein because of specific metabolic pathway of microalgae (Li et al., 2019; Ross et al., 2018).

In N deprived conditions, high lipid accumulations were reported in *Scenedesmus* species (Radakovits et al., 2010). Besides, these same inferences were declared for *Neochloris oleobundans* and *Nannochloropsis* sp. (Courchesne et al., 2009; Gao et al., 2013). However, our results conflict to these papers since The Medium II (N and P depletion) had the lowest lipid content. The effects of N or P's deficiency separately are decreasing the culture's growth and increasing the lipid accumulation, however we researched the combined effects of N and P, so our results may be different from the general trend. The impact of our study can be understandable with this output.

According to our FAMEs results (Table 4) the highest Palmitic acid (C16:0) concentration (21.11 %FAME) is detected in Medium II and the highest total SFAs concentration (24.22 %FAME) in same medium. In every case, many studies declared that the N starvation causes producing higher SFAs. (Zhu et al., 2014; Yodsuwan et al., 2017; Breuer et al., 2012; Anand et al., 2015; Singh et al., 2015; Cointet at al., 2019).

Table 4. FAMEs percentages (% of total fatty acids) of *T. obliquus* biomass harvested from 3 different media.

Name of Fatty Acids	BG 11 (Control Medium)	%50 N+ %50P+ (Medium I)	%50 N-%50P- (Medium II)
Saturated fatty acids (SFAs)			
C14:0 (myristic acid)	2.208±0.02	2.349±0.01	2.097±0.05
C16:0 (palmitic acid)	19.342±0.01	19.211±0.01	21.111±0.01
C18:0 (stearic acid)	1.073±0.02	1.142±0.01	1.019±0.01
Total SFAs	22.623±0.03	22.702±0.01	24.227±0.01
Monounsaturated fatty acids (MUFAs)			
C16:1 (9-hexenoic acid)	2.156±0.05	2.294±0.04	2.048±0.05
C18:1 (oleic acid)	12.513±0.03	13.316±0.02	12.879±0.01
C18:1 t10, t11e 12	4.498±0.04	4.786±0.01	4.273±0.01
Total MUFAs	19.167±0.05	20.396±0.01	19.200±0.03
Polyunsaturated fatty acids (PUFAs)			
C18:2 (linoleic acid, c9 c12)	12.348±0.04	11.589±0.02	11.732±0.01
C18:3 (linolenic acid, c9, c12, c15)	36.291±0.03	36.217 ±0.01	35.582±0.02
Total PUFAs	48.639±0.02	47.806±0.04	47.314±0.04
Others	7.789±0.04	8.291±0.04	7.401±0.03

Data were expressed as mean ± SD. n=3; p<0.001.

Results of the total MUFAs values show that the highest MUFAs concentration (20.39; 19.20; 19.16 %FAME) were in the Medium I, Medium II and Control medium respectively.

Microalgae can deposit high levels of SFAs and MUFAs with a low growth rate (Ji et al., 2011). Our results were compatible with SFAs but not with MUFAs.

Control medium has higher PUFAs concentrations (48.63 %FAME) than Medium I (47.80 %FAME) and Medium II (47.31 %FAME). When comparing of the growth rate and PUFAs concentrations, the results were similar to study of Pribly et al., (2014) which declared the high growth rate causing high PUFAs concentration.

4. CONCLUSION

The aim of this study is to give an idea of the combined effects of N and P in microalgae culture growth parameters and protein-amino acid; lipid-FAMEs profiles of *T obliquus*. Microalgae has high growth rates in different culture conditions and their biomasses might be an alternative source for bioproducts because of their secondary metabolites contents in the cells. Also, microalgae can be used as an alternative sustainable green energy source due to their high lipid content. In the same time, the culture media with high amount of N and P accelerate the culture growth and nutrient deficient cultures are more suitable for lipid and fatty acids production. However, physical factors such as light quantity and intensity, temperature, pH should be evaluated together with nutrient concentrations. This phenomenon is known well but the reactions of different microalgae with same conditions may change. The species variety is as important as cultural conditions. Therefore, the number of studies conducted for this purpose should be increased and studies with different species should be brought to the literature.

For that purpose, *T. obliquus,* on which the combined effects of N and P had not been investigated before, was chosen as the study material. All data which obtained from this study and the studies should be used in the future to commercialize algae for better usage in biotechnological sustainable applications.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship contribution statement

Fusun Akgul: Investigation, Resources, Visualization, Software, Formal Analysis, and Writing - original draft. **Riza Akgul:** Investigation, Methodology, Supervision, and Validation.

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