

Phenotypic Plasticity, Growth, Biochemical Composition, Total Phenolic Content, and Antioxidant Activity of Microalga *Desmodesmus multivariabilis* Under Different Nitrogen and Phosphorus Concentrations



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F. Akgül^{a,*} and S. Kösel^b

^aBurdur Mehmet Akif Ersoy University, Faculty of Science and Arts, Department of Molecular Biology and Genetic, Burdur, Turkey

^bBurdur Mehmet Akif Ersoy University, Institute of Science, Burdur, Turkey

doi: <https://doi.org/10.15255/CABEQ.2023.2199>

Original scientific paper
Received: March 15, 2023
Accepted: July 25, 2023

The use of microalgae in biotechnological studies is increasing day by day because of their ability to produce and store secondary metabolites under extreme conditions. When the culture conditions such as light, nutrients, pH, and temperature are modified, the synthesis and storage amount of these secondary metabolites change. In this study, the effects of different nitrogen and phosphorus concentrations on the morphological changes, growth, biochemical composition, total phenolic content, and antioxidant activity of *Desmodesmus multivariabilis* were investigated.

The study results show that changing of the nitrogen and phosphorus concentration in the microalgae culture medium is an effective strategy for changing growth, biochemical composition, total phenolic content, and antioxidant activity parameters of microalgae. All culture conditions should be detected, depending on the substance desired to be obtained from microalga and its biotechnological application. Therefore, this study is substantial as it is the first study on this concept with *D. multivariabilis*, and for obtaining economically important bioactive compounds of this species, this paper will play a key role.

Keywords

DPPH, Fatty Acid Methyl Esters, lipid, microalgae, morphological changes, nitrogen

Introduction

Algae are prokaryotic or eukaryotic primary producers that live in a wide variety of habitats from aquatic to terrestrial environments and make important contributions to the food chain with photosynthesis¹. In addition to being the basis of the aquatic food chain, they are the oxygen generators of the ecosystem, producing approximately 70 % of the oxygen in the atmosphere². The use of biological materials in technology has increased which emphasize the importance of their sustainable use in biotechnology³. Microalgae can produce bioactive molecules that can be used to produce materials with economic value in various industrial areas, including cosmetics, agriculture, the pharmaceutical industry, food and feed, and wastewater treatment⁴. For this reason, studies on microalgal production and microalgae biotechnology are increasing daily.

Microalgae can tolerate various temperatures, salt concentrations, pH values, and light intensities⁵.

This adaptation process has gained them the ability to produce and store secondary metabolites to protect them from adverse environmental conditions. While culturing microalgae, optimum growing conditions can be used as well as some stress conditions. These stress conditions seriously affect the biochemical composition of microalgae, the growth rate of the culture, and the secondary metabolite production rate⁶. The main stress parameters affecting microalgae cultivation include factors such as nutrients (N, P, etc.), light, pH, and salinity⁷, applied differently in different applications.

Nitrogen (N) is an important building block of various biomolecules such as proteins, nucleic acids, and chlorophyll. Nitrogen content in the microalgae culture medium strongly influences the specific growth rate and biochemical composition of microalgae. In addition, nitrogen affects lipid metabolism, fatty acid, and amino acid composition⁸. The nutrient salt concentration also affects the antioxidant activity of microalgae⁹.

Phosphorus (P) is an essential nutrient that affects primary production chains in aquatic habitats

*Corresponding author: E-mail: fakgul@mehmetakif.edu.tr; tel.: 00 90 248 2133075

and is essential for all biological metabolic activities. It is a constructive-regulatory element in the structure of cell membranes, nucleic acids, enzymes, hormones, basic energy molecules, ATP and GTP. Phosphorus, the second limiting element after nitrogen for microalgae¹⁰, also has significant effect on microalgae growth and metabolism¹¹. In its deficiency, there is a change in the biochemical composition of the cells and the synthesis and accumulation of neutral lipid increases.

Different species of *Desmodesmus* sp. are frequently used in algal biotechnology studies, but there are very few studies using *Desmodesmus multivariabilis*. A few recent studies of this species investigated the bioremediation potential of nitrogen, phosphorus, and sulfur in wastewater under mixotrophic conditions¹², and the potential of obtaining optimum biomass and fatty acid methyl esters in low carbon wastewater¹³, as well as examined culture growth in the presence of solid calcium carbonate¹⁴.

In this study, *D. multivariabilis* was cultured under different nitrogen (0.98 mmol L⁻¹; 2.94 mmol L⁻¹ (BBM); 8.82 mmol L⁻¹) and phosphorus concentrations (0.573 mmol L⁻¹; 1.72 mmol L⁻¹ (BBM); 5.16 mmol L⁻¹) for 24 days, and culture growth parameters such as cell number (CN), optical density (OD), dry weight (DW) and, chlorophyll-a (Chl-a) were monitored. The biochemical composition, antioxidant activity, and total phenolic content of the microalgae cultured in each modified culture were investigated. This species has never been used for the purpose of this study, and in this respect, it is the first in this study field.

Materials and methods

Microalgae strain and culture conditions

The microalga *Desmodesmus multivariabilis* used in this study was isolated from a pond of Bolayır (40°30'57.6"N, 26°46'48.4"E), Çanakkale, Turkey. Micrographs of the microalgae (Fig. 1 and Fig. 2) were obtained with a light microscope (Euromex OX.3035, Holland) and scanning electron microscope (FEI QUANTA FEG 250, Netherlands). The microalga was deposited in Burdur Mehmet Akif Ersoy University Microalgae Culture Collection (MAKUMACC) with strain number 43.

It was identified morphologically as *Acutodesmus deserticola*¹⁵. Accuracy of morphological identification has been researched by 18S rRNA gene sequence analysis. The universal eukaryotic primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used to amplify. The obtained 559 bp gene sequence was analyzed using the BLASTn (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) search algorithm and aligned to the neighbors. This microalga was identified as *Desmodesmus multivariabilis* (NCBI Accession number OK642362.1) with 99 % similarity^{16,17}.

The isolate was cultured in Bold Basal Medium¹⁸ (BBM) and pH adjusted to 8.0.

Each sterile culture medium was transferred into flasks in 5 L volumes, and inoculation was made in the same number of cells (app. 5.5·10⁶) from the stock culture. 5 L inoculated liquid cultures were kept in a climate cabinet at 24 ± 4 °C,

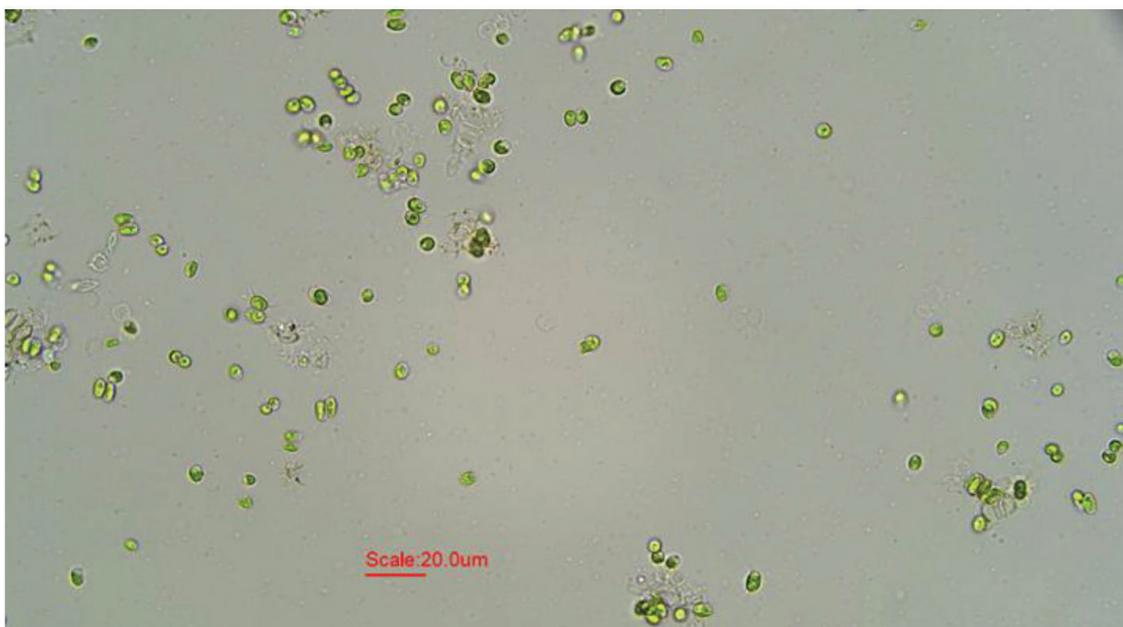


Fig. 1 – Light micrographs (40X) of *D. multivariabilis* in BBM (Scale bar: 20.0 μm)

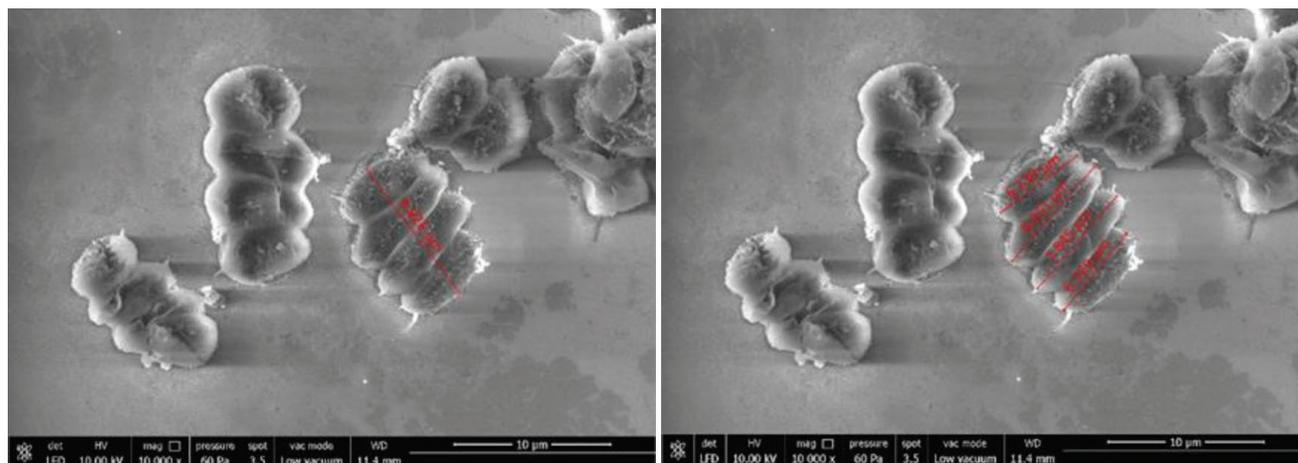


Fig. 2 – Scanning electron micrographs of *D. multivariabilis* (Scale bar: 10 μm)

Table 1 – Nitrogen and phosphorus concentration and N:P ratios of 5 different media

Culture media	N concentration (mmol L ⁻¹)	P concentration (mmol L ⁻¹)	N:P
BBM	2.94	1.72	1.7
N ⁺	8.82	1.72	5.12
N ⁻	0.98	1.72	0.57
P ⁺	2.94	5.16	0.57
P ⁻	2.94	0.573	5.13

BBM: Control group; N⁺: nitrogen excess; P⁺: phosphorus excess; N⁻: nitrogen limitation; P⁻: phosphorus limitation.

200 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ light intensity and 12/12 (light/dark) photoperiod. The mixing and aeration process was done with air pumps (glass pipets) with 500 mL min⁻¹ aeration capacity.

Experimental design

The experiments detected and compared the algal growth parameters, biochemical composition, and DPPH (diphenyl-1-picrylhydrazyl) scavenging activity of the *D. multivariabilis* under different nitrogen and phosphorus concentrations. For this purpose, algae in Bold Basal Medium (BBM) was chosen as the control group. The modified media used in the study and the N, P concentrations and N:P ratios of media are shown in Table 1.

Detection of culture growth parameters

Cell number (10⁴ cells per mL) was determined by direct counting with a Thoma hemocytometer every 3 days during the culture period (24 days). The data obtained was used to calculate the specific growth rate and doubling time.

Specific growth rate (μ (d⁻¹)); doubling time (t_d), and biomass productivity (BP) values were calculated as follows¹⁹:

$$\text{Specific growth rate } (\mu) = \ln(N_t/N_0)/(t_t - t_0) \quad (1)$$

where N_t is the number of cells at the end of log phase, N_0 is the number of cells at the start of log phase, t_t is the final day of log phase, and t_0 is the first day of the log phase.

$$\text{Doubling time } (t_d) = 0.6931/\mu \quad (2)$$

$$\text{BP (g L}^{-1} \text{d}^{-1}) = (X_2 - X_1)/(t_2 - t_1) \quad (3)$$

where X_1 and X_2 are the DW value (g L⁻¹) on days t_1 (starting point of cultivation), and t_2 (end point of cultivation), respectively.

Optical density (OD) was used to determine the cell density. Simultaneously with cell counts, spectrophotometric measurements (Shimadzu UV-1650) were performed at 680 nm to determine the OD. A correlation was established between the cell numbers and the optical density²⁰.

To detect the dry weight (DW) (mg L⁻¹) on the same days when the cell number and optical density were determined, 10-mL samples were taken from the homogeneity cultures, filtered with filter paper (Macherey Nagel, 640 d Ø 125 mm), and dried in the oven at 40 °C, and determined by the following equation²¹.

$$\text{Dry weight} = \text{final weight of the filter} - \text{start weight of the filter (mg L}^{-1}) \quad (4)$$

Chlorophyll a (Chl-a) was extracted in 99 % methanol and measured simultaneously with the cell number, and optical density was determined to use the equations of Lichtenthaler²².

Biochemical compositions analysis

Nitrogen content was analyzed by Dumas combustion²³ method. The crude protein content of microalgae was estimated using (nitrogen · 6.25) as a conversion factor²⁴. To detect the amino acid profile, the HPLC (Shimadzu Prominence) method was used with some modifications²⁵. To detect the crude lipid content of microalgae, the Soxhlet extraction

method was used²⁶. The profile of fatty acid methyl esters was analyzed using the gas chromatography (AGILENT 5975 C, AGILENT 7890A GC) equipped with column DB WAX (50_0.20 mm, 0.20 mm). The application conditions were determined according to Secilmis and Bardakci²⁷.

Microalgae biomass was extracted and purified according to the method described by Cortesi *et al.*²⁸ The qualitative and quantitative evaluations of phenolic compounds were performed by analyzing phenolic extracts with HPLC using gallic acid as a standard. The HPLC system consists of a Beckman chromatograph equipped with 250x4.6 mm C18 Ultrasphere-Ocs column; eluents were detected in 278 nm. The mobile phase used is 2 % acetic acid against (B) methanol in water²⁸.

DPPH scavenging activity

DPPH radical scavenging activity of samples was determined using ascorbic acid as positive control³⁰. Different concentrations (12.5–800 µg mL⁻¹) methanol extracts of microalgae biomass were prepared. 800 µL sample was added to 200 µL 0.1 mM DPPH solution and vortexed for 1 min, then left at room temperature for 30 minutes in the dark. The absorbance of all the sample solutions was measured at 517 nm (BioTek Epoch, USA). The scavenging activities (%) were determined by the following formula³¹.

$$\text{Scavenging activity (\%)} = [(A_0 - A_{\text{sample}})/A_0] \cdot 100$$

where A_0 is the absorbance of the DPPH+methanol, A_{sample} is the absorbance of the sample+methanol+DPPH.

Statistical analysis

All experiment groups were replicated three times. The reported values are the mean ± standard deviation of three values ($P < 0.05$). Data were analyzed using one-way analysis of variance (ANOVA) using Minitab Statistical Software (2016) (Microsoft, USA).

Results and discussion

Microscopic observations

While cell counting of microalgae cultured in different media was performed every other day, detailed microscopic observations were also made (Fig. 3; Table 2). *Desmodesmus* genus, a member of *Scenedesmeacea*, is a pleomorphic strain, which can change the number of cells and coenobium under different environmental factors such as nutrient concentration, light intensity, photoperiod etc³².

In this study, 1–2 celled colony, coenobium had more smaller cells, long and prominent spines, and parietal chloroplast in whole cell were observed in N– medium. Beside this, P limitation caused observing 2–4 celled coenobia and more larger cells.

Consistent with these results; Pancha *et al.* detected that N limitation causes changes in the cell number of colony, 2–4 celled coenobia and an elongation of the spines of *Scenedesmus* strain³³. These results are compatible with Gavis *et al.*, who observed that 1–2 celled coenobia are present in conditions of nitrogen or phosphorus excess, while in contrast, N and P limitation stimulates colony formation. In addition, they show that cell sizes are increasing with N limitation³⁴. In contrast to our results, larger cells were observed in P– medium. This was detected in the study with *Desmodesmus* sp., where the number and length of these spines also decreased with N limitation. These authors also observed disintegration of coenobia of 2 or 4 cells³⁵.

The microalgae cells under N stress conditions, reuses N-rich compounds such as chlorophyll-a for more effective photosynthesis and for accumulating energizing biomolecules. This showed that lipid is the first produced and stored in the plastids under nutrient limitation conditions³⁶. Therefore, the chloroplasts of cells in the N– medium were parietal and filled in the whole cell. These observations are compatible with our results. Additionally, in some studies nutrient limitation caused degradation of chloroplast and photosystem damage³⁷; however, this situation was shown in the cells of our P– medium.

In our study, N and P limitation caused phenotypic changes. These morphological changes are the results of “phenotypic plasticity” that occurs in response to unfavorable environmental conditions in microalgae³⁸. This situation was also observed in our samples, *D. multivariabilis* cells showed morphological changes as protection from adverse environmental conditions (Fig. 3; Table 2).

Culture growth parameters

Cell number values

Cell number was determined by taking 2-mL samples from each culture every 3 days during the 24-day cultivation period. The maximum cell number value was detected in BBM medium as $3775 \cdot 10^4$ cell per mL at 21st day of culture (Fig. 4). The top growth acceleration of culture was shown in N– medium; in any case, this medium had the highest SGR value ($\mu = 0.44 \text{ d}^{-1}$) (Table 3). However, N– medium began to reduce the cell number values earlier than others like P– medium. N– ($2645 \cdot 10^4$ cell per mL) and P– ($2250 \cdot 10^4$ cell per mL) media

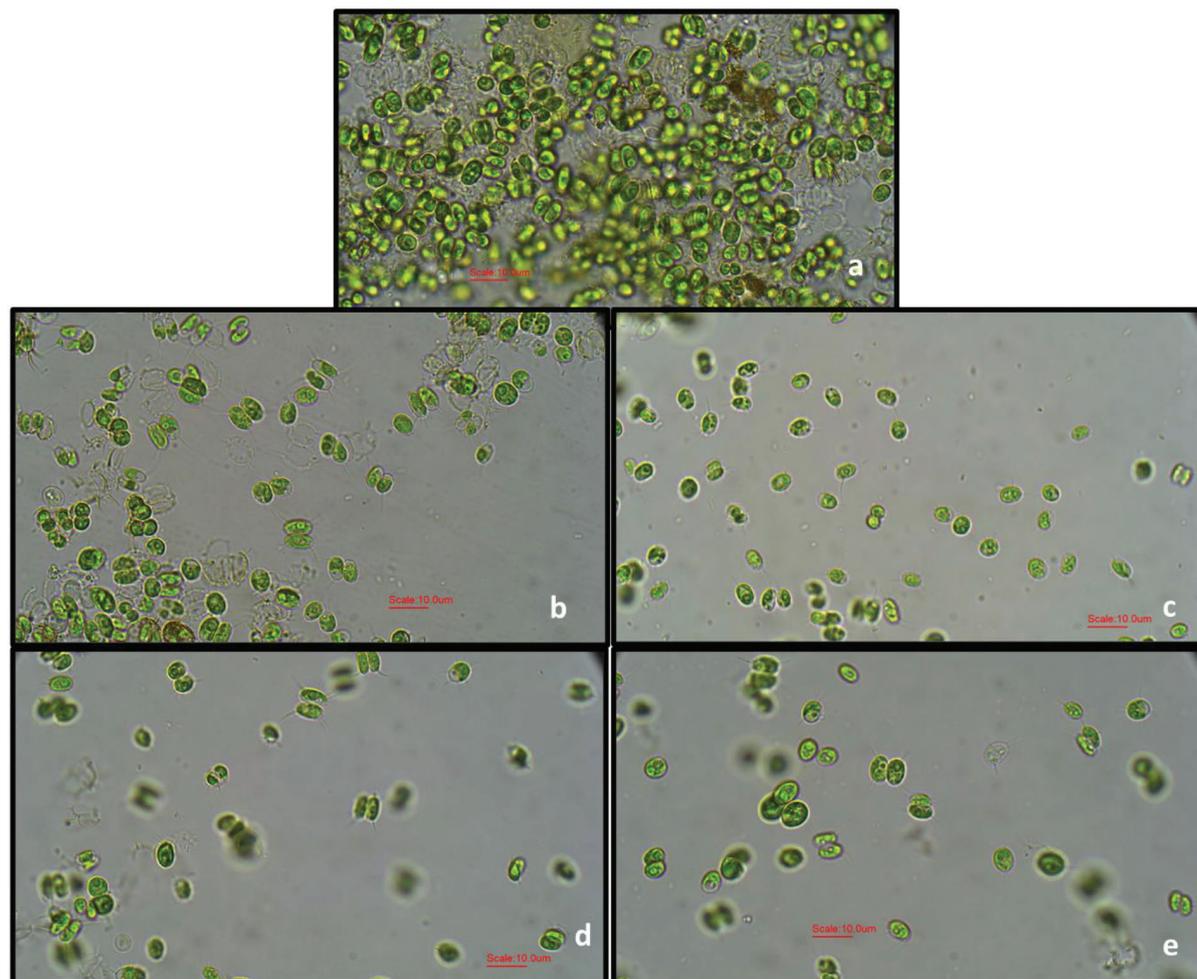


Fig. 3 – Light micrographs (100X) of *D. multivariabilis* in five different culture media. a) in BBM medium; b) in N^+ medium; c) in N^- medium; d) in P^+ medium; e) in P^- medium (Scale bar: 10 μ m, and cultivation time: 24 days; BBM: Control group; N^+ : nitrogen excess; P^+ : phosphorus excess; N^- : nitrogen limitation; P^- : phosphorus limitation).

Table 2 – Morphological characteristics of *D. multivariabilis* microalgae cells and colonies in 5 different culture media

Media	Number of cells per coenobium	Chloroplast shape and structure	Presence of spine	Cell size ¹⁵
BBM	1, 2 and 4	Reticular and does not fill the whole cell.	Rarely occur, often absent.	Average size
N^+	2 and 4	Parietal and fills the whole cell.	Present in almost all colonies.	Average size
N^-	1 and 2	Parietal and fills the whole cell.	Present and prominent in all colonies.	Smaller
P^+	1 and 2	Reticular and does not fill the whole cell.	Present in almost all colonies.	Average size
P^-	2 and 4	Parietal and fills the whole cell.	Present in almost all colonies.	Larger

BBM: Control group; N^+ : nitrogen excess; P^+ : phosphorus excess; N^- : nitrogen limitation; P^- : phosphorus limitation.

reached to the maximum cell number value on day 17. On the other hand, other cultures started to decrease the cell number values from day 21. Our microscopic observations show that the smallest cells are in N^- media, and normally, the cell number and specific growth rate values are highest in this media.

In contrast to our results, research on the *Scenedesmus* culture growth in different nitrogen

media detected that N-rich conditions had higher specific growth rate than the N-depleted medium³⁹. The authors stated that nitrogen limitation or starvation leads to decrease in growth rate^{39–41}. In contrast, high nitrogen supply in culture media leads to stress-induced damage to photosystem II, therefore increases in cell number values (microalgae growing) are inhibited⁴². This situation was compatible with our results.

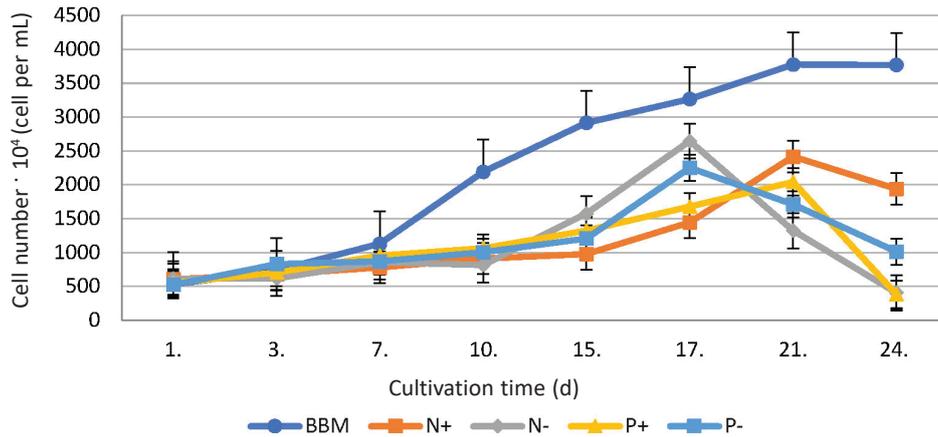


Fig. 4 – Cell number results of *D. multivariabilis* in five different culture media during the cultivation period. Error bars represent the standard deviation among the three replicates of each culture condition ($n=3$, $p<0.05$; BBM: Control group; N⁺: nitrogen excess; P⁺: phosphorus excess; N⁻: nitrogen limitation; P⁻: phosphorus limitation).

Table 3 – Specific growth rate (SGR), doubling time (t_d), and Biomass Productivity (BP) values of *D. multivariabilis* culture in five different media

Media	SGR ($\mu(d^{-1})$)	Doubling time (t_d)	Biomass productivity
BBM	0.33	2.05	0.00032
N ⁺	0.29	2.31	0.00028
N ⁻	0.44	1.54	0.00017
P ⁺	0.34	1.99	0.00034
P ⁻	0.25	2.68	0.00033

BBM: Control group; N⁺: nitrogen excess; P⁺: phosphorus excess; N⁻: nitrogen limitation; P⁻: phosphorus limitation.

Optical density (OD) values

Initial absorbance was determined as 0.5 abs at 680 nm for all cultures. As parallel to cell number values, the high OD values were recorded in BBM medium earlier than in the other cultures (Fig. 5). In general, high OD values were observed on day 17, for control group (2.06 abs); P⁻ (2.04 abs); N⁺ (1.35 abs); P⁺ (1.33 abs) and N⁻ (1.11 abs), respectively. This ranking shows that P limitation leads to increase in OD values. According to our microscopic observation results, the cells in the P⁻ medium are larger than the cells in other media. Therefore, it

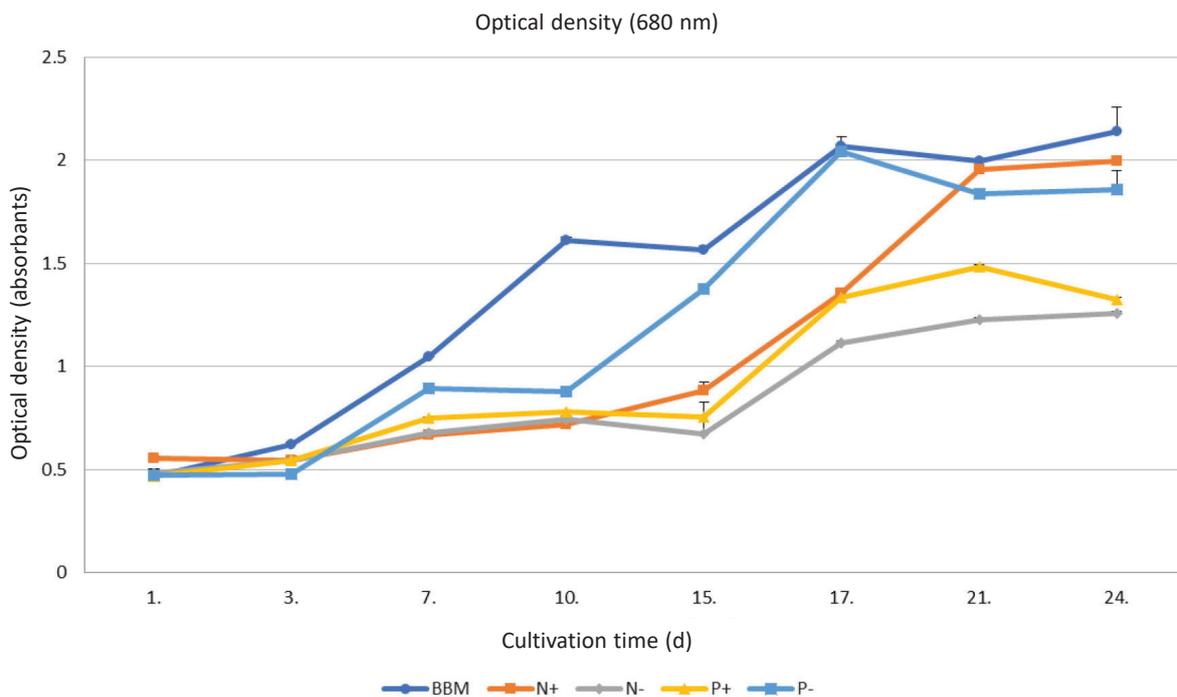


Fig. 5 – OD_{680} of *D. multivariabilis* in five different culture media during the cultivation period. Error bars represent the standard deviation among the three replicates of each culture condition ($n=3$, $p<0.05$; BBM: Control group; N⁺: nitrogen excess; P⁺: phosphorus excess; N⁻: nitrogen limitation; P⁻: phosphorus limitation).

is not surprising that the highest OD values were observed in this medium.

Goiris *et al.* stated that N and P limitation created a low optical density for *Chlorella*, *Tetraselmis* and *Phaeodactylum*⁴³. Fakhry and Makhraby found results similar to ours, and observed low optical density values in *Nannochloropsis salina* with nitrogen deficiency⁴⁴. It can be understood that nutrient limitation causes optical density to decrease, but in order to obtain a clearer result and make a general judgement, all culture growth parameters should be evaluated together.

Dry weight values

Similar to our OD values, maximum dry weight value was recorded in BBM medium (0.0080 mg L⁻¹) on the last culture day (Fig. 6). The highest values were in P- (0.0077 mg L⁻¹), P+ (0.0074 mg L⁻¹), N+ (0.0066 mg L⁻¹), N- (0.0046 mg L⁻¹) media on the day 21, respectively. Our biomass productivity (BP) results (Table 3) show that P+ medium (0.00034) had the highest BP value, and then P- (0.00033); N+ (0.00028) and N- (0.00017), respectively. It is clear from the results that N limitation inhibited biomass productivity.

Compatible with our results, it was detected that the biomass content decreased with a decreasing nitrogen concentration³³. In addition, it can be understood that biomass productivity significantly decreased under strong N-limitation in all species except for *Nannochloropsis oceanica*⁴⁵.

Chlorophyll-a (Chl-a) values

Chl-a value was measured during the culture period. Maximum Chl-a results (8.654 mg L⁻¹) were obtained in BBM medium on day 15, N- medium had minimum results (2.825 mg L⁻¹) on the same day (Fig. 7). The values of P- medium were generally high and the highest value (8.165 mg L⁻¹) was on day 21. On the same day, N+ medium (8.485 mg L⁻¹) and P+ (8.037 mg L⁻¹) medium had the maximum Chl-a value. P and N excess caused an increase in the Chl-a value, and on the other hand, N-limitation caused decreasing decrease.

Nagappan and Kumar declared in their study with some Chlorophyta members that nitrogen deficiency increases in the total chlorophyll content⁴⁶. In another study with *Desmodesmus* sp., it was stated that nitrogen deprivation causes a decrease in chlorophyll content⁴⁷. There are already many articles with different microalgae species advocating the same view on this subject^{48,49}.

As well as the effect of nitrogen and phosphorus concentration on microalgae culture growth, the N:P ratio also has an effect. Therefore, when evaluating the results, it is necessary to look at the N and P concentration, and also the N:P ratio. The elemental composition of the microalgae cell can guide the optimal N:P ratio of the culture medium. The empirical formula for microalgae cell is C₁₀₆H₂₆₃O₁₁₀N₁₆P (N:P ratio 7.2:1)⁵⁰. In another study, for *Chlorella vulgaris* optimum ratio is 8:1⁵¹ and found the similar results (optimum ratio 5:1–8:1) for *Scenedesmus*

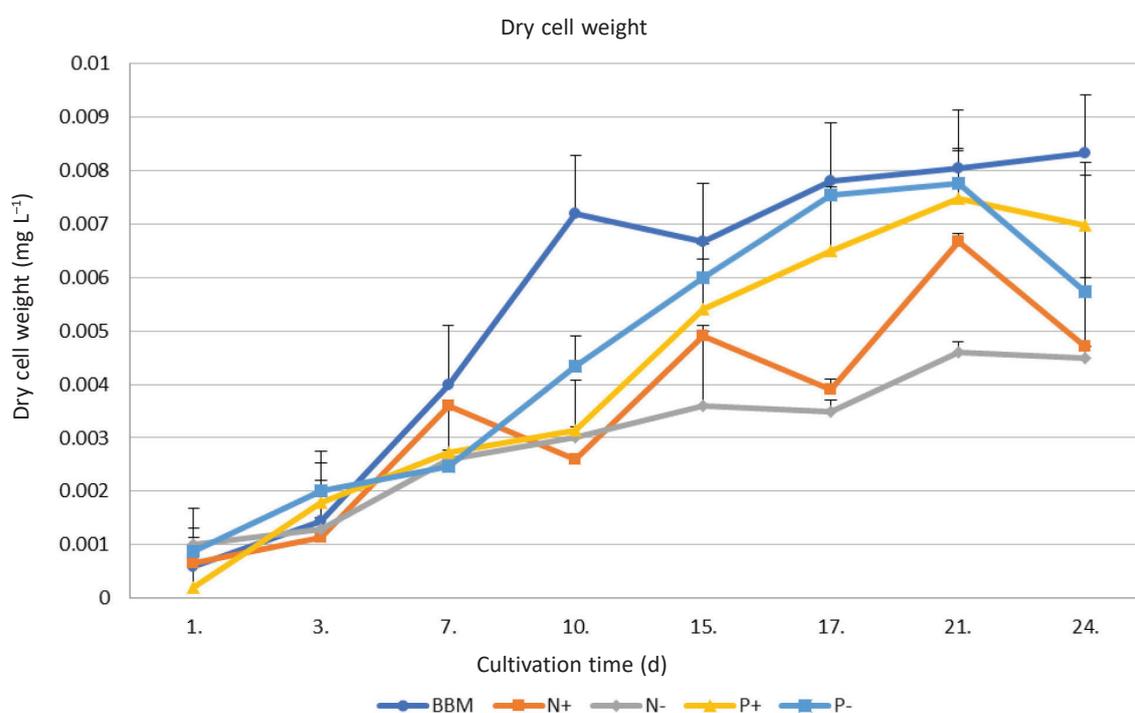


Fig. 6 – Dry weight (DW) of *D. multivariabilis* in five different culture media during the cultivation period. Error bars represent the standard deviation among the three replicates of each culture condition ($n=3$, $p<0.05$; BBM: Control group; N⁺: nitrogen excess; P⁺: phosphorus excess; N⁻: nitrogen limitation; P⁻: phosphorus limitation).

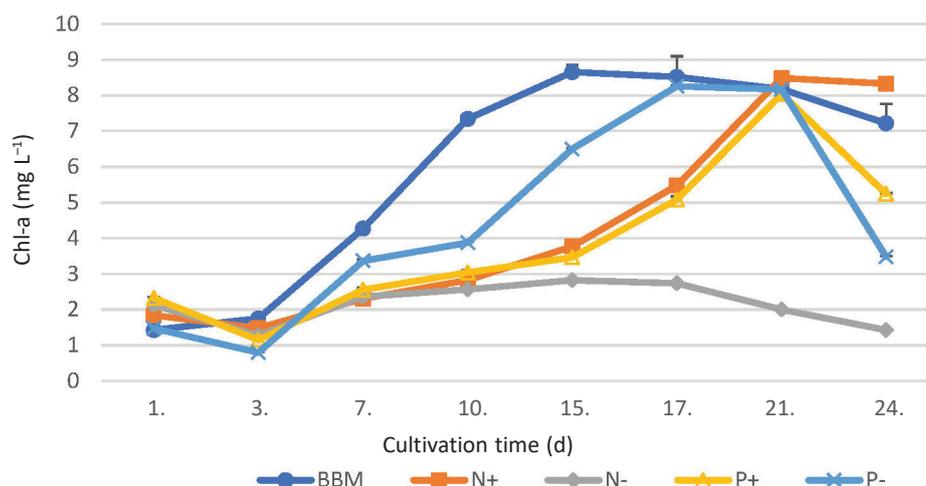


Fig. 7 – Chlorophyll-a (Chl-a) values of *D. multivariabilis* in five different culture media during the cultivation period. Error bars represent the standard deviation among the three replicates of each culture condition ($n=3$, $p<0.05$; BBM: Control group; N⁺: nitrogen excess; P⁺: phosphorus excess; N⁻: nitrogen limitation; P⁻: phosphorus limitation).

sp⁵². Our results show that for the maximum SGR was in N⁻ and maximum BP was in P⁻ and the N:P ratios of both media was ~5:1 (Table 1 and Table 3).

The N and P uptake ratios and the N:P ratio in the culture should be evaluated together and the most appropriate result should be found. To determine N and P uptake, N and P concentrations can be measured every other day during the culture process. It would be more meaningful if the N:P ratio is evaluated together with these results. This will help us understand which element is used more and how the metabolism is affected. Microalgae can change their nutrient uptake ratios according to N:P ratios of culture medium. Xin *et al.* emphasized this issue in their study with *Scenedesmus*, and stated that microalgae metabolism was directly affected by the N:P ratio and the uptake rate of N and P by the cell⁵².

The data obtained from the BBM medium, which is generally used for the culture of Chlorophyta members and selected as the control group in this study, was high when the culture growth parameters data were evaluated together. When the experimental media are evaluated relative to each other, it is evident that the N⁻ medium exhibits the largest SGR value, while the P⁻ medium shows the largest BP value. If a rapid growth is desired in a short time with *D. multivariabilis*, N restriction should be implemented; however, if the goal is to obtain more biomass, P restriction should be applied.

Determination of biochemical composition

Total protein content

Nitrogen is an essential element for amino acid and protein synthesis. In the case of N deficiency, metabolic pathways change; the citric acid cycle is

inhibited, photosynthesis, protein synthesis, and cell division are reduced⁵³. Fig. 8 shows that the highest protein amount (53.22 % DW) was detected in N⁺ media. This is an expected result since the nitrogen concentration is high in this medium. Thus, the highest protein values were observed in the following order: P⁺ (42.52 % DW); P⁻ (41.62 % DW); BBM (41.01 % DW); N⁻ (37.41 % DW). It can be said that the excess of nitrogen and phosphorus triggered protein synthesis. This is compatible with results of the study with *Picocystis salinarum*⁵⁴. Similar results were recorded in the study of Cobos *et al.*, who reported a decrease in protein content of *Acutodesmus obliquus*, *Ankistrodesmus* sp. and *Chlorella lewinii* with N limitation⁵⁵.

Amino acids profiles

The amino acids most abundant in algal cells are Arg, Ala, Val, Phe, Tyr, Asp, Glu and Lys⁵⁶. It can be observed from Table 4 that these amino acids are also present in *D. multivariabilis*. The highest amount of Arg (4608.359 $\mu\text{g g}^{-1}$) was detected in the biomass obtained from the BBM medium selected as the control group. When examining the modified media, it is evident that the N limitation sharply reduces the amount of Arg (82.639 $\mu\text{g g}^{-1}$). Arg is an amino acid that is usually high in microalgae, and similar results were obtained in another study⁵⁷. In cases of nutrient stress, Arg undertakes the tasks of regulating cell physiology and protecting the cell from osmotic changes, preventing aggregation of soluble proteins⁵⁸. Therefore, it is quite normal to decrease the Arg amount when there is a limitation of nitrogen. In order to cope with the negative effects of nitrogen stress, the amount of amino acid Arg is increased.

On the other hand, the amounts of Ala (21.342 $\mu\text{g g}^{-1}$), Val (86.986 $\mu\text{g g}^{-1}$), Phe (4.083 $\mu\text{g g}^{-1}$), Tyr

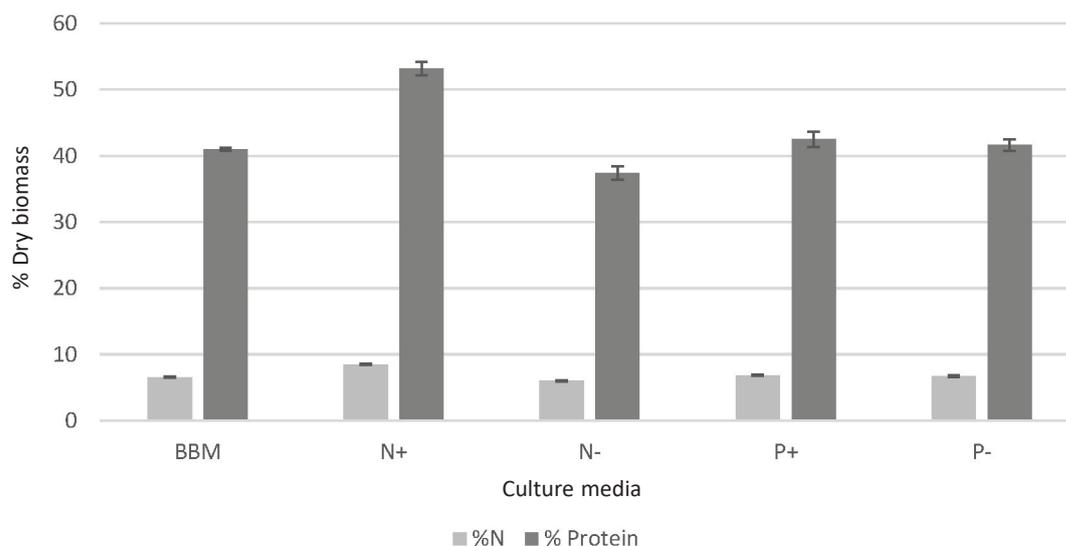


Fig. 8 – % Nitrogen and total protein content (%) in *D. multivariabilis* biomass harvested from five different media. A difference was considered significant at the level of $p < 0.05$ ($n=3$; BBM: Control group; N⁺: nitrogen excess; P⁺: phosphorus excess; N⁻: nitrogen limitation; P⁻: phosphorus limitation).

Table 4 – Amino acids compositions and amounts ($\mu\text{g g}^{-1}$ DW) in *D. multivariabilis* biomass harvested from five different media

Amino acids / Media	BBM	N ⁺	N ⁻	P ⁺	P ⁻
Arginine (Arg)	4608.359±3.96	1464.895±0.05	82.639±0.19	777.073±0.07	88.282±2.00
Alanine (Ala)	14.301±1.85	4.640±0.16	3.430±0.21	21.342±0.22	5.750±0.12
Valine (Val)	61.435±1.04	65.741±0.12	63.353±0.21	86.986±0.028	64.827±0.17
Phenylalanine (Phe)	0.956±0.08	0.850±0.10	0.549±0.23	4.083±0.019	2.638±0.09
Tyrosine (Tyr)	0.436±0.11	0.639±0.20	0.460±0.21	0.878±0.033	0.422±0.11
Aspartic acid (Asp)	1.458±0.10	4.826±0.19	5.885±0.09	13.334±0.204	4.764±0.13
Glutamic acid (Glu)	147.067±1.60	139.615±1.20	238.573±2.184	640.367±0.181	146.145±0.103
Lysine (Lys)	0.012±0.001	0.025±0.002	0.019±0.004	0.018±0.006	0.016±0.001

Data are expressed as mean \pm SD, $n=3$. There is a significant difference among all amino acid types of each group, $p < 0.05$.

BBM: Control group; N⁺: nitrogen excess; P⁺: phosphorus excess; N⁻: nitrogen limitation; P⁻: phosphorus limitation.

(0.878 $\mu\text{g g}^{-1}$), Asp (13.334 $\mu\text{g g}^{-1}$) and Glu (640.367 $\mu\text{g g}^{-1}$) amino acids were found to be higher in the P⁺ medium compared to the other media. This result shows that increasing phosphorus concentration caused an increase in amino acid percentages.

Amino acids, which act as stress reducers in the cell, are stored under abiotic stress⁵⁹. In addition to being the building blocks of proteins, amino acids are the precursors of nitrogenous compounds such as nucleic acids, polyamines, and quaternary ammonium compounds. Under abiotic stress conditions, *de novo* protein synthesis is inhibited, protein turnover and proteolytic activity increase, thus the amount of free amino acids increases^{60,61}. This kind of variation in the amino acid profile is the result of these metabolic events. Therefore, it is important to analyze metabolic pathways in such studies, but we did not analyze the amino acid metabolism pathway in this study. It is clear from our results that this

microalga can be used as an amino acid supplement, and in which medium it should be cultured to obtain amino acids.

Total lipid content

The amount of lipid and its productivity of microalgae vary according to the nutrient concentration of the culture medium and environmental conditions⁶². Our study recorded the highest lipid amount (7.1 % DW) in N⁺ media, followed by BBM (6.6 % DW), N⁻ (5.7 % DW), P⁺ (5.2 % DW), P⁻ (3.5 % DW), respectively (Table 5). These results suggest that N starvation stimulates the lipid productivity, as nitrogen is the major component for many metabolic pathways.

Generally, nitrogen deficiency in the culture medium of microalgae leads to an increase in lipid content. Many studies with different algae species

Table 5 – Total lipid content in *D. multivariabilis* biomass harvested from five different media. A difference was considered significant at the level of $p < 0.05$.

Media	BBM	N ⁺	N ⁻	P ⁺	P ⁻
Lipid content (% DW)	6.6±0.1	7.1±0.15	5.71±0.03	5.2±0.05	3.51±0.03

BBM: Control group; N⁺: nitrogen excess; P⁺: phosphorus excess; N⁻: nitrogen limitation; P⁻: phosphorus limitation.

Table 6 – Fatty acids compositions and percentage (% of total fatty acids) in *D. multivariabilis* biomass harvested from five different media

	BBM	N ⁺	N ⁻	P ⁺	P ⁻
<i>Saturated Fatty Acids (SFAs)</i>					
Lauric acid methyl ester; 12:0	2.633±0.008	2.573±0.010	2.299±0.011	2.298±0.050	1.250±0.120
Pentadecanoic acid methyl ester; 14:0	12.636±0.093	15.736±0.090	11.834±0.093	18.153±0.144	17.506±0.169
Palmitic acid methyl ester; 16:0	3.345±0.106	5.640±0.171	2.743±0.090	5.660±0.216	11.026±0.025
Stearic acid methyl ester; 18:0	2.234±0.012	1.893±0.018	1.938±0.03	1.834±0.160	0.933±0.012
Total SFAs	20.848±0.102	25.842±0.202	18.814±0.098	27.945±0.402	30.715±0.234
<i>Monounsaturated fatty acids (MUFAs)</i>					
Oleic acid methyl ester; ω9; 18:1	10.206±0.103	8.471±0.194	10.487±0.040	4.216±0.111	16.198±0.004
<i>Polyunsaturated fatty acids (PUFAs)</i>					
Linoleic acid methyl ester; ω6; 18:2	6.814±0.188	11.982±0.017	35.605±1.421	27.771±0.212	6.925±0.363
Linolenic acid methyl ester; ω3; 18:3	33.645±0.370	39.707±0.268	24.647±1.947	31.166±1.003	40.393±1.910
5.8.11.14-Eicosatetraenoic acid methyl ester; 20:4	9.355±0.904	6.340±0.210	4.515±0.967	4.524±0.271	3.304±0.216
Cyclopentane tridecanoic acid methyl ester	20.601±1.241	9.297±0.901	7.192±0.219	5.441±1.021	3.050±0.995
Total PUFAs	70.415±0.232	67.326±1.23	71.959±2.21	68.902±2.31	53.672±2.132

Data are expressed as mean ± SD, n=3. There is a significant difference among all amino acid types of each group, $p < 0.05$.

BBM: Control group; N⁺: nitrogen excess; P⁺: phosphorus excess; N⁻: nitrogen limitation; P⁻: phosphorus limitation.

support this finding, such as in *Scenedesmus* sp.³³; in *Phaeodactylum tricornutum*⁶³; in *Chlorella reinhardtii*⁶⁴.

However, our results are different from these general results. We detected the highest lipid content (7.1 % DW) in the biomass harvested from N⁺ medium. Similar to our results, An et al. stated in their study with *Scenedesmus obliquus* that lipid productivity increased with increasing nitrogen⁶⁵. Similarly, in another study, Li et al. reported that lipid productivity in *Scenedesmus* LX1 was high in the medium with high nitrogen density, and lower in the medium with low nitrogen density⁶⁶. They explained this situation with biomass productivity results parallel to the lipid productivity results.

In many studies with *Scenedesmaceae*, it is stated that phosphorus restriction increases lipid content^{67–69}. However, in our study, the lowest lipid content was determined in P⁻ medium. This shows that the phosphorus is mostly utilized in the synthesis of phospholipids and added to the cell structure, as reflected by the high values of culture growth parameters, and larger cell sizes in the P⁻ medium.

These results suggest that lipid accumulation is directly affected by photosynthesis and respiration reactions, synthesis of organic matter, and conversion mechanisms of this organic matter to different

organic substances. Therefore, all metabolic pathways of the microalgae cell should be examined in detail in order to draw more conclusive findings in studies conducted for this purpose. In addition, the type of nitrogen added to the culture medium, as well as its addition or non-addition during the culture, also affect lipid productivity depending on the amount of nutrients.

FAMES profiles

In microalgae, C16:0, C18:0; C18:1; C18:2 C20:2 fatty acids are frequently observed^{70,71}. However, modifications can be made in the composition of the culture medium, depending on the type of fatty acid desired to be obtained. Especially N and P restrictions change the fatty acid profile in microalgae. There are many publications on this concept^{39,69,72,73}.

Based on Table 6, nitrogen and phosphorus concentrations play an important role in the fatty acids profile of *D. multivariabilis*. According to general view, percentages of PUFAs are high in all media. In detail, the highest total amount of SFAs (30.715 % of total FAMES) and MUFAs (16.198 % of total FAMES) were detected in P⁻ medium. On the other hand, it is evident that nitrogen starvation

led to the highest PUFAs amount (71.959 % of total FAMES)

In this study, P restriction was highly effective in altering fatty acid diversity and increasing some of them. P limitation causes cells to constantly divide or store high-energy molecules such as lipid⁷⁵. Our high cell growth parameters and low lipid content in P– medium indicate that the former occurred in *D. multivariabilis*.

For biodiesel production, a high concentration of MUFAs (~50 %) is desired^{76,77}, and our results show that this microalga is not very suitable for use in biodiesel production. On the other hand, PUFAs are very important in human nutrition due to their benefits for cardiovascular, brain, and nervous system development^{78,79}. It is evident from Table 6 that *D. multivariabilis* is a rich source of PUFAs; moreover, it increases the PUFA content even more with N limitation. These results show that this microalga can be exploited in human food products for its nutraceutical benefits when cultured in nitrogen-limited media.

Total phenolic content and antioxidant activity

Based on Fig. 9, the phenolic content of the microalgae biomass cultured in BBM medium selected as the control group was the highest (0.74 mg GAE kg⁻¹ KM). On the other hand, a sharp decrease was observed in the biomasses harvested from other media. The lowest phenolic content was detected in biomass harvested from N– (0.006

mg GAE kg⁻¹ KM); P+ (0.033 mg GAE kg⁻¹ KM); N+ (0.065 mg GAE kg⁻¹ KM); P– (0.087 mg GAE kg⁻¹ KM) media, respectively. These results show that the excess or limitation of N and P lead to a decrease in total phenolic content, and N limitation reduces this content much more.

Phenolic compounds are an important group of antioxidant substances in terrestrial plants⁸⁰. Many articles have investigated the amount of phenol and antioxidant activity together in microalgae^{81,82}. To the best of our knowledge, the number of studies researching the relationship between nutrient stress and the amount of phenolic components is very poor, and papers on this topic are very recent. One of them stated that phenolic content decreased with N limitation⁹. In another study with *Acutodesmus dimorphus*, polyphenol content decreased under nitrogen deprivation⁸³. Goiris *et al.* detected a decrease in phenolic content and antioxidant activity with nutrient limitation, particularly N limitation⁴³.

It is evident from Fig. 10 that DPPH scavenging activity is higher at lower extract concentration. When the extracts obtained from all media are compared, it is observed that at 87 µg mL⁻¹, 175 µg mL⁻¹, and 350 µg mL⁻¹, the P– medium had the highest DPPH scavenging activities (75.83 % SCV; 74.34 % SCV; 70.76 % SCV, respectively). In general, it can be concluded that the P limitation increased the DPPH scavenging activity.

In their study with *Isochrysis* sp. and *P. tricornutum*, Jeyakumar *et al.* stated that DPPH scavenging

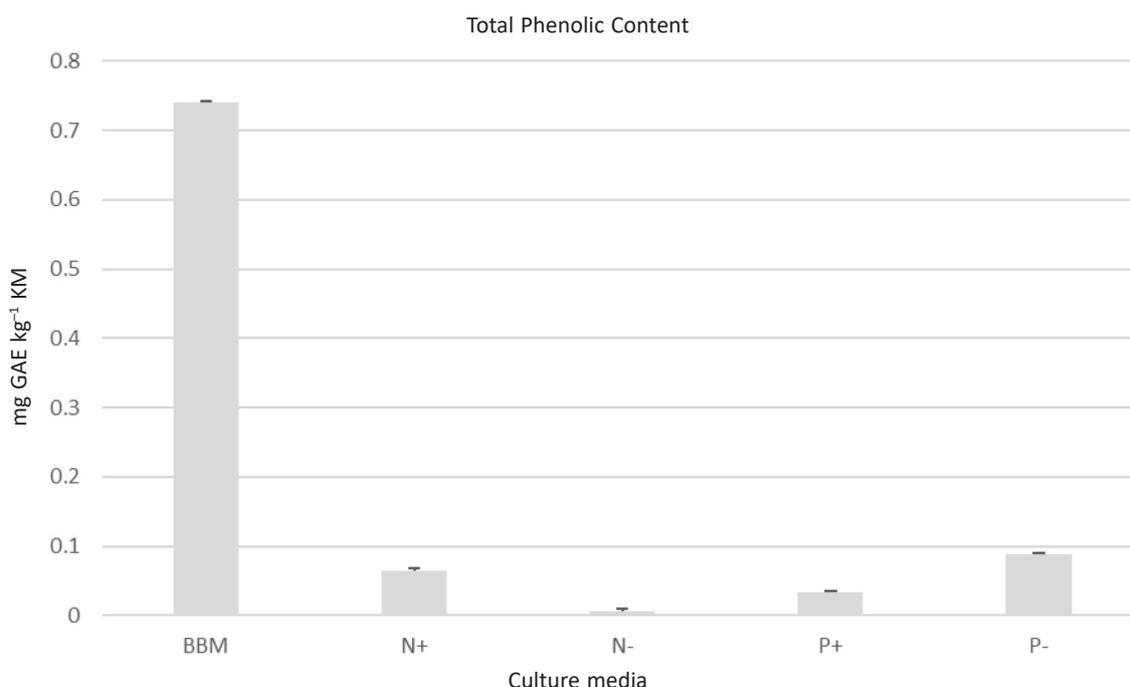


Fig. 9 – Total phenolic content in *D. multivariabilis* biomass harvested from five different media. A difference was considered significant at the level of $p < 0.05$ ($n = 3$; BBM: Control group; N+: nitrogen excess; P+: phosphorus excess; N–: nitrogen limitation; P–: phosphorus limitation).

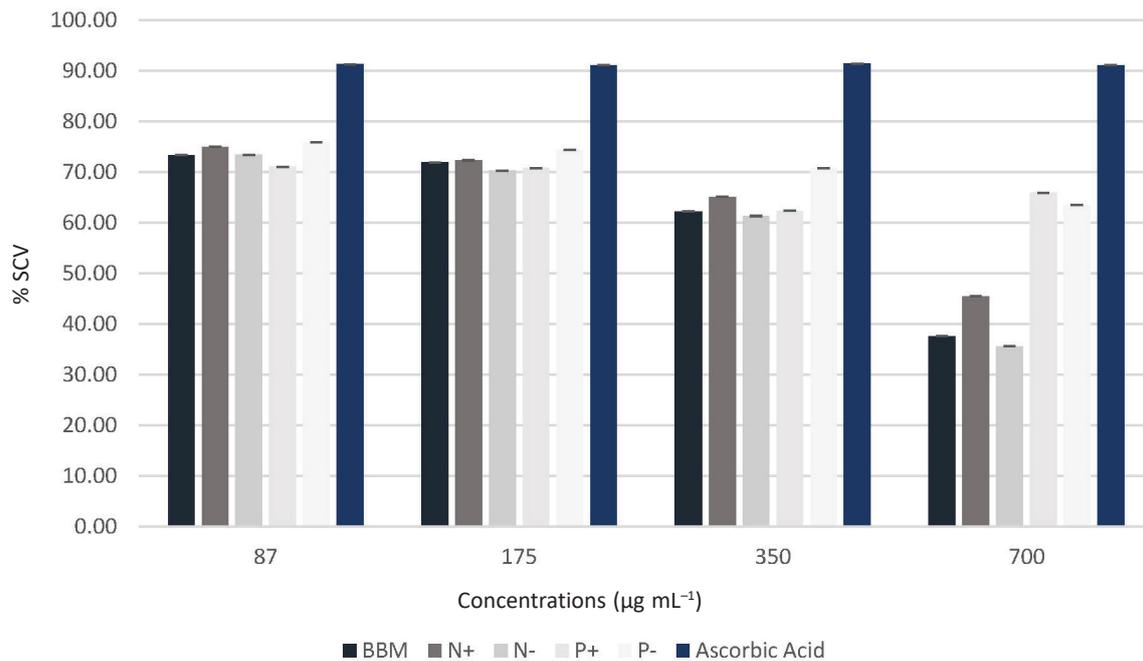


Fig. 10 – DPPH scavenging activity of *D. multivariabilis* biomass harvested from five different media at different concentrations. A difference was considered significant at the level of $p < 0.05$ ($n=3$; BBM: Control group; N⁺: nitrogen excess; P⁺: phosphorus excess; N⁻: nitrogen limitation; P⁻: phosphorus limitation).

ing activity was higher (85 %) in nitrogen-depleted conditions than in BBM (64 %) and nitrogen-repleted (75 %) media⁸⁴. In another study with *Dunaliella salina*, similar results were reported high DPPH scavenging activity in N⁻ medium⁸⁵. The antioxidant activity of some microalgae cultured in different wastewater compositions was investigated, and it was reported that highest antioxidant activity was observed under nitrogen depletion⁸⁶. Our results are consistent with this study; it is a general truth that nutrient deficiency leads to an increase in antioxidant activity.

When the total phenol content and antioxidant activity results are evaluated together, it becomes evident, that the phenolic content decreases while antioxidant activity increases in the absence of nitrogen and phosphorus. It is known that the phenolic content increases under stress conditions in land plants⁸⁷ and macroalgae⁸⁸, but the reason for its decrease in microalgae remains unknown. Therefore, comprehensive research into the phenol metabolic pathway is necessary. In our opinion, the total phenolic content also increased under stress conditions in microalgae. However, the cell then used the phenolic substance to protect itself from oxidation. To understand this, in future studies, it is necessary to measure the amount of phenol at the beginning and end of the logarithmic phase during culture. Therefore, the number of studies on phenol and nutrient stress should be increased. With these results, this study has filled the gap in this field in the literature.

Conclusion

Nitrogen and phosphorus are the most important elements for biota because they are the major components of the cell's basic structural units, such as proteins, enzymes, DNA, RNA, and ATP; in N or P; lead the major changes in culture growth parameters values and biochemical composition of microalgae. The most efficient tool for manipulating these values of microalgae is modification of the nutrient concentrations of the culture media.

In summary, with nitrogen and phosphorus concentration modification, phenotypic plasticity was observed in *D. multivariabilis*. Cell number of colony, chloroplast shape and structure, spine length, and cell sizes were recorded differently in each media. In addition, the culture growth parameters changed such that the N⁻ medium exhibited the highest SGR value and the P⁻ medium showed the highest BP value. If a rapid growth is desired in a short time with this species, N restriction is recommended. However, if the goal is to obtain more biomass, it can be suggested that P restriction should be applied. In studies, increasing nitrogen should be considered to achieve higher lipid and protein ratios in *D. multivariabilis*. Furthermore, FAMES composition changed according to nitrogen and phosphorus concentration. To obtain more SFAs and MUFAs, P limitation should be applied; for more PUFAs, N limitation is recommended. According to our results, nutrient modifications should also be made according to the desired amino acid type. Fur-

thermore, it is evident from our results that nutrient concentration changes both the total phenol content and antioxidant activity.

In future studies, the N:P ratio should be considered to determine the rate of N and P uptake into the cell. For this purpose, these values should be known by nutrient determination at different stages of the culture. In addition, according to the results of this study, metabolic pathway studies with transcriptomic analyzes should be performed, and the stress physiology of microalgae should be fully elucidated.

DISCLOSURE OF CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ACKNOWLEDGEMENT

This work was supported by Burdur Mehmet Akif Ersoy University [number of project: 0784-YL-21]. We thank Dr. Rıza Akgül for culture studies and light micrographs.

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