Effects of Nitrogen Starvation on Growth and Biochemical Composition of Some Microalgae Species

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Abstract

Nitrogen is one of the most important nutrient sources for the growth of microalgae. We studied the effects of nitrogen starvation on the growth responses, biochemical composition and fatty acid profile of *Dunaliella tertiolecta, Phaeodactylum tricornutum* and *Nannochloropsis oculata*. The lack of nitrogen caused changes in carbohydrate, protein, lipid and fatty acid composition in all examined microalgae. The carbohydrate content increased 59% in *D. tertiolecta*, while the lipid level increased 139% in *P. tricornutum* under nitrogen stress conditions. Nitrogen starvation increased the oligosaccharide and polysaccharide contents of *D. tertiolecta* 4.18-fold and 3.77-fold, respectively. Furthermore, triacylglycerol (TAG) levels in *N. oculata* and *P. tricornutum* increased 2.3-fold and 7.4-fold, respectively. The dramatic increase in the amount of TAG is important for the use of these microalgae as raw materials in biodiesel. Nitrogen starvation increased the amounts of oligosaccharides and polysaccharides of *D. tertiolecta*, while increased eicosapentaenoic acid (EPA) in *N. oculata* and docosahexaenoic acid (DHA) content in *P. tricornutum*. The amount of polyunsaturated fatty acids (PUFAs), EPA, DHA, oligosaccharides and polysaccharides in microalgal species can be increased without using the too costly nitrogen source in the culture conditions, which can reduce the most costly of living feeding.

Introduction

Microalgae, due to their valuable biochemical content, are preferred as raw materials in many areas (Sathyamoorthy and Rajendran 2022), such as animal feed (Borowitzka 1997; Ansari et al. 2021), biofuel (Borowitzka 2010; Moshood et al. 2021; Venkata Subhash et al. 2022), nutraceutical (Barkia et al. 2019; Zanella and Vianello 2020) and functional foods (Andrade 2018). Microalgae play an important role, particularly in fish and shrimp farming due to their protein, lipid and carbohydrate contents in addition to their size (Baharuddin et al. 2016). Microalgae have the potential to replace traditional aquaculture feeds due to their rich nutritional content and accumulation of triglycerides and protein in the muscle. The use of microalgae as feed in aquaculture increases resistance to diseases, omega 3 content and skeletal quality while reducing nitrogen output to the environment (Becker 2013). The amount of polyunsaturated fatty acids, particularly eicosapentaenoic acid (EPA), arachidonic acid (ARA), and docosahexaenoic acid (DHA), in their food content is very important for the normal growth and survival of fish larvae (Reitan et al. 1997).

There is general agreement that the biochemical composition of microalgae varies depending on species, light, temperature, salinity, pH and culture conditions (Behrens and Kyle 1996; Becker 2013; Yaakob et al. 2021). Photosynthetic efficiency, biomass productivity, protein, lipids, carbohydrates, fatty acids, vitamins and pigment accumulation are affected under stress conditions (Tebbani et al. 2014; Panis and Carreon 2016). In addition, under environmental stress conditions, microalgae cells synthesize neutral lipids in the form of triacylglycerol (TAG) (Scott et al. 2010). TAGs are highly valuable biological molecules due to their importance in health, agriculture, chemical industry and biological systems. They offer an efficient way to store carbon and energy (Li-Beisson et al. 2021). Many microalgal species contain high amounts of lipids with TAG content, which is important in biodiesel production (Borowitzka and Moheimani 2013).

Some microalgal species can also produce lipids in the form of TAG, similar to vegetable oils, with EPA and DHA that are important for nutrition (Klok et al. 2014).

Nitrogen is the major element in microalgae culture, as it plays a role in protein, DNA and chlorophyll synthesis. Nitrogen restriction or starvation can affect the growth of microalgae, the synthesis of protein and carbohydrates and particularly increase lipid accumulation (Benvenuti et al. 2015; Shen et al. 2016; Zarrinmehr et al. 2020). Lipid and carbohydrate accumulation increased in *Chlorella zonfingiensis* cells under nitrogen starvation conditions and carbohydrate synthesis occurred before lipid synthesis (Zhu et al. 2014). Janssen et al. (2019) have reported that *Nannochloropsis gaditana* accumulates TAG in response to nitrogen starvation.

The focus of the present study was to investigate the effects of nitrogen starvation (nitrogen-free culture medium, N^-) on the growth responses, carbohydrate, protein, lipid contents and fatty acid profiles of *Dunaliella tertiolecta*, *Phaeodactylum tricornutum* and *Nannochloropsis oculata*. In addition, the fold changes in the amount of TAG, oligosaccharide and polysaccharide were investigated by Fourier transform infrared (FTIR) spectroscopy. FTIR is a useful method for identifying the functional groups of biological samples (Arif et al. 2021), such as microalgal biomass (Stehfest et al. 2005) and intra/extracellular metabolites (Kosa et al. 2017). Considering all data obtained from the trials, knowledge and advice are given about which microalgal species can be used in which sector or sectors.

Materials & Methods

Microalgal species and experimental design

Dunaliella tertiolecta K-0591, *Phaeodactylum tricornutum* RCC2967 and *Nannochloropsis oculata* 849/1 were obtained from the Norwegian Culture Collection of Algae (NORCCA), the Roscoff Culture Collection (RCC) and the Culture Collection of Algae and Protozoa (CCAP), respectively. *D. tertiolecta* and *N. oculata* were maintained in F/2 culture medium (Guillard and Ryther 1962; Guillard 1975), while *P. tricornutum* was maintained in F/2 culture medium with silica.

Microalgal culture stocks (5 days old) were used to initiate the experiments. The initial optical density value of each trial was adjusted to 0.1. F/2 medium was used for the control group experiment, while F/2 without $NaNO_3$ was used for the nitrogen starvation group experiment (N^-). Microalgal species were cultured in 500 mL flasks containing 200 ml of culture medium for 10 days. All trials were constantly illuminated (100 µmol photons m^{-2} s^{-1}) and shaken three times a day. Cultivation was carried out at 20°C for *P. tricornutum* and 23°C for *D. tertiolecta* and *N. oculata.* All trials were performed in triplicate.

Optical density, cell number, dry weight and FT-IR were measured at 0, 3, 5, 7, and 10 days of the experiments, while total protein, carbohydrate, lipid and fatty acid profiles were determined at 0, 5, and 10 days under optimal and nitrogen starvation conditions. The algal sample was centrifuged at 10000 rpm at 4°C for 10 min, and the supernatant was discarded and then washed three times with distilled water.

The pellets were transferred to tubes and dried in a lyophilizer. Dried microalgae samples were used for FT-IR, biochemical composition and fatty acid profile analyses.

Growth Measurement

Optical density was measured by UV- spectrophotometry in triplicate at 625 nm for *P. tricornutum* and 680 nm for *D. tertiolecta* and *N. oculata.* The specific growth rate (SGR) was calculated using the following formulas (Godoy-Hernández and Vázquez-Flota 2006; Cirik and Gökpınar 2008):

$$SGR\ (\mu) = \ln(X_2/X_1)/(t_2-t_1)$$

where X_2 is the optical density of the test group at t_2 and X_1 is the optical density of the control group at t_1. The cell number was determined using a Neubauer counting chamber with homogeneous sampling. Dry weight measurements were determined according to Boussiba et al. (1992).

Ft-ir Analysis

The values corresponding to the 4000 – 400 cm^{-1} spectral range of the dried microalgae samples were determined by FT-IR (Bruker Vertex 70 ATR). FT-IR analyses were performed by Tekirdağ Namık Kemal University Central Research Laboratory (NABİLTEM). Using the data corresponding to the wavelengths read, changes in the amounts of TAG (1744 cm^{-1}/1652 cm^{-1}), oligosaccharide (1145 cm^{-1}/1652 cm^{-1}) and polysaccharide (1045 cm^{-1}/1652 cm^{-1}) were determined (Angün 2013).

Biochemical Composition Analyses

The total protein content of the biomass was determined by the Bradford method (Bradford 1976), while the total carbohydrate content was assessed by the phenol—sulfuric acid method (Dubois et al. 1956) and the sulfo-phospho-vanillin method was used for the total lipid analysis (Mishra et al. 2014). Fatty acid samples were analyzed by GC-FID. To determine the location of the peaks, analyses were performed on an HP-88 100 m fatty acid column using supelco-fame mix 37. Methanolic potassium hydroxide and isooctane were used for sample preparation (Gumus et al. 2015). The fatty acid composition of microalgal species was determined by the Ege University Central Research Test and Analysis Laboratory Application and Research Center (EGE-MATAL).

Statistical analysis

All trials were performed in three replications and the data were expressed as the standard deviation of the mean. Levene's test was applied for homogeneity of variances. Differences among the experimental groups were determined by Tukey's test after one-way analysis of variance (ANOVA). Differences between groups were calculated with the independent sample t test at a 95% confidence interval. Pearson's

correlations were used to determine the direction of relationships between variables. IBM SPSS 25.0 and Microsoft Excel 2016 software were used to evaluate the obtained data.

Results

Cell growth characteristics

Changes in the optical density, cell number and dry weight of *D. tertiolecta, N. oculata* and *P. tricornutum* under control as well as nitrogen starvation (N^-) conditions are shown in Fig. 1. The lack of nitrogen caused slower growth in all microalgal species. Optical density, cell number and dry weight measurements that provide information about growth support each other. The specific growth rates (μ) of *D. tertiolecta, N. oculata* and *P. tricornutum* on the 5th day of the experiments in the control groups were 0.119 (day), 0.155 (day), and 0.223 (day), respectively, while they were 0.083 (day), 0.122 (day), and 0.226 (day) under N^- conditions, respectively.

Figure 1. Changes in the optical density, cell number and dry weight of *D. tertiolecta, N. oculata, P. tricornutum.* Values are presented as the mean ± standart deviation (n = 3) square: control group, triangle: nitrogen starvation group (N^-)

FTIR analysis

FTIR spectra indicate characteristic bands that reflect the biochemical composition of the sample. Qualitative and quantitative differences in the molecular composition of the sample are revealed by the location and intensity of these characteristic bands (Ferro et al. 2019). Fold changes in TAG, oligosaccharide and polysaccharide are shown in Fig. 2. The coefficient calculations were determined by rate the data obtained from the nitrogen starvation cultures to the data of the control group. The amounts of oligosaccharides and polysaccharides increased by 4.18-fold and 3.77-fold, respectively on the 7th day in the N^- group of *D. tertiolecta*. Nitrogen starvation caused a 2.3-fold increase in TAG levels and increased polysaccharide accumulation approximately 2-fold on the 10th day of *N. oculata* culture. In FTIR analyses of *P. tricornutum*, the amounts of TAG and polysaccharide increased 7.4-fold and 2.4-fold in the nitrogen-free conditions compared to the control conditions on the 7th day of the experiment. At the same time, the amount of oligosaccharides increased 5.3-fold in the nitrogen starvation group compared to the control group on the 10th day for *P. tricornutum*.

Figure 2. Fold changes of TAG, oligosaccharide and polysaccharide

Effect of nitrogen starvation on biochemical composition

The changes in carbohydrate, protein and lipid amounts of microalgal species under nitrogen starvation are presented in Table 1. Under nutrient stress, microalgae convert photosynthetic carbon flux into different pathways to produce energy-rich carbohydrates and lipids, which compete with each other during their synthesis (Siaut et al. 2011; Pancha et al. 2014). Carbohydrate production increased in the N^-

groups in *D. tertiolecta* and *P. tricornutum* but not in *N. oculata* cells. The maximum values of 5509.85 ± 265.5 and 5241.1 ± 259.2 μg mL^{-1} were observed under nitrogen starvation in *D. tertiolecta* and *P. tricornutum* on the 5th day of cultivation, while these were 1346.6 ± 30.6 μg mL^{-1} in the control group in *N. oculata* on the 10th day of cultivation. Pearson's correlation showed that the cell number for *N. oculata* had a strong positive correlation with carbohydrates (r:1.00), and a positive correlation was observed between optical density and carbohydrates in *D. tertiolecta* (r:0.998). *D. tertiolecta* had the highest protein level of 538.416 ± 14.5 μg mL^{-1} on the first day of the experiment. Protein accumulation decreased in *N. oculata* under nitrogen starvation conditions but increased in *D. tertiolecta* and *P. tricornutum* species compared to the control group on the last day of the experiment. A negative correlation was observed between dry weight and MUFAs in *D. tertioecta* under nitrogen stress conditions (r:0.997). Lipid content was increased under nitrogen starvation for all examined species. The highest lipid levels were determined on the 10th day in *D. tertiolecta, N. oculata* and *P. tricornutum* at 285.638 ± 3.7, 295.479 ± 5.3 and 562.234 ± 19.8 μg mL^{-1}, respectively. The Pearson correlation coefficient was 0.997, indicating a strong correlation between lipid content and cell number in *P. tricornutum* under nitrogen starvation.

Table 1
Biochemical composition of *D. tertiolecta, N. oculata* and *P. tricornutum*

Microalgae		Mean ± standard deviation (µg mL^{-1})				
		Day 0th	Day 5th		Day 10th	
		Control/N^-	Control	N^-	Control	N^-
Dunaliella tertiolecta	**Carbohydrate**	1177,6 ± 35,9	4027,35 ± 100,0[a]	5509,85 ± 265,5[b]	953,1 ± 44,2[c]	1517,1 ± 46,9[d]
	Protein	538,416 ± 14,5	408,589 ± 7,7[a]	321,337 ± 9,9[b]	401,658 ± 13,0[c]	441,015 ± 12,4[d]
	Lipid	143,085 ± 5,4	227,128 ± 8,4[a]	162,500 ± 7,9[b]	186,702 ± 5,6[c]	285,638 ± 3,7[d]
Nannochloropsis oculata	**Carbohydrate**	246,6 ± 11,7	190,85 ± 12,5[a]	140,6 ± 2,8[b]	1346,6 ± 30,6[c]	510,6 ± 26,6[d]
	Protein	56,485 ± 9,5	211,436 ± 6,6[a]	84,579 ± 4,8[b]	130,495 ± 12,4[c]	84,084 ± 12,3[d]
	Lipid	72,074 ± 3,5	150,798 ± 47,5[a]	134,043 ± 5,3[a]	253,989 ± 3,2[b]	295,479 ± 5,3[c]
Phaeodactylum tricornutum	**Carbohydrate**	879,85 ± 191,0	2963,35 ± 67,6[a]	5241,1 ± 259,2[b]	4118,35 ± 127,8[c]	4598,1 ± 35,8[d]
	Protein	106,547 ± 2,6	258,960 ± 6,1[a]	178,144 ± 20,7[b]	247,079 ± 12,2[c]	384,084 ± 23,1[d]
	Lipid	77,926 ± 4,6	205,053 ± 7,5[a]	368,617 ± 13,7[b]	235,106 ± 17,7[c]	562,234 ± 19,8[d]

Effect of nitrogen starvation on the fatty acid profile

The fatty acid profile of *D. tertiolecta* and the contents of saturated fatty acids (SFAs) and mono- and polyunsaturated fatty acids (MUFAs and PUFAs) are shown in Table 2. The dominant fatty acids of *D. tertiolecta* are palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1 cis), linoleic acid (C18:2 cis), linolenic acid (C18:3n3), eicosatrienoic acid (C20:3n3), eicosapentaenoic acid (C20:5) and docosahexaenoic acid (C22:6). C20:5 increased from 14.88–16.32%, and C18:1cis and C18:2cis increased from 5.48–6.51% and from 8.80–9.39%, respectively under nitrogen starvation conditions on the 10th day in *D. tertiolecta*. Under nitrogen-free conditions on the 5th day of culture, C18:0 and C22:6 increased from 5.34–6.24% and from 9.44–9.86%, respectively. In contrast, C18:3n3 decreased from 9.59–8.47% on the 5th day of the experiment.

A positive correlation was observed between PUFA and lipid amount in *P. tricornutum* (r:0.998) and a negative correlation was determined in *N. oculata* (r:0.998). A negative Pearson correlation was

determined between lipid and SFA levels in *D. tertiolecta* under nitrogen starvation. Additionally, a negative correlation was observed between dry weight and SFA level in *P. tricornutum* under nitrogen stress conditions (r:0.999).

Table 2
The fatty acid profile of *D. tertiolecta*

	Mean ± standard deviation (%)				
	Day 0th	Day 5th		Day 10th	
	Control/N^-	Control	N^-	Control	N^-
Tridecanoic (C13:0)	1.108 ± 0.069	1.109 ± 0.016	1.801 ± 0.000	1.722 ± 0.090	1.700 ± 0.020
Myristic (C14:0)	1.810 ± 0.063	1.611 ± 0.075	1.649 ± 0.000	1.742 ± 0.124	1.878 ± 0.071
Pentadecanoic (C15:0)	1.859 ± 0.040	1.378 ± 0.072	1.587 ± 0.000	1.289 ± 0.092	1.601 ± 0.048
Palmitic (C16:0)	12.485 ± 0.323	12.487 ± 0.113	12.384 ± 0.000	12.680 ± 0.292	12.516 ± 0.332
Palmitoleic (C16:1)	2.661 ± 0.146	2.377 ± 0.207	2.759 ± 0.000	2.705 ± 0.026	2.644 ± 0.116
Heptadecanoic(C17:0)	1.603 ± 0.029	1.157 ± 0.042	1.350 ± 0.000	1.410 ± 0.032	1.343 ± 0.122
cis-10-heptadecanoic (C17:1)	2.492 ± 0.045	2.354 ± 0.140	2.260 ± 0.000	2.490 ± 0.047	2.312 ± 0.063
Stearic (C18:0)	6.496 ± 0.039	5.345 ± 0.129	6.249 ± 0.000	5.810 ± 0.064	5.170 ± 0.219
Oleic (C18:1 cis)	6.525 ± 0.044	6.630 ± 0.178	6.093 ± 0.002	5.480 ± 0.147	6.516 ± 0.048
Linoleic (C18:2 cis)	8.677 ± 0.116	8.565 ± 0.056	8.757 ± 0.000	8.805 ± 0.115	9.395 ± 0.241
Arachidic (C20:0)	2.447 ± 0.152	2.667 ± 0.170	2.681 ± 0.004	2.777 ± 0.126	2.652 ± 0.150
cis-11-eicosenoic (C20:1)	2.697 ± 0.044	2.508 ± 0.098	2.587 ± 0.001	2.374 ± 0.069	2.644 ± 0.115
Linolenic (C18:3n3)	9.234 ± 0.057	9.591 ± 0.047	8.476 ± 0.002	8.862 ± 0.135	8.452 ± 0.115
cis-11,14-eicodadienoic (C20:2)	3.548 ± 0.157	3.625 ± 0.148	3.875 ± 0.000	3.830 ± 0.065	3.503 ± 0.352
cis-8,11,14-eicosatrienoic (C20:3n6)	4.281 ± 0.218	4.672 ± 0.163	4.879 ± 0.000	4.816 ± 0.087	4.509 ± 0.218
cis-11,14,17-eicosatrienoic (C20:3n3)	8.241 ± 0.158	8.733 ± 0.075	7.877 ± 0.000	8.517 ± 0.068	7.538 ± 0.149

	Mean ± standard deviation (%)				
	Day 0th	Day 5th		Day 10th	
	Control/N^-	Control	N^-	Control	N^-
cis-5,8,11,14,17-eicosapentaenoic (C20:5)	14.389 ± 0.088	15.751 ± 0.120	14.869 ± 0.000	14.881 ± 0.135	16.328 ± 0.269
cis-4,7,10,13,16,19-docosahexaenoic (C22:6)	9.448 ± 0.025	9.441 ± 0.293	9.868 ± 0.000	9.811 ± 0.064	9.299 ± 0.066
SFAs	27.809	25.754	27.701	27.429	26.859
MUFAs	14.374	13.868	13.699	13.050	14.117
PUFAs	57.817	60.378	58.601	59.521	59.023

The profile of fatty acids in *N. oculata* under different culture conditions is shown in Table 3. The dominant fatty acids in *N. oculata* are myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1 cis), linoleic acid (C18:2 cis), linolenic acid (C18:3n3), eicosatrienoic acid (C20:3n3), eicosapentaenoic acid (C20:5) and docosahexaenoic acid (C22:6). Under nitrogen starvation conditions on the 10th day, C18:1cis increased from 4.29–7.18% conspicuously. Furthermore, C20:1 increased only on the last day of the experiment under stress conditions, while C18:2cis increased on both the 5th and 10th days of culture. C22:6 decreased from 23.43–22.43% in the absence of nitrogen on the 5th day.

Table 3
The fatty acid profile of *N. oculata*

	Mean ± standard deviation (%)				
	Day 0th	Day 5th		Day 10th	
	Control/N^-	Control	N^-	Control	N^-
Tridecanoic (C13:0)	1.212 ± 0.035	1.397 ± 0.042	1.517 ± 0.043	1.400 ± 0.034	1.758 ± 0.000
Myristic (C14:0)	5.102 ± 0.021	4.611 ± 0.069	5.080 ± 0.068	4.696 ± 0.237	5.066 ± 0.000
Pentadecanoic (C15:0)	1.181 ± 0.076	1.355 ± 0.000	1.433 ± 0.136	1.634 ± 0.028	1.282 ± 0.090
cis-10-pentadecanoic (C15:1)	1.171 ± 0.090	1.292 ± 0.052	1.208 ± 0.037	1.343 ± 0.126	1.569 ± 0.118
Palmitic (C16:0)	9.177 ± 0.139	9.818 ± 0.087	9.777 ± 0.016	9.343 ± 0.133	9.340 ± 0.137
Palmitoleic (C16:1)	2.790 ± 0.122	2.249 ± 0.001	2.591 ± 0.082	2.061 ± 0.078	2.465 ± 0.089
Heptadecanoic (C17:0)	2.111 ± 0.034	2.226 ± 0.141	2.425 ± 0.100	3.108 ± 0.149	2.195 ± 0.086
cis-10-heptadecanoic (C17:1)	1.361 ± 0.081	1.406 ± 0.021	1.203 ± 0.086	1.410 ± 0.039	1.596 ± 0.213
Stearic (C18:0)	4.994 ± 0.057	4.658 ± 0.152	4.562 ± 0.058	3.470 ± 0.024	3.619 ± 0.054
Oleic (C18:1 cis)	4.285 ± 0.060	4.306 ± 0.059	4.505 ± 0.097	4.292 ± 0.092	7.189 ± 0.088
Linoleic (C18:2 cis)	4.688 ± 0.100	4.169 ± 0.093	4.804 ± 0.102	4.353 ± 0.164	4.601 ± 0.066
Arachidic (C20:0)	2.801 ± 0.063	2.721 ± 0.094	2.673 ± 0.113	4.415 ± 0.238	2.161 ± 0.104
cis-11-eicosenoic (C20:1)	3.490 ± 0.057	3.457 ± 0.310	3.170 ± 0.091	3.523 ± 0.092	3.621 ± 0.051
Linolenic (C18:3n3)	7.389 ± 0.055	6.495 ± 0.064	6.620 ± 0.165	7.109 ± 0.106	6.484 ± 0.068
cis-11,14- eicodadienoic (C20:2)	3.209 ± 0.106	3.784 ± 0.003	3.170 ± 0.161	3.107 ± 0.025	3.292 ± 0.249
cis-8,11,14-eicosatrienoic (C20:3n6)	2.344 ± 0.000	2.902 ± 0.079	2.759 ± 0.151	2.609 ± 0.065	2.624 ± 0.020

	Mean ± standard deviation (%)				
	Day 0th	Day 5th		Day 10th	
	Control/N^-	Control	N^-	Control	N^-
cis-11,14,17-eicosatrienoic (C20:3n3)	7.181 ± 0.078	7.621 ± 0.074	7.130 ± 0.069	7.301 ± 0.035	6.948 ± 0.097
cis-13,16-docosadienoic (C22:2)	0.586 ± 0.053	0.578 ± 0.014	0.674 ± 0.019	0.580 ± 0.009	0.755 ± 0.045
cis-5,8,11,14,17-eicosapentaenoic (C20:5)	12.591 ± 0.06	11.655 ± 0.32	12.264 ± 0.054	12.164 ± 0.075	11.800 ± 0.049
cis-4,7,10,13,16,19-docosahexaenoic (C22:6)	22.338 ± 0.328	23.301 ± 0.108	22.435 ± 0.154	22.082 ± 0.286	21.634 ± 0.443
SFAs	26.577	26.786	27.467	28.066	25.422
MUFAs	13.097	12.710	12.677	12.629	16.441
PUFAs	60.326	60.504	59.856	59.306	58.137

The effect of nitrogen starvation on the fatty acid profile of *P. tricornutum* is represented in Table 4. PUFAs constituted 47.18% of the total fatty acids in the control group on the 10th day of the experiment and 50.21% in the nitrogen-deprived group. The dominant fatty acids in *P. tricornutum* were palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1 cis), linolenic acid (C18:3n3), eicodadienoic acid (C20:2), eicosapentaenoic acid (C20:5) and docosahexanoic acid (C22:6). Under nitrogen starvation conditions, C16:0 increased from 14.58–15.59% and from 14.30–14.82% on the 5th day and 10th day of the experiment, respectively. A lack of nitrogen caused C16:1, C18:3n3 and C22:2 to increase from 4.63–6.02%, from 5.64–6.43% and from 4.49–5.36%, respectively on the last day. On the 5th day, C22:6 increased from 6.74–8.82% distinctly in the nitrogen-starved group.

Table 4
The fatty acid profile of *P. tricornutum*

	Mean ± standard deviation (%)				
	Day 0th	Day 5th		Day 10th	
	Control/N^-	Control	N^-	Control	N^-
Caprylic (C8:0)	1.474 ± 0.341	1.829 ± 0.066	1.493 ± 0.107	1.224 ± 0.074	1.308 ± 0.042
Tridecanoic (C13:0)	1.671 ± 0.177	1.935 ± 0.032	1.915 ± 0.048	1.745 ± 0.139	1.262 ± 0.005
Myristic (C14:0)	4.592 ± 0.310	3.887 ± 0.142	3.642 ± 0.123	4.689 ± 0.190	4.280 ± 0.051
Pentadecanoic (C15:0)	1.352 ± 0.093	1.485 ± 0.055	1.396 ± 0.007	1.504 ± 0.025	1.767 ± 0.026
Palmitic (C16:0)	12.948 ± 2.436	14.582 ± 0.207	15.594 ± 0.380	14.300 ± 0.120	14.825 ± 0.085
Palmitoleic (C16:1)	5.785 ± 0.844	4.861 ± 0.178	4.489 ± 0.626	4.638 ± 0.135	6.028 ± 0.118
Heptadecanoic (C17:0)	4.443 ± 0.079	4.055 ± 0.095	4.460 ± 0.124	6.059 ± 0.095	4.707 ± 0.030
cis-10-heptadecanoic (C17:1)	3.363 ± 0.689	2.798 ± 0.071	3.520 ± 0.092	3.198 ± 0.040	2.844 ± 0.031
Stearic (C18:0)	5.630 ± 0.091	5.701 ± 0.035	4.777 ± 0.142	4.855 ± 0.044	4.769 ± 0.122
Elaidic (C18:1 trans)	2.793 ± 0.064	2.201 ± 0.065	2.898 ± 0.057	2.508 ± 0.013	1.164 ± 0.029
Oleic (C18:1 cis)	6.332 ± 0.391	8.520 ± 0.145	7.174 ± 0.113	8.095 ± 0.041	6.829 ± 0.070
Linoleic (C18:2 cis)	4.751 ± 0.074	4.625 ± 0.073	4.659 ± 0.180	4.486 ± 0.068	4.565 ± 0.110
Linolenic (C18:3n3)	6.159 ± 0.359	6.559 ± 0.134	4.345 ± 0.170	5.646 ± 0.153	6.430 ± 0.080
Heneicosanoic (C21:0)	1.600 ± 1.848	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
cis-11,14- eicodadienoic (C20:2)	5.140 ± 0.584	5.685 ± 0.243	6.432 ± 0.245	6.017 ± 0.041	6.557 ± 0.453
Behenic (C22:0)	1.233 ± 1.432	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000

	Mean ± standard deviation (%)				
	Day 0th	Day 5th		Day 10th	
	Control/N^-	Control	N^-	Control	N^-
cis-8,11,14-eicosatrienoic (C20:3n6)	3.188 ± 0.429	3.595 ± 0.084	3.693 ± 0.261	3.204 ± 0.021	3.663 ± 0.149
cis-13,16-docosadienoic (C22:2)	4.992 ± 0.421	4.369 ± 0.080	4.700 ± 0.018	4.495 ± 0.041	5.361 ± 0.034
cis-5,8,11,14,17-eicosapentaenoic (C20:5)	15.747 ± 0.195	16.570 ± 0.007	15.990 ± 0.330	16.615 ± 0.077	16.802 ± 0.068
cis-4,7,10,13,16,19-docosahexaenoic (C22:6)	6.808 ± 0.059	6.742 ± 0.064	8.825 ± 0.239	6.722 ± 0.094	6.838 ± 0.069
SFAs	34.941	33.475	33.276	34.377	32.919
MUFAs	18.274	18.380	18.080	18.439	16.865
PUFAs	46.785	48.146	48.644	47.184	50.216

Discussion

Microalgae are used in many industrial areas, such as aquaculture (Nagappan et al. 2021), food (Torres-Tiji et al. 2020), cosmetics (De Luca et al. 2021), biodiesel (Chisti 2007), agriculture (González-Pérez et al. 2022), pharmacy (Yarkent et al. 2020), medicine (Sathasivam et al. 2019) and wastewater treatment (Li et al. 2019). Microalgae are nutrient-rich organisms used in aquaculture hatcheries (Spolaore et al. 2006). Microalgal cultures attract attention due to their importance in terms of sustainability in fish and shrimp farming (Cheng et al. 2020). Pigments such as carotene and astaxanthin obtained from microalgae increase the immunity of aquacultured animals and prevent the use of antibiotics and drugs for treatment (Singh et al. 2017). Therefore, it is important to utilize microalgae biotechnology in aquaculture to benefit the environment (Li et al. 2021).

The productivity of biomass and lipids in microalgae cells is affected by environmental factors as well as the type and amount of nutrients (Zhu et al. 2016; Yalcin 2020). In the present study, the optical density, cell number and dry weight of *D. tertiolecta*, *P. tricornutum* and *N. oculata* were higher in the control group than in the nitrogen-starved group on the last day of the trial. The growth of microalgae cells is related to the nitrogen concentration in the culture medium. In the case of nitrogen depletion, a general slowdown in growth and an increase in lipid accumulation occurs in the cells (Van Vooren et al. 2012). Although nitrogen stress has a high effect on the photosynthetic apparatus of cells, this may limit the growth of cells but provides an increase in valuable fatty acids (Kamalanathan et al. 2016). The number of cells in *N. oculata* cultures increased approximately 2.6-fold in the control group compared to the nitrogen-starved group on the 10th day of the experiment. This was reported by Dianursanti et al. (2018), who

stated that the reduction of nitrogen from the culture medium reduces the growth rate as well as the inhibition of photosynthesis due to the decrease in chlorophyll formation in cells. Nitrogen concentration affects the cell metabolism of *D. tertiolecta* and can easily affect cell growth (Song et al. 2016). Researchers have reported that nitrogen-starved diatom species provide a high amount of lipid accumulation, stop cell division and increase cell density slightly (Kaixian and Borowitzka 1993; Larson and Rees 1996; Falkowski et al. 1998). Similar results were obtained in *P. tricornutum*, the only diatom species examined in the current study. Mock and Kroon (2002) reported that loss of pigment and protein amounts was observed in the Antarctic region, where nitrogen is limited.

In the present study, TAG content increased approximately 2-fold in *D. tertiolecta* in nitrogen-free medium and decreased after the 3rd day. Under nitrogen-free culture medium, the oligosaccharide and polysaccharide contents increased 4.18-fold and 3.77-fold, respectively on the 7th day and started to decrease in the following days. Similar to our study, a study has reported that *D. tertiolecta* accumulates high amounts of starch in the presence of nitrogen or nitrogen-free conditions, while it accumulates low amounts of TAG (Tan et al. 2016). Nitrogen starvation has proven to increase the C/N ratio due to decreased amino acid synthesis and protein levels, resulting in increased carbon storage in polysaccharides and/or lipids (Dean et al. 2010). *N. oculata* increased the TAG content in nitrogen-free culture conditions by 2.3-fold compared to the control group, while the amounts of oligosaccharides and polysaccharides were increased by 1.26-fold and 1.91-fold, respectively. In the study of Shan Ahamed et al. (2022), the amount of TAG in *Nannochloropsis* cells increased less (1.15-fold) in the absence of nitrogen than in the present study. Ma et al. (2016) and Martin et al. (2014) reported that TAG accumulation increased in *N. oculata* cells under nitrogen stress, similar to the results of our study. Tan et al. (2016) reported that while *N. oceanica* increased the amount of TAG in nitrogen-deficient medium, as in the *N. oculata* species used in this study, contrary to our study, it lacked starch, which is a storage compound. In our study, TAG accumulation in the nitrogen-starved group increased 7.4-fold compared to the control group in *P. tricornutum*. Alonso et al. (2000) reported that the amount of TAG increased 1.08-fold under nitrogen-limited conditions of *P. tricornutum*. Breuer et al. (2012) reported that TAG efficiency in *P. tricornutum* increased 4-fold under nitrogen stress compared to the control group. In our research, the oligosaccharide and polysaccharide contents of *P. tricornutum* increased 5.3-fold and 2.4-fold, respectively in the nitrogen-free group compared to the control group.

D. tertiolecta cells increased the amount of carbohydrates under nitrogen stress by 36% and 59%, respectively on the 5th and 10th days of the experiment. Similar to our results, Slocombe et al. (2015) and Nikookar et al. (2005) have reported that *D. tertiolecta* is among the best species for carbohydrate production. Hong et al. (2017) have also reported that the carbohydrate content of species exposed to nitrogen deprivation is increased. However, the same study, which contradicts our study, has reported that fatty acid accumulation did not change compared to the first day. It is thought that the results are contradictory due to the use of different culture conditions. *D. tertiolecta* tends to prefer starch rather than TAG for chemical energy accumulation, and therefore, *D. tertiolecta* cells rapidly accumulate starch in response to nitrogen-deficient culture conditions (Tan et al. 2016). In our study, carbohydrate accumulation in *N. oculata* decreased in nitrogen starvation condition compared to the control group.

Hong et al. (2017) stated that the amount of carbohydrates gradually decreased until the end of their research. Another study determined that in the absence of nitrogen in *N. oculata*, there was a decrease in the amount of protein and chlorophyll a, whereas the accumulation of carbohydrates increased from 24.1–29% (Paes et al. 2016). In the current study, carbohydrate amounts in *P. tricornutum* were 76% and 11% higher in nitrogen-starved groups on the 5th and 10th days of the experiment group compared to the control group, respectively.

While the amount of protein in *D. tertiolecta* and *P. tricornutum* was lower in the nitrogen-free group on the 5th day of the experiment than in the control group, it was higher in the nitrogen-free group than in the control group on the 10th day of the experiment. This can be attributed to the accumulation of metabolic wastes in the medium and self-destruction of cells exposed to nitrogen starvation for a long time. This change in protein content contradicts the study of Kamalanathan et al. (2016). *N. oculata* reduces protein accumulation in the absence of nitrogen, which is similar to the result obtained by Paes et al. (2016).

Many studies have shown that *Dunaliella* species respond to nitrogen deficiency by increasing lipid accumulation in their cells (Lombardi and Wangersky 1995; Chen et al. 2011). On the last day of the experiment, we found that the amount of lipids in *D. tertiolecta* increased by 52% in the nitrogen starved group compared to the control group. Although lipid accumulation was low in the first week of the experiment under nitrogen starvation conditions, it increased after the 9th day, which is similar to the results of Jiang et al. (2012). Rizwan et al. (2022) stated that the amounts of lipids and carbohydrates increased by approximately 1.4-fold and 1.6-fold in *Dunaliella* exposed to nitrogen stress in the dark, respectively, while these rates were approximately 2-fold and 4.7-fold, respectively according to the initial value in our study. In *N. oculata*, lipid accumulation was 16% higher in the nitrogen-free group than in the control group on the 10th day of the experiment. Many researchers have reported that nitrogen deficiency causes lipid accumulation in *N. oculata* cells (Rodolfi et al. 2009; Su et al. 2011; Bajwa et al. 2018). *N. oculata* is one of the most commonly used microalgal species in the culture of marine fish larvae. This research shows that increasing the oil content of *N. oculata* under nitrogen stress conditions can contribute to the energy requirements of fish larvae. In *P. tricornutum*, on the 5th and 10th days of the experiment, lipid amounts were 79% and 139% higher in the nitrogen-starved group than in the control group, respectively. Buono et al. (2016) and Yodsuwan et al. (2017) emphasized that *P. tricornutum* responds to nitrogen stress by increasing lipid accumulation, as in our study.

The nutritional value of microalgae is determined by its protein content followed by the accumulation of polyunsaturated fatty acids (PUFA) fatty acids such as docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and arachidonic acid (ARA) (Reitan et al. 1997). These fatty acids are very important because they cannot be easily synthesized in the laboratory. The fatty acid composition is related to light intensity, culture medium, temperature and pH (Daroch et al. 2013). EPA and DHA are essential for the growth and survival of marine fish larvae (Shah et al. 2018). In the present study, microalgal species were especially rich in PUFAs. PUFAs play important roles in processes such as thermal adaptation, regulation of membrane fluidity and permeability, oxygen and electron transport in cellular and tissue metabolism (Cardozo et al. 2007; Lee et al. 2014). The fact that the PUFA content of *D. tertiolecta* cultures was

approximately 60% of all fatty acids in each trial group and on all days of measurement showed that *D. tertiolecta* is a microalgal species that can be used in food and aquaculture activities. Avidan et al. (2021) in their nitrogen stress study with *D. tertiolecta*, determined that the amount of oleic acid increased 1.4-fold, while it increased approximately 1.2-fold in the present study. Lee et al. (2014) stated that nitrogen limitation or high light intensity increased the levels of SFA and MUFA in *D. tertiolecta* cultures and decreased the level of PUFA. It also reported that nitrogen depletion caused an increase in the degree of saturation of fatty acids in *D. tertiolecta* cells, since PUFAs are particularly sensitive to oxidation, which supports our study. In our study, the SFA content in nitrogen-starved cells increased on the 5th day of the experiment, while MUFA increased on the 10th day and PUFA content decreased at the same time. Lee et al. (2014) reported that the dominant fatty acids in *D. tertiolecta* are linolenic acid (C18:3), palmitic acid (C16:0), hexaadecatetraenoic acid (C16:4), linoleic acid (C18:2) and oleic acid (C18:1), while eicosapentanoic acid (C20:5), palmitic acid (C16:0), docosahexanoic (C22:6), eicosantronoic (C20:3n3), linolenic acid (C18:3n3) and linoleic acid (C18:2 cis) were determined in the current study.

In our study, although the EPA value increased by 5% under nitrogen stress in *N. oculata*, Hodgson et al. (1991) and Reitan et al. (1994) emphasized that the decrease in the EPA ratio during the lipid-inducing phase may be related to additional nutritional deficiencies. Additionally, Gong et al. (2013) and Xiao et al. (2013) reported that although fatty acid accumulation increased in *N. oculata* cells, there was no change in the total amount of EPA. Olofsson et al. (2014) stated that saturated and monounsaturated fatty acids increased from 76% to approximately 90% of total fatty acids in *N. oculata* under nitrogen-limited conditions, while this rate increased from 39.7–41.8% in our study. Xu et al. (2001), Zhila et al. (2005) and Su et al. (2011) reported that *N. oculata* cells showed a decrease in linolenic acid and an increase in oleic acid when exposed to nitrogen stress. In our study, nitrogen stress caused an increase in both linoleic acid and oleic acid. While *N. oculata* increased the amount of oleic acid (C18:1cis) 1.7-fold under nitrogen starvation, it increased 1.07-fold in another study (Shan Ahamed et al. 2022). Contrary to this study, Qiao et al. (2016) stated that saturated fatty acids and monounsaturated fatty acids increased in *P. tricornutum* cells exposed to nitrogen stress. However, we determined that the amount of PUFA increased under nitrogen starvation conditions, while the fatty acids of SFA and MUFA decreased. In another study conducted with *P. tricornutum*, the amounts of palmitic acid (C16:0), palmitoleic acid (C16:1) and EPA (C20:5) were determined on the 21st day of culture exposed to nitrogen deprivation at 17.73 ± 8.40%, 13.07 ± 6.03%, and 1.81%±1.25%, respectively (Yodsuwan et al. 2017). These were 14.82 ± 0.08%, 6.028 ± 0.11%, and 16.80 ± 0.06% in our study on the 10th day of the experiment.

Conclusions

Microalgae are recognized as pioneer organisms in the aquaculture, food, medicine and biofuel industries. Each microalgae needs different or similar culture conditions for growth. A change in culture conditions causes differences in the biochemical composition of microalgae. These differences cause the death of species in some cases or the synthesis or increase of molecules that can be used as raw materials in various sectors. In our study, nitrogen starvation increased the amount of oligosaccharides and polysaccharides in *D. tertiolecta* and increased the EPA and DHA contents in *N. oculata* and *P.*

tricornutum. The fact that the amount of PUFA, EPA, DHA, oligosaccharides, and polysaccharides in microalgal species can be increased without using the costliest nitrogen source in the culture conditions can reduce the cost of feeding in aquaculture. In addition, *N. oculata* and *P. tricornutum* can be used as raw materials for biodiesel, as they increase the amount of TAG by 2.3-fold and 7.4-fold, respectively in nitrogen-free conditions.

Declarations

Competing interests: The authors no conflict of interest to declare.

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Authors' contributions: The experiments were designed by Pınar A. Şirin and Serpil Serdar. Laboratory studies were carried out by Pınar A. Şirin. Biochemical analyses were performed by Pınar A. Şirin and Serpil Serdar. The manuscript was written by Pınar A. Şirin. Serpil Serdar reviewed and editing the manuscript. All authors read and approved the manuscript.

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Figures

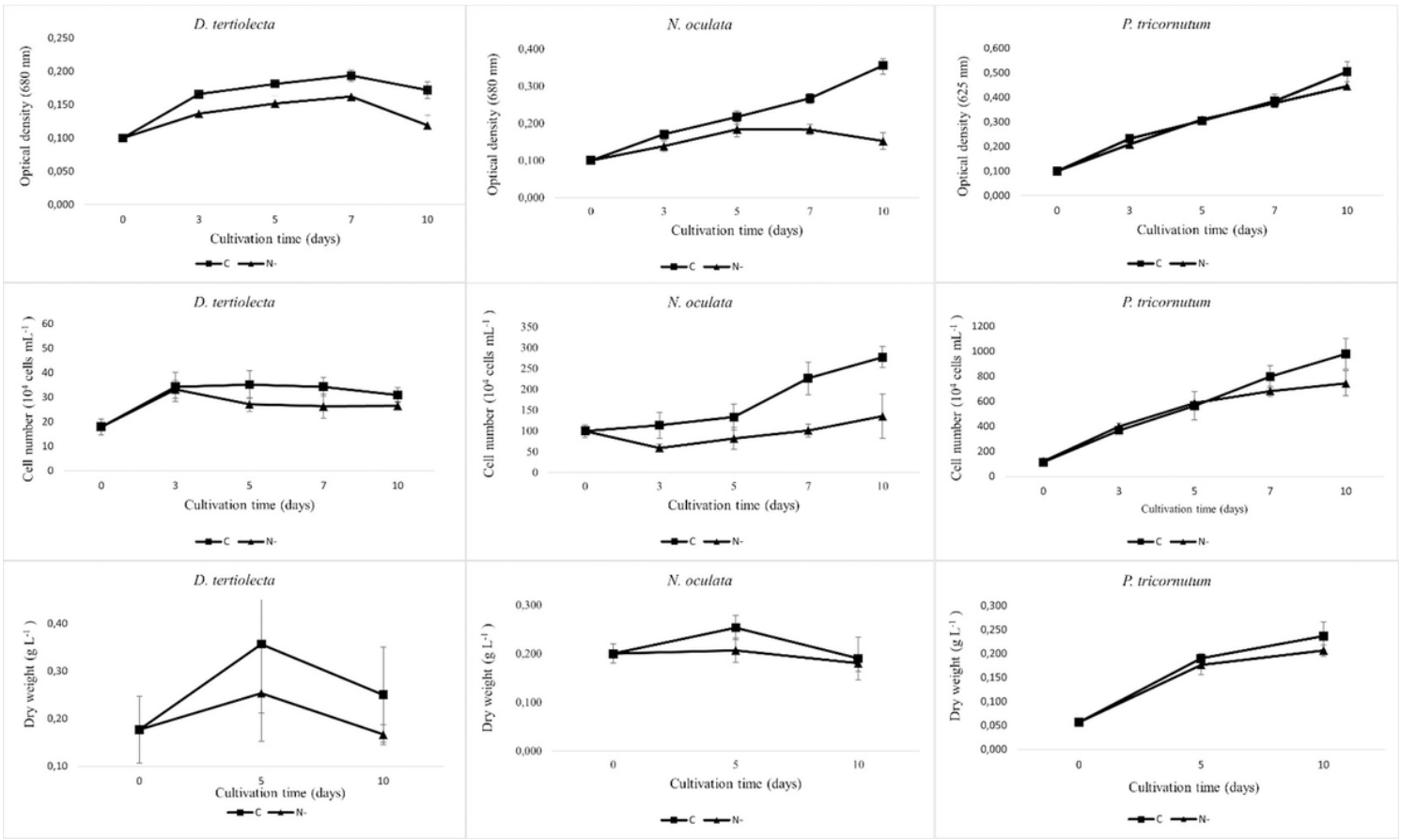

Figure 1

Changes in the optical density, cell number and dry weight of *D. tertiolecta, N. oculata, P. tricornutum.* Values are presented as the mean ±standart deviation (n=3) square: control group, triangle: nitrogen starvation group (N^-)

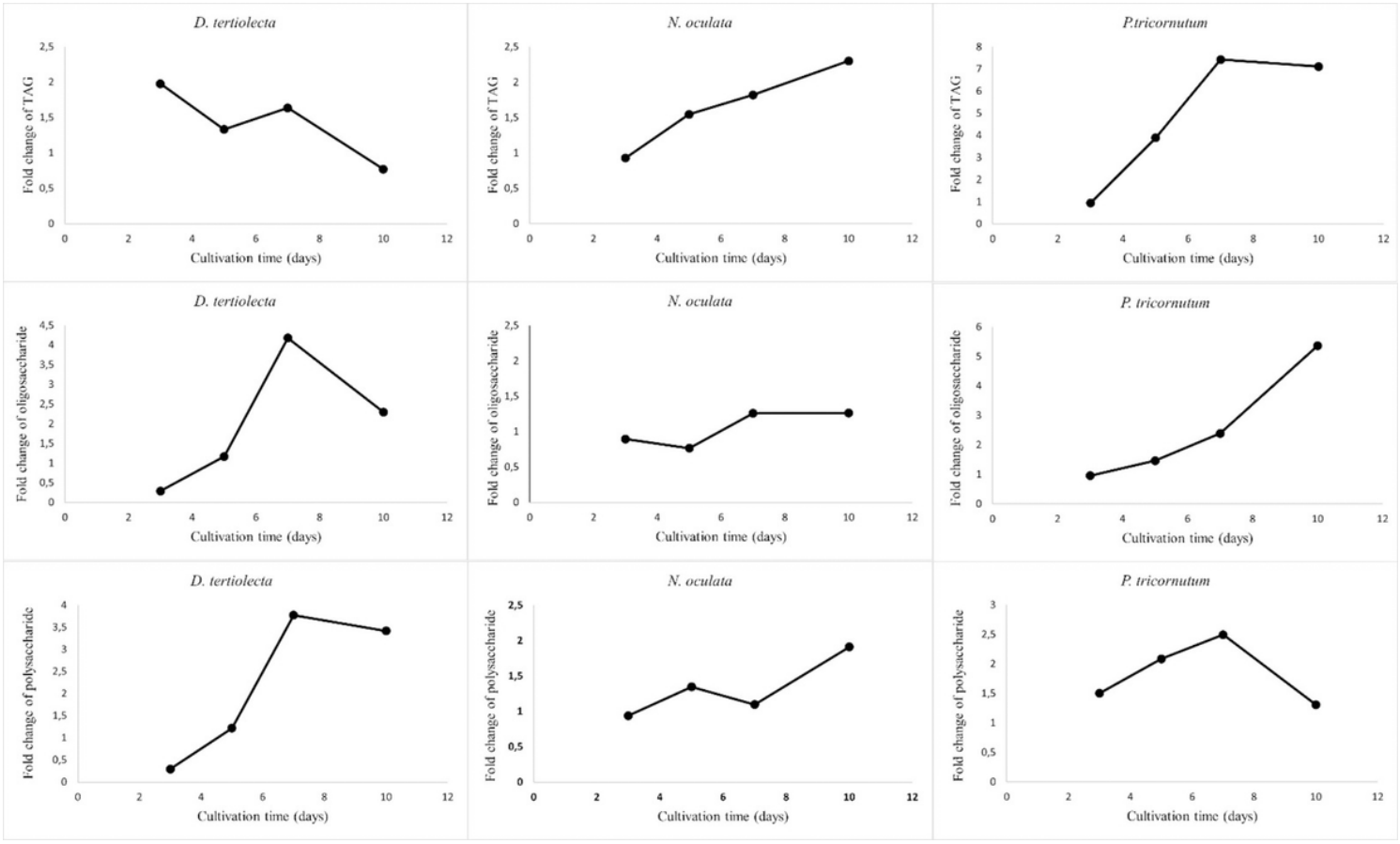

Figure 2

Fold changes of TAG, oligosaccharide and polysaccharide