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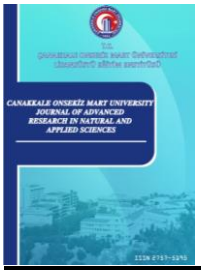

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The Effect of Nitrogen and Phosphorus Limitations at Different Salt Ratios on Growth and Biochemical Composition of *Tetraselmis suecica* (Chlorodendrophyceae)

Cananur Sisalan Pihava[1], Leyla Uslu[2,*]

[1,2]Fisheries Faculty, Çukurova University, Balcalı Campüs, Adana, Türkiye

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Research Article

Abstract − Algae are plant organisms that produce organic molecules in the aquatic environment and absorb carbon. Since algae are photosynthetic organisms, they give oxygen to the environment. Algae, one of the most important living resources of the seas, are used in many fields such as food, agriculture, cosmetics, medicine, pharmacy, industry and also as a biofuel source thanks to the metabolites they store in the cell. In the study carried out to determine the effects of nitrogen and phosphorus limitations at different salinity rates on the chlorophyll *a*, dry weight, optical density, protein, lipid and fatty acid contents of the microalgae *Tetraselmis suecica* from the class Chlorodendrophyceae cultured in the laboratory conditions, at ‰15, 30 and 45 salinity rates 50% N and 50% P reductions were applied. The lowest growth was detected in the culture containing 50% N(-). The highest lipid ratio was determined as 39.8±1% in the 50% N(-) group, while the closest ratio was 34.6% in the 50% P(-) group. The highest polyunsaturated fatty acids were determined in the group containing 50% P(-) at all salinity values. The protein value was determined as 22.3% in the 50% P(-) group and 15.7% in the 50% N(-) group at ‰30 salinity.

Keywords *– Fatty acids, growth, lipid, protein, N limitation, P limitation, salinity, tetraselmis suecica.*

1. Introduction

Various factors affect the development of algae; In addition to factors such as light, temperature, pH, salinity is one of the most important environmental factors affecting phytoplankton development, metabolism and distribution (Alsull et al., 2012). Marine phytoplanktonic organisms have a very good tolerance to salinity changes. According to the salinity in the natural environment, many algae species also grow well at lower or higher salinities. Although many algae grow in the ‰12-44 salinity range, they prefer the optimum ‰20-24 salinity. However, there are algae species that can survive even at ‰140 salinity, where most of them cannot survive (Ben-Amotz, & Avron, 1983; Borowitzka, & Borowitzka, 1988). E.g; While *Isochrysis galbana* shows maximum growth at ‰15, *Tetraselmis suecica* prefers ‰25-35 salinity (Vonshak, & Tomaselli, 2000). Microalgae, like land plants, require light, carbon dioxide, water and inorganic salts for growth. Although their production seems to be costlier when compared to agricultural crops, it is more advantageous in that they can be divided within hours and produced throughout the year (Siaut et al, 2007; Grobbelaar, 2004). Reducing the cost of algae production is possible with the use of sunlight, seawater and carbon dioxide from greenhouse gases. The main elements used in algae production are nitrogen (N), phosphorus (P), sodium (Na), iron (Fe). Since most of these elements are in seawater, it can reduce the cost of the culture medium in the production of marine microalgae (Mirón et al., 2003). After carbon, the most important nutrient for living mass production is N. In general, N constitutes between 1% and 10% of their dry weight (Gökpınar and Cirik, 1991). N is one

[1] cananursisalan@gmail.com
[2] hizarcil@cu.edu.tr
[*]Corresponding Author

of the most important factors affecting growth and biochemical composition in algae cultures. The lack of N in the culture medium causes a decrease in the amount of dry matter and chl *a*, and an increase in carotene and lipids (Sukenik et al., 1989). Chemical and physical factors in the culture medium can also affect the content of fatty acids. Increased lipid content has been observed in many microalgae species grown with N restriction. In the late 1940s, N starvation was found to be effective on fat storage and a lipid level of 70-85% in dry weight was reported. *Dunaliella* sp. and some algae species, such as *T. suecica*, contain low lipids and generally produce carbohydrates rather than lipids. Besides N, some other nutritional deficiencies can also cause an increase in lipid content (Piorreck and Pohl, 1984). P is an important nutrient for algae and a component of molecules such as phospholipids and nucleic acids. In plants, P plays an important role in many critical metabolic processes, especially photosynthesis and respiration (Plaxton and Tran, 2011). The presence of P in the aquatic environment is especially important for algae and other aquatic organisms. The use of P in seawater not only affects cell volume, nutritional status, photosynthetic activity, and other biochemical and physiological properties of algal cells, but also affects the composition and quantity of the marine phytoplankton community (Benitez-Nelson, 2000; Paytan and McLaughlin, 2007). Two of the most important problems in our world today are undoubtedly environmental pollution and increasing energy needs. Environmentally-friendly production, sustainable environment and sustainable green economy have started to form the agenda of countries. The use of microalgae in environmental applications is increasing and microalgae technology is developing rapidly. The use of microalgae in preventing water pollution and in bioenergy is seen as an important ecological investment for the future. For this purpose, many studies need to investigate the environmental uses of rapidly developing microalgae technology and its potential in meeting energy needs (Sisman-Aydin, 2019). In this study, the effects of 3 different salinity (‰15, 30 and 45) and nutrient deficiencies (50% N(-) and 50% P(-)) on growth, protein, lipid and fatty acid changes in *T. suecica* were investigated.

2. Materials and Methods

2.1. Inoculum source and cultivation conditions

T. suecica (UTEX LB 2286) used in the study was obtained from UTEX Culture Laboratory. The study was carried out in Çukurova University Fisheries Faculty Algal Biotechnology laboratory. *T. suecica* belonging to the class Chlorodendrophyceae was used in the study. *T. suecica* is a green, four flagellated and motile marine microalgae. It is usually 10 µm long by 14 µm wide. *Tetraselmis* is a promising microalgae species for biofuel use due to its rapid growth rate and high lipid content (Alonso et al., 2012).

In the experiment, the F/2 culture medium (Guillard, 1973) was used as the medium. The experiment was carried out in a laboratory environment. Cultures were replicated first in flasks and then in balloons and sufficient amount of inoculum was prepared. In the experiment, N and P deficiency were tested at 3 different salinity ratios. Salinity ratios were adjusted to be ‰15, 30 and 45 and a control group for each salt concentration was formed. In other groups, experiments were established by making 50% N and 50% P deficiency. The inoculation rate to be used in the trial was adjusted to be 20% and the trial was set up in 5L volumes. From the beginning of the experiment to the last day of the experiment, samples were taken from the trial dishes in order to determine the optical density (OD), biomass and chl *a* value.

2.2. Biomass (Dry weight), Chlorophyll *a* (Chl *a*) and Optical Density (OD)

Dry weight amount was determined according to Vonshak, 1997. To determine the amount of Chl *a* throughout the experiment, 5 ml samples were taken from the balloons of each treatment group. The samples taken were filtered through GFC filter. 10 ml of 95% ethanol was added to the samples. Samples were left in the refrigerator (+4°C) for 24 hours in the dark after shaking. At the end of the extraction period, the upper clear part was taken and the absorption values were measured at 649 and 665 nm in the visible spectrophotometer. The results were calculated using the following formulas (Eq. 2.1.) (Kulkarni, & Nikolov, 2018).

$$Chl\ a\ (\frac{mg}{g}) = 13.36 * Abs665nm - 5.19 * Abs649nm \quad (2.1)$$

In order to measure OD, after the cultures were mixed homogeneously daily, 3 ml of sample was taken with the help of a pipette. The samples taken from the tubes were placed in quartz cuvettes and the visible spectrophotometer was read at a wavelength of 680 nm (Wong, & Franz, 2013).

2.3. Lipid, fatty acids and protein analysis

Lipid analysis was performed according to the method applied by Bligh and Dyer (1959). 0.2g homogenized sample was mixed with a Warring blender after adding 120 ml of methanol/chloroform (1/2). Then, these samples were added to 20 ml of 0.4% $CaCl_2$ solution and filtered through filter paper (Scleicher & Schuell, 5951/2 185 mm) and kept in an oven at 105 ˚C for 2 hours and then filtered into flasks that were tared. These balloons were closed in such a way that their mouths were not airtight and kept in a dark environment for 1 night, and the next day, the top layer consisting of methanol-water was removed with the help of a separating funnel. Chloroform from the chloroform-lipid part remaining in the balloons was evaporated using a rotary evaporator in a water bath at 60 ˚C. Then, the balloons were kept in the oven at 90 ˚C for 1 hour, allowing all the chloroform in them to evaporate, and cooled to room temperature in a desiccator and weighed on a precision balance with 0.1 mg sensitivity. The following formula was used to calculate the lipid ratio (Eq. 2.2).

$$Lipid\% = \frac{(Ballon\ tare(g) + Lipid(g)) - (Ballon\ tare(g)) * 100}{amount\ of\ sample(g)} \quad (2.2)$$

From the extracted lipid, fatty acid methyl esters were made according to the method of Ichihara et al. (1996). 4mL of 2M KOH and 2mL of n-heptane were added to 25 mg of extracted lipid sample. Then, it was vortexed for 2 minutes at room temperature and centrifuged at 4000 rpm for 10 minutes, and the heptane layer was analyzed by gas chromatography (GC).

2.3.1. Gas chromatographic conditions

Fatty acid content was analyzed using a GC Clarus 500 instrument (Perkin–Elmer, USA), a flame ionization detector (FID) and an SGE (60mx0.32mm ID BPX70x0.25 μm, USA) column. Injector and detector temperatures were adjusted as 260ºC and 230ºC, respectively. In the meantime, the oven temperature was kept at 140ºC for 8 minutes, then it was increased by 4ºC every minute until 220ºC, from 220ºC to 230ºC by 4ºC every minute and kept at 230ºC for 15 minutes, the analysis was completed after a total of 45.5 minutes. By controlling the sample size at 1 µl and the carrier gas at 16ps, the split current ratio of 40.0mL/min (1:40) was used. Fatty acids were determined depending on the arrival times of the FAME mixture consisting of standard 37 components (Supelco 37 F.A.M.E. Mix C4-C24 Component, Catalogue No. 18919).

Total crude protein was made in the Kjeldahl apparatus according to the Kjeldahl method (AOAC, 1998). The amount of protein was calculated according to the formula in the following equation (Eq. 2.3)

$$\%N = \frac{14.01 * (A - B) * M}{g * 10} * 100$$

$$\%Protein = \%N * 6.25 \quad (2.3)$$

A: The amount of HCl consumed for the sample

B: The amount of HCl consumed for the blind

M: Acid molarity

g: Sample quantity

3. Result and Discussion

In this study, which was carried out to determine the effect of nutrient deficiency on the growth and biochemical structure of *T. suecica* in the Çukurova University Fisheries and Algal Biotechnology Laboratory, at ‰15, 30 and 45 salinities; 50% N and 50% P deficiencies were tried and the trial was completed on different days. In the experiment, chl *a*, dry weight analysis, OD, protein, lipid and fatty acid analyzes were performed.

3.1. Optical Density (OD)

Optical densities were determined in order to monitor the growth of *T. suecica*, which was cultured in environments where salt concentrations of ‰15, 30 and 45 were applied and N and P were reduced by 50%. When the growth curve of *T. suecica* cultures at ‰45 salt concentration is examined, it is seen that the logarithmic phase starts with 50% N(-), 50% P(-) with inoculation, and a lag phase lasting approximately one day in the control group (Figure 1). While the OD values were lower in the 50% P-reduced culture than in the control group, the lowest OD values were determined in the 50% N-reduced culture. The highest OD values in cultures treated with 50% P(-) and 50% N(-) were determined as 0.376±0.01 and 0.269±0.02, respectively.

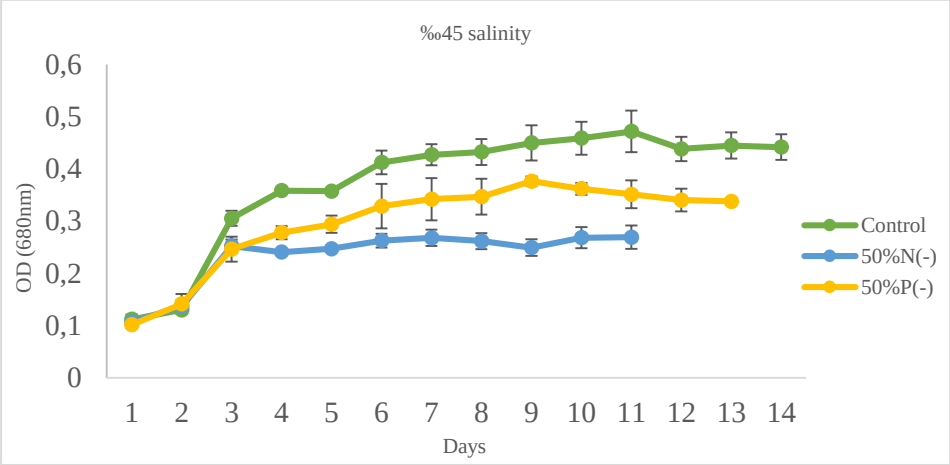

Figure 1. OD values of *T. suecica* groups at ‰45 salinity.

When the growth curve of *T. suecica* cultures at ‰30 salt concentration is examined, it is seen that the logarithmic phase begins with the inoculation in 50% N(-), 50% P(-) and control groups (Figure 2). As seen in the growth curve, OD values in 50% N(-), 50% P(-) and control groups show a similar increase until the 4th day, and after the 4th day, the OD values in 50% N(-), 50% P(-) cultures decrease is observed. After the 6th day of growth, the growth was lower in the culture containing 50% N(-). The highest OD values in cultures treated with 50% P(-) and 50% N(-) were determined as 0.377±0.02 and 0.291±0.007, respectively.

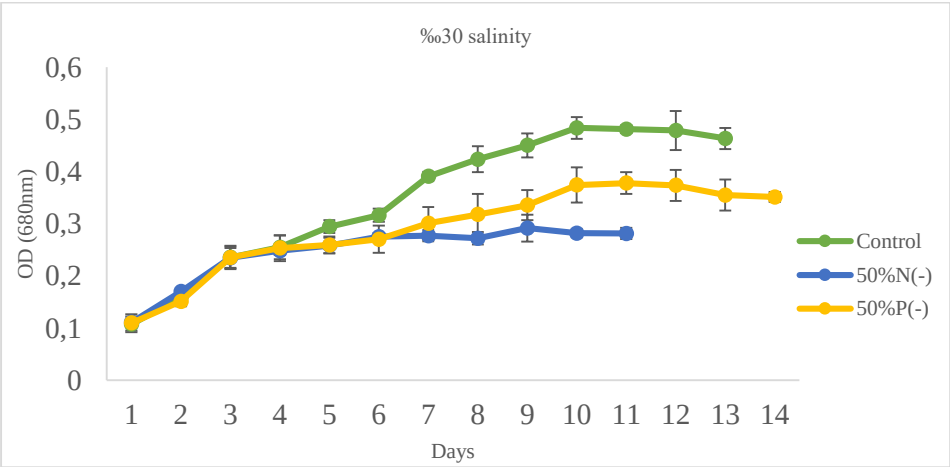

Figure 2. OD values of *T. suecica* groups at ‰30 salinity.

When the growth curve of *T. suecica* cultures at ‰15 salt concentration is examined, 50% N(-), 50% P(-) and in the control group, the logarithmic phase started with inoculation and as seen in the growth curve, 50% N(-), 50% P(-)) and the control group showed a similar increase in OD values until the 3rd day. From the 3rd day of growth, a decrease in OD values was observed in 50% P(-) cultures compared to the control group, and the lowest OD values were recorded in 50% N(-) cultures (Figure 3). While the highest determined OD value was 0.402±0.01 in the control group, the highest OD values were determined as 0.350±0.01 and 0.311±0.03 in cultures treated with 50% P(-) and 50% N(-), respectively.

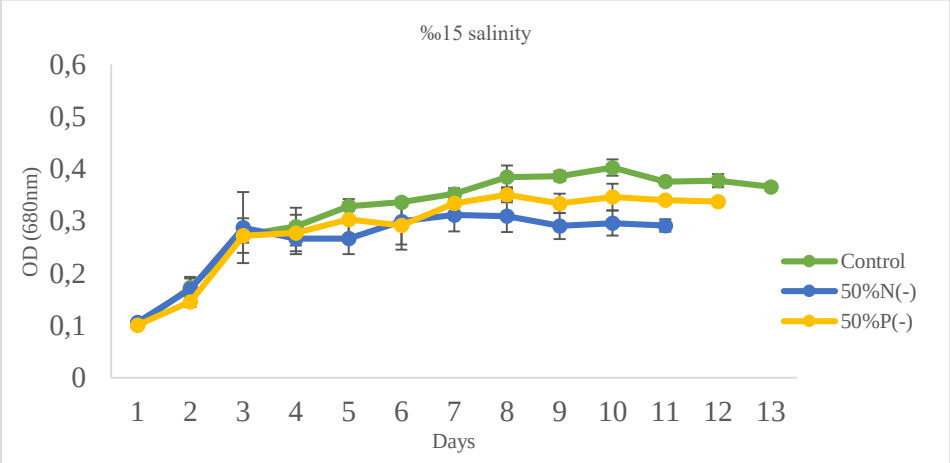

Figure 3. OD values of *T. suecica* groups at 15‰ salinity.

At ‰15, 30 and 45 salt concentrations, OD values were higher in cultures treated with 50% P(-) compared to 50% N(-) cultures. It is observed that the salinity ratios applied in cultures treated with N and P deficiency have no effect.

3.2. Dry Weight

The amounts of dry weight in the groups applied 50% N(-) and 50% P(-) at ‰45, 30 and 15 salt concentrations are as summarized in the table below (Table 1). As seen in the table, the lowest dry weight values at all three salinity concentrations were determined in the culture applied with 50% N(-). The amount of dry weight in the culture treated with 50% P(-) was higher than the group treated with 50% N(-) and lower than the control group.

Table 1
Dry weight (dw) of groups (gL^{-1})

Salinity (‰)		**Control**	**50% N(-)**	**50% P(-)**
45	dw_{first}	0.045±0.004	0.042±0.01	0.403±0.002
	dw_{last}	0.218±0.001^{a}	0.128±0.01^{c}	0.164±0.001^{b}
	$dw_{max.}$	0.223±0.01^{a}	0.128±0.01^{c}	0.184±0.004^{b}
30	dw_{first}	0.043±0.007	0.045±0.008	0.044±0.002
	dw_{last}	0.229±0.005^{a}	0.134±0.02^{c}	0.173±0.02^{b}
	$dw_{max.}$	0.240±0.006^{a}	0.139±0.01^{c}	0.185±0.03^{b}
15	dw_{first}	0.040±0.001	0.042±0.001	0.039±0.003
	dw_{last}	0.178±0.003^{a}	0.139±0.006^{c}	0.163±0.003^{b}
	dw_{max}	0.197±0.008^{a}	0.150±0.01^{c}	0.170±0.007^{b}

There is a statistical difference between the means of lines symbolized by the letters a, b, c ($p<0.05$).

3.3. Chlorophyll *a*

Consistent with the OD measurements and dry weight amounts in the treatment groups, it was determined that chl *a* values were higher in the 50% P(-) applied group at ‰15, 30 and 45 salt concentrations than in the 50% N(-) applied group and lower than the control group. ‰The chl *a* value of the group administered 50% P(-) at 30 salt concentration was 0.198 mgL^{-1}, the chl *a* value of the group with 50% N deficiency was higher than 0.121 mgL^{-1}, and the chl *a* value of the control group was lower than 0.261 mgL^{-1} has been. ‰30 salinity was the salt concentration with the highest chl *a* values in all three groups (Table 2).

Table 2
Chl *a* amounts of all groups (mgL^{-1})

Salinity(‰)		**Control**	**50% N(-)**	**50% P(-)**
	$Chla_{first}$	0.064±0.001	0.067±0.01	0.062±0.002
45	$Chla_{last}$	0.148±0.002[a]	0.089±0.01[c]	0.111±0.001[b]
	$Chla_{first}$	0.063±0.007	0.067±0.008	0.064±0.002
30	$Chla_{last}$	0.261±0.005[a]	0.121±0.02[c]	0.198±0.0[b]
	$Chla_{first}$	0.068±0.001	0.061±0.001	0.065±0.003
15	$Chla_{last}$	0.138±0.003[a]	0.09±0.006[c]	0.114±0.003[b]

There is a statistical difference between the means of lines symbolized by the letters a, b, c ($p<0.05$).

3.4. Protein Values

A significant effect of 50% N(-) and 50% P(-) treatments on protein content were found at ‰15, 30 and 45 salt concentrations applied in the experiment ($p<0.05$). The highest protein value was determined in ‰30 salinity groups, while the lowest values were determined in ‰45 salinity groups. While the high protein rate at ‰30 salinity was determined as 22.3% in the 50% P(-) applied group, the protein rate was determined as 20.1% at ‰30 salinity in the 50% N(-) applied group. The highest protein content was recorded at 26.8%, ‰30 salinity in the control group (Table 3).

Table 3
Protein amounts of the groups (%)

Salinity(‰)	**Control**	**50%N(-)**	**50%P(-)**
45	20.6±2[c]	10.3±1[c]	16.4±2[c]
30	26.8±1[a]	15.7±1[a]	22.3±1[a]
15	25.3±1[b]	12.8±2[b]	20.1±2[b]

There is a statistical difference between the means of lines symbolized by the letters a, b, c ($p<0.05$).

3.5. Lipid Amount

Salinity, N(-) and P(-) treatments applied in the experiment were found to have a significant effect on lipid content ($p<0.05$). The highest lipid value was found to be 39.8% in the group treated with ‰30 salinity and 50% N(-) (Table 4).

Table 4
Lipid amounts of the groups (%)

Salinity(‰)	**Control**	**50%N(-)**	**50%P(-)**
45	21.6±2[c]	33.2±2[b]	30.4±2[b]
30	23.7±3[a]	39.8±1[a]	34.6±1[a]
15	22.9±1[b]	31.1±2[c]	29.3±1[b]

There is a statistical difference between the means of lines symbolized by the letters a, b, c ($p<0.05$).

3.6. Fatty acids

The fatty acid changes of the groups treated with nutrient deficiencies at different salinities are given in Tables 5, 6 and 7. Among the saturated fatty acids (SFA) C16:0 (palmitic acid) in the groups cultured at ‰45 salinity, the highest N(-) group was determined. Monounsaturated fatty acids (ΣMUFA) were higher in the 50% P(-) group compared to the control group, while it was lower in the 50% N(-) group and this group was statistically different from the other groups ($p<0.05$). C16:1 (palmitoleic acid) from MUFA was found in the group with the highest P (-), while C18:1ω9 (Oleic acid) showed similar values and lower in both groups compared to the control group. Polyunsaturated fatty acids (ΣPUFA) were found in the group with the highest P(-) and the lowest N(-), and differences were determined between the groups ($p<0.05$). C18:3ω3 (α- Linoleic acid) was the most dominant fatty acids within the PUFA group. While C18:2ω6c (linoleic acid) from PUFA was found to be similar in both groups, C18:3ω3 (α- Linoleic acid) was determined as 11.155% in the group with the highest P(-). In the phosphorus deficient group, EPA was determined as the highest group with 4.425%. Accordingly, total unsaturated fatty (ΣUFA) acids with 45.41% were highest in the group with 50% P(-) and the lowest in the group with 50% N(-) (Table 5).

Table 5
Fatty acid profile of *T. suecica* (at ‰45 salinity)

Fatty Acids	**Name**	**Control(‰45)**	**%50N(-)**	**%50 P(-)**
C6:0	Caproic acid	1.125±0.075	0.23±0.03	0.34
C8:0	Caprilic acid	1.26±0.01	1.37	1.25
C10:0	Capric acid	1.86±0.05	2.86±0.02	2.055±0.05
C11:0	Undecylic acid	0	0.03	0
C12:0	Lauric acid	2.035±0.045	3.34	2.31±0.01
C14:0	Myristic acid	1.77±0.06	2.945±0.015	1.995±0.005
C16:0	Palmitic acid	29.24±0.68	24.12±0.04	22.2±0.05
C17:0	Margaric acid	0.51±0.01	0.72	0.65
C18:0	Stearic acid	4.41±0.1	4.925±0.005	7.61±0.01
C20:0	Arachidic acid	2.055±0.045	1.655±0.005	3.565±0.075
C22:0	Behenic acid	0.35±0.01	0.525±0.005	0.06
C24:0	Lignoceric acid	0.03	0.04	0.05
ΣSFA		**44.64±1.085^{a}**	**42.76±0.12^{b}**	**42.08±0.2^{b}**
C14:1	Myristoleic acid	1.15±0.03	1.1	1.07
C15:1	Ginkgolic acid	0.115±0.005	0	0.185±0.005
C16:1	Palmitoleic acid	2.13±0.05	2.385±0.025	2.805±0.015
C17:1		1.725±0.045	1.35±1.3	1.835±0.015
C18:1ω9	Oleic acid	14.79±0.27	12.42±0.01	12.685±0.055
C18:1ω7	Vacsenic acid	3.805±0.045	5.79±0.011	4.9±0.08
C20:1ω9	Gondoic acid	0.21±0.01	0.23	0.255±0.025
C22:1ω9	Erucic acid	0.055±0.005	0.06	0.595±0.005
C24:1ω9	Nervonic acid	0.15±0.06	0.305±0.005	0.13±0.01
ΣMUFA		**24.13±0.52^{a}**	**23.64±1.351^{b}**	**24.46±0.21^{a}**
C18:2ω6c	Linoleic acid	5.02±0.12	4.44±0.02	4.39±0.01
C18:3ω6	γ- Linoleic acid	0.71±0.02	1.22±0.01	0.245±0.012
C18:3ω3	α- Linoleic acid	7.85±0.11	8.34±0.05	11.155±0.03
C20:2	Eicosadienoic acid	0.04	0.06	0.045±0.005
C20:4ω6	Arachidonic acid	0.14	0.225±0.005	0.135±0.005
C20:3ω6	Dihomo-γ-linolenic acid	0.03	0.05	0.485±0.005
C20:5ω3	Eicosapentaenoic acid (EPA)	3±0.07	4.05±0.03	4.425±0.005
C22:6ω3	Docosahexaenoic acid (DHA)	0.07	0.11	0.07
ΣPUFA		**16.68±0.32^{b}**	**15.13±0.11^{c}**	**20.95±0.072^{a}**
ΣUFA		**40.81**	**38.77**	**45.41**
Defined		**85.45±0.65**	**81.53±0.52**	**87.49±0.16**

There is a statistical difference between the means of the columns symbolized by the letters a, b, c ($p<0.05$).

Among the groups cultured at ‰30 salinity, SFA was the opposite of that at ‰45 salinity and was determined in the group with the highest N reduction. In the other two groups, the results were similar ($p>0.05$). ΣMUFA, on the other hand, was found to be the highest in the P(-) group and the lowest in the N(-) group, and all three groups differed statistically ($p<0.05$). Oleic acid is the predominant fatty acid in MUFA and it was determined the highest in the N(-) group with 15.72%, while it was found in the control group with 11.595%. In the PUFA group, the dominant fatty acid was α-Linoleic acid, as at ‰45 salinity. At this salinity, α- Linoleic acid was determined as 10.93% in the group with the highest P(-). EPA was determined in the group with the lowest control and the highest P(-). While ΣPUFA was 21.35% in the group with the highest P(-), total UFA acids were determined with the highest 48% in the control group (Table 6).

Table 6
Fatty acid profile of *T. suecica* (at 30‰ salinity)

Fatty Acids	**Name**	**Control(‰30)**	**50% N(-)**	**50% P(-)**
C6:0	Caproic acid	0	1.69±0.04	1.88
C8:0	Caprilic acid	1.45±0.01	1.52	1.47±0.02
C10:0	Capric acid	3.33±0.01	2.42±0.01	2.37±0.015
C12:0	Lauric acid	3.9±0.01	2.675±0.005	2.57±0.025
C14:0	Myristic acid	3.41±0.02	2.3±0.01	2.24±0.01
C16:0	Palmitic acid	21.885±0.035	24.425±0.025	22.24±0.285
C17:0	Margaric acid	0.21	0.465±0.005	0.88±0.015
C18:0	Stearic acid	6.6±0.02	5.64±0.01	8.33±0.09
C20:0	Arachidic acid	2.765±0.005	2.15	3.27±0.06
C22:0	Behenic acid	0.08±0.001	0.65±0.005	0.05
C24:0	Lignoceric acid	0.06	0.065±0.005	0.06
ΣSFA		**43.85±0.121^{b}**	**44±0.115^{b}**	**45.46±0.52^{a}**
C14:1	Myristoleic acid	1.235±0.005	0.85	0.69±0.005
C15:1	Ginkgolic acid	0.175±0.005	0	0.18±0.005
C16:1	Palmitoleic acid	4.915±0.015	1.565±0.005	1.76±0.02
C17:1		3.13±0.01	2.045±0.015	2.04±0.02
C18:1ω9	Oleic acid	11.595±0.005	15.72±0.03	13.69±0.0165
C18:1ω7	Vacsenic acid	6.015±0.105	4.92±0.06	4.3±0.11
C20:1ω9	Gondoic acid	0.245±0.005	0.15	0.17
C22:1ω9	Erucic acid	0.295±0.005	0.69±0.02	0.6±0.01
C24:1ω9	Nervonic acid	0.225±0.005	0.365±0.005	0
ΣMUFA		**27.83±0.16^{a}**	**26.30±0.135^{b}**	**23.43±0.7^{c}**
C18:2ω6c	Linoleic acid	3.76±0.02	4.56±0.04	4.45±0.06
C18:3ω6	γ- Linoleic acid	0.235±0.015	0.125±0.005	0.13
C18:3ω3	α- Linoleic acid	8.7±0.02	9.59	10.93±0.125
C20:2	Eicosadienoic acid	0	0.05	0.06
C20:4ω6	Arachidonic acid	0.83±0.01	0.805±0.005	0.14
C20:3ω6	Dihomo-γ-linolenic acid	0.29	0.065	0.72±0.01
C20:5ω3	Eicosapentaenoic acid (EPA)	3.11±0.01	4.39	4.87±0.08
C22:6ω3	Docosahexaenoic acid (DHA)	0.1±0.04	0.06	0.05
ΣPUFA		**20.23±0.115^{b}**	**19.64±0.05^{c}**	**21.35±0.275^{a}**
ΣUFA		**48.06**	**45.95**	**44.78**
Defined		**91.91±0.132**	**89.95±0.1**	**90.34±0.49**

There is a statistical difference between the means of the columns symbolized by the letters a, b, c ($p<0.05$).

ΣSFA at ‰15 salinity was determined as 46.615% in the N(-) group and all groups differed from each other ($p<0.05$). The most dominant fatty acid in the SFA group was palmitic acid, and the highest N(-) was determined in the group applied. MUFA group fatty acids were the most in the N(-) group (24.29%), and oleic acid was the most dominant fatty acid in all groups. Oleic acid was found to be 10.335% in the group with the lowest P(-). The most dominant fatty acid in ΣPUFA was α- Linoleic acid as in the other groups, and it was determined as 7.235% in the group with the lowest P(-). Linoleic acid (7.47%) and EPA (5.425) were also determined in the group with the highest P(-). ΣPUFA was determined with the highest rate of 21.335% in the P(-) group, and there was a difference between the groups ($p<0.05$). Total UFA were found to be 41.44% in the control group and 41.49% in the P group, while it was 44.125% in the N group (Table 7).

Table 7
Fatty acid profile of *T. suecica* (at ‰15 salinity)

Fatty Acids	**Name**	**Control(‰15)**	**50% N(-)**	**50% P(-)**
C6:0	Caproic acid	0.26±0.04	2.34±0.042	2.02±0.32
C8:0	Caprilic acid	1.51±0.02	2.375±0.005	1.865±0.035
C10:0	Capric acid	3.58±0.04	3.56±0.03	3.43±0.04
C12:0	Lauric acid	4.205±0.045	3.94±0.05	3.89±0.01
C14:0	Myristic acid	3.71±0.05	3.4±0.07	3.415±0.025
C16:0	Palmitic acid	18.315±0.215	23.335±0.095	20.04±0.04
C17:0	Margaric acid	0.39±0.01	0.185±0.005	0.74
C18:0	Stearic acid	7.645±0.085	4.705±0.065	5.69±0.01
C20:0	Arachidic acid	3.88±0.07	2.02±0.01	3.95±0.01
C22:0	Behenic acid	0.06±0.03	0.7	0.06
C24:0	Lignoceric acid	0.03	0.045±0.005	0.04
∑SFA		**43.71±0.605^{c}**	**46.61±0.377^{a}**	**45.31±0.49^{b}**
C14:1	Myristoleic acid	0.73±0.01	0.795±0.025	0.745±0.005
C15:1	Ginkgolic acid	0	0	0
C16:1	Palmitoleic acid	2.44±0.02	1.955±0.005	2.29
C17:1		3.295±0.025	3.065±0.045	2.985±0.005
C18:1ω9	Oleic acid	11.34±0.11	11.9±0.12	10.335±0.035
C18:1ω7	Vacsenic acid	3.44±0.06	3.315±0.135	3.505±0.065
C20:1ω9	Gondoic acid	0.325±0.045	2.21±0.01	0.15±0.01
C22:1ω9	Erucic acid	0.305±0.225	0.6±0.01	0.06
C24:1ω9	Nervonic acid	0.515±0.115	0.45±0.07	0.385±0.005
∑MUFA		**22.39±0.6^{b}**	**24.29±0.42^{a}**	**20.45±0.125^{c}**
C18:2ω6c	Linoleic acid	4.055±0.065	4.53±0.05	7.47
C18:3ω6	γ- Linoleic acid	0.84±0.069	1.46±0.02	0.175±0.025
C18:3ω3	α- Linoleic acid	9.095±0.225	8.665±0.075	7.235±0.015
C20:2	Eicosadienoic acid	0.05	0.05	0.09
C20:4ω6	Arachidonic acid	0.088±0.05	0.145±0.005	0.215±0.005
C20:3ω6	Dihomo-γ-linolenic acid	0.335±0.005	0.445±0.005	0.52
C20:5ω3	Eicosapentaenoic acid (EPA)	4.56±0.05	4.49±0.03	5.425±0.005
C22:6ω3	Docosahexaenoic acid (DHA)	0.03	0.05	0.205±0.005
∑PUFA		**19.05±0.464^{c}**	**19.83±0.185^{b}**	**21.33±0.05^{a}**
ΣUFA		**41.44**	**44.12**	**41.79**
Defined		**85.15±0.55**	**90.74±0.32**	**87.10±0.22**

There is a statistical difference between the means of the columns symbolized by the letters a, b, c ($p<0.05$).

As a result, among all three salinity groups, total UFA were determined highest in ‰30 salinity N(-) and lowest in ‰45 salinity N(-) group. While MUFA fatty acids were found at ‰30 salt concentration in the group made with 50% N(-) the highest, PUFA fatty acids were found in the groups made with P(-) at ‰30 and ‰15 salinity the highest. The lowest PUFA values were determined at ‰45 salinity N(-), and the highest values were determined in all salinity groups in the groups with P(-) (Table 8).

Table 8
Fatty acid values of all groups (%)

	Control			50% N(-)			50% P(-)		
	‰45	**‰30**	**‰15**	**‰45**	**‰30**	**‰15**	**‰45**	**‰30**	**‰15**
SFA	44.645^{c}	43.85^{d}	43.71^{de}	42.76^{e}	44^{d}	46.61^{a}	42.08^{f}	45.46^{b}	45.31^{b}
MUFA	24.13^{c}	27.83^{a}	22.39^{e}	23.64^{d}	26.30^{b}	24.29^{c}	24.46^{c}	23.43^{d}	20.45^{f}
PUFA	16.68^{d}	20.23^{b}	19.05^{c}	15.13^{e}	19.64^{c}	19.83^{bc}	20.95^{ab}	21.35^{a}	21.33^{a}
UFA	40.81^{e}	48.06^{a}	41.44^{d}	38.77^{f}	45.95^{b}	44.12^{c}	45.41^{b}	44.78^{c}	41.79^{d}

There is a statistical difference between the means of the columns symbolized by the letters a, b, c, d, e, f ($p<0.05$).

4. Discussion

N and P deficiency and different salt concentration treatments, which are stress conditions that stimulate the increase of lipid content of *T. suecica* from the green microalgae group, have been studied in vitro. In this study, the effects of 3 different salinity (‰15, 30 and 45) and nutrient deficiencies (50% N(-) and 50% P(-)) on growth, protein, lipid and fatty acid changes in *T. suecica* were investigated. Environmental factors are effective in the growth and biochemical structure of algae, as well as the type and density of nutrient elements in the culture medium (Brown et al, 1989). It is known that the change in N sources (such as ammonium, nitrate, and nitrite) and their concentrations are effective in the growth and biochemical structure of algae (Gökpınar, 1991; Fidalgo et al, 1995). In general, F/2 medium is used for the growth of marine microalgae in the laboratory environment. $NaNO_3$ is used as N source and $NaH_2PO_4.H_20$ is used as P source in the nutrient medium. This study is based on NO_3-N 882 µmol/l and PO_4 36.3 µmol/l (Guillard, 1973). N limitation by 50% (441 µmol/l) reduction in N source and P limitation by 50% (18.15 µmol/l) reduction in P source. N and P addition rates were among the parameters affecting growth in *T. suecica*. It was observed that there were decreases in OD and biomass amounts in *T. suecica* cultured at all three salinity values where 50% N and P restriction were applied. In a study, it was stated that low N concentration reduced growth in *N. oculata*, but did not affect it in *C. vulgaris* (Converti et al, 2009). It has been reported that when cultured in different N sources and N-free media, the growth of *Ellipsoidion* sp. slows down in N-free media (Xu et al, 2001). Khozin-Goldberg,& Cohen (2006) reported that when the freshwater species *Monodus subterraneus* was cultured in an environment with P restriction, the amount of dry weight decreased with P restriction. Salinity is the most important environmental factor affecting the development, biochemical structure and distribution of algae. It is known that with the decrease in photosynthesis rate of algae cells due to the increase in salinity, protein synthesis from their biochemical content also decreases. At the same time, it has been reported that with the increase in salinity, the growth slows down and there is a significant decrease in production (Zhang et al, 2010). Marine algae have a very high tolerance to salinity changes. It has been stated that many species show good growth at salinities lower than the optimum salinity values. While most of the marine algae prefer ‰12-44, optimum ‰20-24 salinity, some algae species can continue their photosynthetic activities for a while even if the growth rate decreases very much at ‰140 salinity (Ben-Amotz, & Avron, 1983; Borowitzka, & Borowitzka, 1988). While *T. suecica* prefers ‰25-35 salinity, *I. galbana* ‰15, *S. platensis* shows good growth at ‰1 salinity (Vonshak, & Tomaselli, 2000). In this study, the best growth was obtained in the group containing ‰30 salinity. The closest to this was determined at ‰45 salinity. The lowest growth was found at ‰15. *T. suecica* showed good growth in a wide salinity range of 15-45 ppt. It is in agreement with the results reported in previous studies. According to Alsull, & Maznah Omar (2012); At different salinity concentrations (‰20, ‰25, ‰30, ‰33, ‰35 and ‰38), *Tetraselmis* sp. and *Nannochloropsis* sp. achieved the best growth at ‰33 salinity concentration. Durmaz, & Pirinç (2017) reported in their study that the highest cell number of *T. chuii* species was obtained in cultures with a salinity concentration of ‰40, the highest amount of dry weight in culture media adjusted at ‰30 and ‰40 salinity. They stated that *T. suecica* developed in the range of 15-60 ppt (Venckus et al, 2021), 20-60ppt (Pugkaew et al, 2019), 25-35 ppt (Fabregas et al, 1986). In the studies, *T. suecica* was cultured in four different salinity values (15, 30, 60 and 90 ppt). They reported that the highest growth was achieved at 15 ppt salinity, while growth slowed down at other salinity values. They also stated that the highest protein content (56.9%) was found in this group, while the highest lipid ratio was 27.7% at 30ppt salinity. They reported that the protein and lipid content decreased as the salt content increased (Venckus et al, 2021). Pugkaew et al. (2019) also found that when *T. suecica* is cultured under 6 different salinities (10,

20, 30, 40, 50 and 60 ppt), the species can be cultured between 20 and 60 ppt values, but 10ppt is essential for the development of the species. They reported that there is no suitable salinity and that the growth is lower than other salinity rates. At the same time, they reported that the highest growth was at 30 ppt, and the growth at 20, 40, 50 and 60 ppt values was similar to each other. They reported that while the protein content was found at the highest 10 ppt, this ratio decreased as the salinity increased, and the highest lipid content was at 60 ppt. Gu et al. (2012) reported that N. oculata had the highest lipid content at ‰25 and 35 salinity, and the lowest at ‰15 salinity. At the same time, they determined that the protein content decreased with salinity. In our study, the highest protein was found in the control group with ‰30 salinity, while the closest value was obtained in the control group at ‰15 salinity. In this study, highest lipid content was found as 39.8% in the group with the ‰30 salinity and 50% N(-). Salinity is the most important factor affecting microalgal production yield, especially in uncontrolled environments of growing systems. Uncovering the optimal salinity range and high-value biochemical production pathways for algal biomass helps increase production potential and reduce cost (Pugkaew et al, 2018). In our study, it was observed that salinity variations caused a decrease or increase in the amount of biomass by affecting the biochemical structure of *T. suecica*. Elements such as N, P, S, Ca, Mg, Si are essential elements in algae, and these elements are significantly needed in the environment. N (-) and P (-) caused decreases in OD and dry weight values. The highest OD and dry weight amounts obtained in the culture were determined in the control groups at all three salinity values, while the lowest values were determined in the N(-) groups. In their study, Bondiolli et al. (2012) determined that while the dry weight efficiency was 7.8 g $m^{-2}day^{-1}$ at the end of the first week in cultures with N restriction in *T. suecica*, this value was 2.2 g $m^{-2}day^{-1}$ in the second week of the experiment.

The application of N and P deficiency, which is effective in the growth of algae, among the elements used in the nutrient medium, increases the lipid ratio in many algae species and causes a decrease in the protein ratio. In the late 1940s, N starvation in algae was found to be effective on lipid storage, and a lipid level of 70-85% in dry weight was reported. *Dunaliella* sp. or *T. suecica* species, on the other hand, contain low lipid and generally produce carbohydrates rather than lipids (Piorreck et al., 1984). Uslu et al. (2011) cultured *S. platensis* in media containing different amounts of N and obtained the highest lipid content in media containing 100%N(-). They also determined the highest protein ratio in the control group. Mutlu et al. (2011) found the highest lipid in *C. vulgaris* as 35.6% in the 100% N(-) group in their study. In their study, Gouveia et al. (2009) found the maximum total lipid ratio of 56% in N. oleabundans species cultured with N limitation. In their study, Roopnarain et al. (2014) determined that the highest lipid contents were obtained in cultures containing 25%P and lower levels of P in *I. galbana*, which they cultured with different levels of P restriction. *Nephrochlamys yushanlensis* control was cultured with N, N-P and P deficiency and lipid contents were determined. Total lipid contents were found to be 31% control, 58.6% N(-), 34% P(-), and 49% N-P(-), respectively (Maltsev et al, 2021). In this study, both N and P deficiencies caused an increase in lipid ratios and a decrease in protein ratios in all salinity groups. It shows that applied N deficiency increases the amount of lipid more than P deficiency. Nutritional and environmental factors can affect the ratio of fatty acids relative to each other as well as the total lipid content. Many studies have reported that fatty acids or total lipid productivity can be increased when salinity is higher (Takagi and Karseno 2006; Xia et al, 2013; Ho et al, 2014) or lower (Kim et al. 2016a). The main role of fatty acids in algae is related to cell membrane functions and metabolic processes (Guschina and Harwood, 2006). The degree of unsaturation of fatty acids is also an important parameter in the adaptation of algae to environmental conditions. Changes in the lipid fatty acid profile in response to the high salinity of the environment are necessary to retain membrane fluid and prevent its degradation. However, data on the effect of sodium chloride on the fatty acid composition of algal lipids are scarce and conflicting. *Isochrysis* cells grown under high concentrations of NaCl in medium contain increasing proportions of the PUFA C18 and C22 (Ben-Amotz et al. 1985), whereas Renaud, & Parry (1994) found C18:5 in high-salinity Isochrysis sp. and reported a reduction in C22:6. It has been reported that *Dunaliella, Nannochloropsis* and *N. frustulum* contain less UFA at high NaCl concentrations (Renaud and Parry 1994; Xu and Beardall 1997; Hu and Gao 2006). In our study, total SFA were highest in the ‰15 salinity N and P deficiency groups (46% and 45%) and in the ‰30 P deficiency group (45%); Total UFA were highest in the ‰30 salinity control group (48%), and the lowest in the ‰45 salinity N(-) group (38.77%). In their

study, Kim et al. (2016) determined that the UFA values of *Tetraselmis* sp. (35-22ppt) at 2 different salinities were higher at 35 ppt (58%) and SFA values at 22ppt. In our study, the most dominant fatty acids in all groups were palmitic acid (C16:0), oleic acid (C18:1ω9), α- Linoleic acid (C18:3ω3) and EPA (C20:5ω3). The predominant presence of these fatty acids in *Tetraselmis* members has also been reported previously (Griffiths et al, 2012; Adarme-Vega et al, 2014; Kim et al, 2016). Our results indicate that salinity levels have an effect on fatty acids. EPA, which is an important fatty acid in terms of human health, constituted 3-5.425% of fatty acids. Similar but slightly higher EPA levels of 6% to 7% have been reported in *Tetraselmis* sp. (Adarme-Vega et al, 2014). The main fatty acids Palmitic acid (C16:0) and oleic acid (C18:1) found in vegetable lipids are widely used in biodiesel production and are important determinants of biodiesel quality (Kaur et al.2012; Knothe 2008) and in high amounts in *T. suecica*. found. It seems possible that the lipid obtained from *Tetraselmis* can be used as a biodiesel raw material. However, linoleic acid (C18:3) and PUFAs, which greatly reduce the oxidation stability of biodiesel, may require the addition or blending of antioxidants to improve biodiesel quality (Griffiths et al, 2012). Huang et al. (2013) determined in their study that T. *subcordiformis* contains a high amount of 18:3ω3 (17.68-22.22%) at different N concentrations. In our study, 18:3ω3 (α-Linoleic acid) fatty acid (7.235-11.155%) was the most dominant among PUFAs. Reducing the amount of N and P in the microalgae medium causes a decrease in the cell density and protein amount, and due to the inability to produce the energy molecules (ATP and NADPH) that provide chlorophyll synthesis, the amount of chl *a* decreases. (Shifrin, & Chisholm, 1981; Sukenik et al, 1989; Roopnarain et al, 2014). In this study, decreases in the amount of chl *a* and protein in the groups treated with N and P deficiency; Increases in lipid content were observed.

One of the factors affecting the growth and biochemical structure of algae is the period of illumination. As the illumination period gets longer, the growth rate of the algae increases and the cell numbers increase. Studies have reported that there is not much difference in terms of algal development between the 18-hour illumination period and the 12-hour illumination period (Oh et al, 2009). In this study, 16:8 light-dark period and 80 $\mu molm^{-2}s^{-1}$ light intensity were applied to the cultures. In a study, *P. cruentum* species were grown in growth media (12:12 light-dark period and CO_2 carbon source, 6:18 light-dark period and CO_2 carbon source, 18:6 light-dark and CO_2 carbon source, 24 hours dark and glucose. carbon source, 24 hours dark and glycerol carbon source) were cultured and the highest lipid content was found with 19.3% in the 12:12 light-dark period group (Oh et al, 2009). In another study, *I. galbana* was cultured with 24 hours of continuous light and 8 hours of continuous light, and the highest PUFA was obtained in cultures with 8 hours of illumination (Bandarra et al, 2003). It is concluded that these studies and the light-dark period applied in our study positively affect the lipid increase. The biochemical content of the biomass obtained in different phases of the growth period in microalgae cultures also differs. The amount of lipid is related to the age of the algae culture, and the amount of lipid obtained increases as the culture ages. The total amount of lipid obtained in the stationary phase of the culture is higher than that obtained from other phases (Ying et al, 2002). In this study, the culture was harvested after entering the stationary phase and the lipid amounts were examined in this phase. In a study in which nitrate, nitrite and urea were used as N sources in the medium, *I. galbana* was cultured and no difference was found between them in terms of growth. However, the highest lipid ratio was found with urea (42.05%) and nitrite (41.61%) at the end of the stationary phase in the culture harvested at the beginning of the stationary phase of the study. Fatty acids PUFA (62.27%), EPA (27.66%) and DHA (14.13%) were found in the group cultured with the highest urea and at the beginning of the stationary phase. In the group cultured with urea, the protein was found to be the highest (44.96%) in the phase where the logarithmic stage increased (Fidalgo et al, 1998).

5. Conclusion

In the study, different salinity and nutrient deficiencies caused changes in cell densities, chl *a*, dry weight, protein, lipid and fatty acids of microalgae. It was determined that the most suitable salinity value in terms of growth dynamics of *T. suecica* was ‰30. Protein and lipid values were determined at high values at ‰30 salinity, followed by ‰15 salt concentration. In the nutrient deficiency studies, it was determined that the

control groups still had the highest growth dynamics, and the closest group was the 50% P(-) group. The growth was lowest in the N(-) treated groups. When the protein values were examined, the lowest was found in this group. On the contrary, lipid values were found at the highest level in this group, while the lowest was determined in the control group. When lipid enhancement studies are desired, it can be said that the most suitable salinity for this species is ‰30 and it is appropriate to use a medium whose N is reduced by half. UFA were highest in the control group with ‰30 salinity and lowest in the group with high salinity and N(-). PUFA group fatty acids, which we have to take from outside in terms of the health of living things, were at the highest levels in all salinity groups made with P(-). EPA, which is included in PUFA, was also higher in P(-) made groups. Accordingly, it can be said that the reduction of phosphorus in the medium at different levels may cause an increase in PUFA.

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Author Contributions

Cananur Pihava: Collected data and performed the analysis.

Leyla Uslu: Planned, analysed data, conducted experiments and wrote all parts of manuscript.

Conflicts of Interest

There is no conflict of interest.

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