Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol.26 (2): 459 – 475 (2022)
www.ejabf.journals.ekb.eg

The Growth and Biochemical Composition of *Nannochloropsis oculata* under Influence of Different Nitrogen Sources for Aquaculture Live Food

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ARTICLE INFO

Article History:
Received: March 7, 2022
Accepted: March19, 2022
Online: April 16, 2022

Keywords:
Nannochloropsis oculata,
Nitrogen source,
Cell density,
Specific growth rate,
Biochemical composition,
Fatty acids profile

ABSTRACT

This study aimed to investigate the influence of nitrogen from different sources on the growth and biochemical composition of the marine alga *Nannochloropsis oculata*. The effect of different nitrogen sources was addressed, viz. ammonium bicarbonate, ammonium sulfate, ammonium nitrate, ammonium chloride, urea, and sodium nitrate. The highest cell density (22.46 $\times 10^6$ cell mL^{-1}) of *N. oculate*, with a specific growth rate of 0.41 division day^{-1} was obtained with ammonium bicarbonate after eight days of incubation. Meanwhile, the total protein content was significantly increased by 38.28% with urea, although it showed the lowest content of lipid, carbohydrate. Ammonium nitrate showed the highest total content of lipids (43.67%), with a pronounced increase in total carbohydrate content (19.09 %) in sodium nitrate. Palmitic acid (C16:0) and stearic acid (C18:0) were the most abundant saturated fatty acids within all experimental treatments; whereas, palmitoleic acid (C16:1 ω7) and oleic acid (C18:1 ω9c) were the major monounsaturated ones. Both sodium nitrate and ammonium bicarbonate showed a higher content of eicosapentaenoic acid (C20:5 ω3) EPA, while docosahexaenoic acid (C22: 6 ω3) DHA and arachidonic acid (C20: 4 ω6) increased with urea. In conclusion, this study demonstrated the variation of growth, fatty acid profile, and biochemical composition in *N. oculata*, benefiting the production of microalgae for aquaculture live food.

1. INTRODUCTION

Aquaculture is the fastest developing sector of global food production accounting for more than half of the worldwide fish production **(Galappaththi *et al*., 2020; Chan *et al*., 2021)**. It has the ability to meet the food demands of millions of people in the developing countries, who would benefit from low-cost protein **(Hagar, 2014)**. Egypt has the most aquaculture production in Africa with a total production of around 1.8 million tons annually, concentrated in the production of freshwater fish **(Kaleem & Sabi, 2021)**. Recently, Egypt is facing the problem of freshwater scarcity that led to the necessity of mariculture **(Shaalan *et al*., 2018)**.

In this respect, the Egyptian Government has recently established some of the mega mariculture projects, such as the Gholion project in Kafer ElShiekh Governorate (2800 acres), Fayrouz project in Port Said at Shark El Tafriaa (15000 acres), the Diba triangle in Damietta Governorate (103 acres), Suez Canal authority project in the eastern side of Suez Canal and Gargob in Matroh Governorate (7000 acres); they are mainly focusing on sea bream, sea bass, and shrimp cultures **(Feidi, 2018)**. These projects necessitate the production of huge amounts of high-quality, healthy fish larvae by marine hatcheries. However, these marine hatcheries are facing bile of critical challenges, of which the cost and availability of live food are on the top to secure the required fish larvae feeding. Microalgae are widely used in marine hatcheries as live food for zooplankton, bivalves, crustaceans and the early stages of fish larvae **(Villar-Navarro *et al*., 2021)**. In addition, microalgae are one of the most sustainable natural sources that can be utilized in biodiesel production **(Zaki *et al*., 2021)**.

Nitrogen is a fundamental constituent in plant growth, cell structure, and functional processes of microalgae and is considered as an indispensable element of proteins, amino acids, nucleic acids, enzymes, and photosynthetic pigments; these are essential components stimulating algal growth through rapid cell division **(Sajjadi *et al*., 2018)**. Therefore, algae critically require nitrogen to divide, grow and reproduce; microalgae can effectively grow in cultures with the nitrogen of various forms (Table 1), such as ammonium, nitrate, and urea according to algae species **(Zhu *et al*., 2019)**.

Table 1. Nitrogen preferable source/form for growth of different microalgal species

Microalgal species	**Preferable nitrogen source**	**Reference**
Dunaliella salina	ammonium	Norici *et al.* (2002)
Chlorella sp. GN1	nitrate	Feng *et al.* (2020)
Chlorococcum sp.	urea	Fatini *et al.* (2021)
N. oceanica	ammonium bicarbonate	Mahdieh *et al.* (2019)
Chlorella vulgaris	ammonium nitrate	Simsek and Cetin (2019)
Nannochloropsis salina	urea	Campos *et al.* (2014)

The genus *Nannochloropsis* is considered the leading algae in marine hatcheries of a significant importance, and it plays an important role in aquaculture development **(Kaparapu, 2018)**. The microalga *Nannochloropsis oculata* species (2–5 µm) is a unicellular nonmotile marine species, belonging to phylum Ochrophyta and class Eustigmatophyceae **(Adl *et al.*, 2012)**. Some of the *Nannochloropsis* species are utlized in marine hatcheries for rotifer *Brachionus plicatilis* feeding and improving feed ingestion and larval growth **(Li *et al.*, 2020)**, *Artemia franciscana* rich in high amounts of polyunsaturated fatty acids (PUFA), especially EPA

(20:5 ω3) and DHA(22:6 ω3) **(El Khodary *et al*., 2021)**, and the feeding of *cyclops abyssorum divergens* copepods that are suitable prey for the mouth of cultured fish larvae **(El Sayed *et al*., 2021).**

This experiment was designed to investigate the impacts of diversified nitrogen sources on the growth performance pattern of *Nannochloropsis oculata* and determine the physiological responses related to chemical constituents and fatty acid composition. Results of this experimental research would help enhancing the knowledge on *N.oculata* culturing and realizing the best cultivation conditions for large-scale production in the future. This would help in future aqua culturing developmental activities at the national and the global levels and would certainly impact the aquaculture-related economy.

2. MATERIALS AND METHODS

2.1. Inoculation and seawater preparation

The *Nannochloropsis oculata* inoculum originally obtained from SEAFDEC, Iloilo, Philippines, was maintained under the controlled conditions of the algae culture lab. and cultured in seawater collected from El Attaka Port, the Red Sea, Suez (29°54'07.6"N 32°28'00.4"E). The physicochemical characteristics of seawater at the site are presented in Table (2). The salinity of sea water at the site was 43.8±1 g/L and was adjusted to the salinity of 25 g/L **(Hassan, 2002)**, using salinometer (Lovibond Salinity meter, SD320) by diluting seawater with filtrated fresh water. The diluted seawater was filtered twice; the first through a layer of three (5 microns) cotton cartridge bags and then by a filtration disc (0.2 microns) to obtain pathogen-free water. Filtered seawater was sterilized by chlorex 5% for 24 hours then chlorine was eliminated through continuous aeration.

Fig. 1. Algal carboys bags under controlled conditions in the algae culture lab.

Table 2. The Physicochemical characteristics of seawater at the study site

Parameter	Value	Unit
Salinity	43±1	g/L
pH	7.85	-
Dissolved oxygen (DO)	5.06	mg/L
Temperature	29.5	°C
Ammonia (NH_4)	10.18	μM^{-1}
Nitrite (NO_2)	4.01	μM^{-1}
Nitrate (NO_3)	8.53	μM^{-1}

2.2. Culturing procedures of *N.oculata*

Nannochloropsis oculata was cultivated in sterilized seawater supplemented with F/2 culture medium of **Guillard (1975)** containing (75 g/L $NaNO_3$, 5 g/L $NaH_2PO_4.2H_2O$, 1 mL of stock trace metal solution (per 1 Littre; 3.15 g $FeCl_3.6H_2O$, 4.36 g $Na_2EDTA.2H_2O$, 1mL; 9.8 g/L $CuSO4.5H_2O$, 1mL; 6.3 g/L $Na_2MoO_4.2H_2O$, 1mL; 22.0 g/L $ZnSO_4.7H_2O$, 1 mL; 10.0 g/L $CoCl_2.6H_2O$ and 1 mL; 180.0 g/L $MnCl_2.4H_2O$), vitamin B1 100 µg/·L and B12 1000 µg/·L in the form of (Tri-B ampoules). The culture was loaded in 20 litters Carboys bags (Fig. 1) and maintained under controlled conditions of continuous illumination with 100 µmol photons m^{-2} s^{-1} of cool-white fluorescent **(Morretti, 1999)**, salinity (25 ± 2 g/L), and temperature (21 ± 2°C) with continuous aeration.

2.3. Experimental design

The experiment was designed to determine the impact and response of varying nitrogen sources on *N. oculata* cell growth and biochemical composition by replacing sodium nitrate ($NaNO_3$ 0.88 mmol/L) in F/2 medium with other sources of nitrogen; namely, 1) ammonium nitrate (NH_4NO_3), 2) ammonium bicarbonate (NH_4HCO_3), 3) ammonium chloride (NH_4CL), 4) ammonium sulfate ($(NH_4)_2SO_4$) and 5) urea ($CO(NH_2)_2$). In the medium, except for the nitrogen source, the other nutrient components were consistent with the F/2 medium. The initial cell density was adjusted to be about 1×10^6 cell/mL in all the triplicate treatments, and cultures were left to grow and monitor for 15 days.

2.4. The growth pattern of *N. oculata*

2.4.1. Cell density

Algal cell densities were determined by hemocytometer and confirmed with UV-VIS spectrophotometer (model: T60 UV) by measuring the optical densities at 540 nm (**Rocha *et al*., 2003)**.

2.4.2. Specific growth rate (μ) and doubling time (T_d)

The maximum specific growth rate (μ, division/day) and doubling time (T_d) were calculated according to the following equations recommended by **Durmaz and Erbil (2017)**.

$$\mu \text{ (division/day)} = \ln(X_2/X_1)/(t_2-t_1)$$

$$t_d \text{ (doubling/day)} = \ln 2/\mu = (0.693)/\mu$$

Where, X_1=cell concentration at time t_1, and X_2= cell concentration at time t_2.

2.5. Biochemical constituent analysis

2.5.1. Total protein and carbohydrate

Total protein and carbohydrate were extracted after pigment extraction, following the method of **Payne and Stewart (1988)**. Total soluble protein was quantitatively determined using the method of **Bradford (1976)**, using bovine serum albumin as a standard protein. Carbohydrate content was estimated by the method of phenol-sulfuric acid as reported by **Kochert (1973)** using glucose as a standard reference.

2.5.2. Total lipid and Fatty acid methyl ester

Total lipid was extracted as described in the study of **Ren *et al.* (2017)**. According to the modified method of **Zahran and Tawfeuk (2019)**, fatty acids were determined by converting the oil to fatty acid methyl esters, adding 1.0 mL of hexane to 15 mg of oil, followed by 1.0 mL of sodium methoxide (0.4 mol). After 30 seconds of vortexing, the mixtures were allowed to settle for 15 minutes. The upper phase containing the FAMEs was collected and analyzed by gas chromatography (GC-FID, Perkin Elmer Auto System XL), equipped with a flame ionization detector (FID). ZB-Wax fused silica capillary column was employed as the packing column (60 m x 0.32 mm i.d). Helium was used as carrier gas at a flow rate of 1 $mL \cdot min^{-1}$. The injector and detector temperatures were set at 250°C and 250°C, respectively. Ultimately, the fatty acids were recorded as percentages of total fatty acids.

2.6. Statistical analysis

Version 22 of the Statistical Package for Social Science (SPSS) was used to analyze the resulting data **(Allen *et al.*, 2014)**. According to **Duncan (1955)**, ANOVA differences between means were assigned as significant at $P \leq 0.05$ via the least significant difference (LSD) for the multiple ranges of post hoc comparisons to determine the differences among the means of replication. The results were presented as mean ± standard deviation (SD).

RESULTS

3.1. Effect of different nitrogen sources on growth parameters of *N.oculata*

3.1.1. Cell density

The cell density of *N. oculata* was strongly promoted by the various nitrogen sources after the first inoculation day (Table 3). The algal growth was greatly affected by NH_4HCO_3 of 22.46 × 10^6 cell/mL and· $NaNO_3$ of 21.60 × 10^6 cell/mL, reaching its peak on the ninth day of the incubation. However, the growth of *N. oculata* was decreased significantly ($p < 0.05$) in cultures enriched with $(NH_4)_2SO_4$ and CO $(NH_2)_2$, with the lowest cell density compared to other treatments (Table 3). A level of moderate effect on the algal growth was determined when either NH_4NO_3 or NH_4CL was used as a nitrogen source. Optical density is presented in Fig. (2).

Table 3. Effect of different nitrogen sources on cell density (× 10^6) of *N. oculata* during 15 days incubation

Day	$NaNO_3$	$(CO(NH_2)_2)$	NH_4NO_3	NH_4CL	$(NH_4)_2SO_4$	NH_4HCO_3
0	١.00	١.00	١.00	١.00	١.00	١.00
1	1.31 ± 0.06 ^{c}	1.48± 0.02 ^{c}	2.80 ± 0.04 ^{a}	1.46 ± 0.03 ^{c}	2.00 ± 0.20	1.35 ± 0.10 ^{c}
3	3.23 ± 0.15 ^{c}	3.60 ± 0.20 ^{b}	4.25 ± 0.25 ^{a}	3.08 ± 0.18 ^{c}	3.07 ± 0.14 ^{c}	2.96 ± 0.22 ^{c}
5	6.61 ± 0.03 ^{b}	7.32 ± 0.11 ^{a}	6.37 ± 0.03 ^{bc}	5.49 ± 0.13 ^{d}	5.71 ± 0.25 ^{d}	6.19 ± 0.24 ^{c}
٧	10.27 ± 0.16^{a}	5.91 ± 0.02 ^{e}	7.25 ± 0.15 ^{d}	5.95 ± 0.36 ^{e}	9.37 ± 0.21 ^{b}	7.98 ± 0.02 ^{c}
٩	21.60 ± 0.53^{b}	14.20 ± 0.20^{e}	15.87 ± 0.03 ^{d}	17.65 ± 0.15 ^{c}	13.91 ± 0.02^{e}	22.46 ± 0.16 ^{a}
١١	19.58 ± 0.15^{a}	13.34 ± 0.11^{d}	15.47 ± 0.13 ^{c}	12.23 ± 0.13 ^{e}	9.88 ± 0.12 ^{f}	18.41 ± 0.11 ^{b}
١٣	18.07 ± 0.07^{a}	9.40 ± 0.17 ^{f}	12.96 ± 0.01^{c}	11.28 ± 0.07^{d}	9.68 ± 0.01 ^{e}	16.53 ± 0.20^{b}
١5	16.47 ± 0.07^{a}	5.55 ± 0.20 ^{e}	10.33 ± 0.29 ^{c}	10.33 ± 0.02 ^{c}	9.55 ± 0.26 ^{d}	14.60 ± 0.06 ^{b}

Data are presented as mean ± standard deviation. Different superscripts within the same row indicate significant differences among treatments at level of $p \leq 0.05$, $a > b > c > d > e > f$).

3.1.2. Optical density

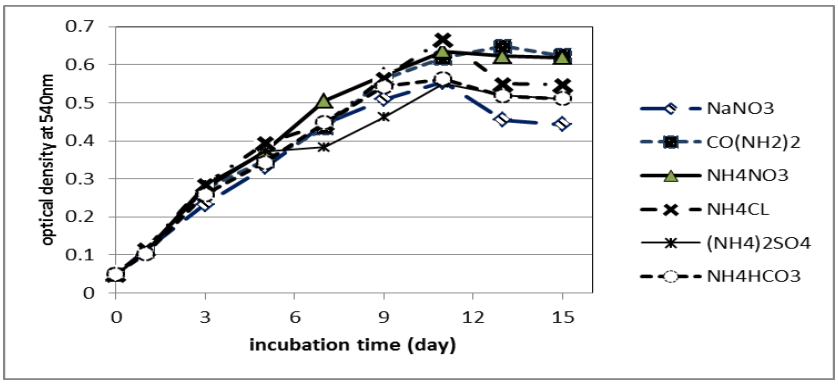

Fig. 2. Effect of different nitrogen sources on optical density at 540 nm of *N. oculata* during the incubation period

3.1.3. Specific growth rate (μ) and doubling time (TD)

Variations in specific growth rate (μ) and doubling time (TD) of *N. oculata* among the different treatments are shown in Fig. (3). NH_4HCO_3 and NH_4CL were determined to achieve the highest significant growth rate ($p \leq 0.05$), with the lowest doubling time of 1.71 and 1.74 doubling/ day, respectively. While, cultures with $(NH_4)_2SO_4$ recorded the lowest specific growth rate (0.25 division/ day) with 2.76 doubling/ day. Results showed no significant difference ($p > 0.05$) either in specific growth rate or the doubling time for the cultures enriched with ($CO(NH_2)_2$) and NH_4NO_3.

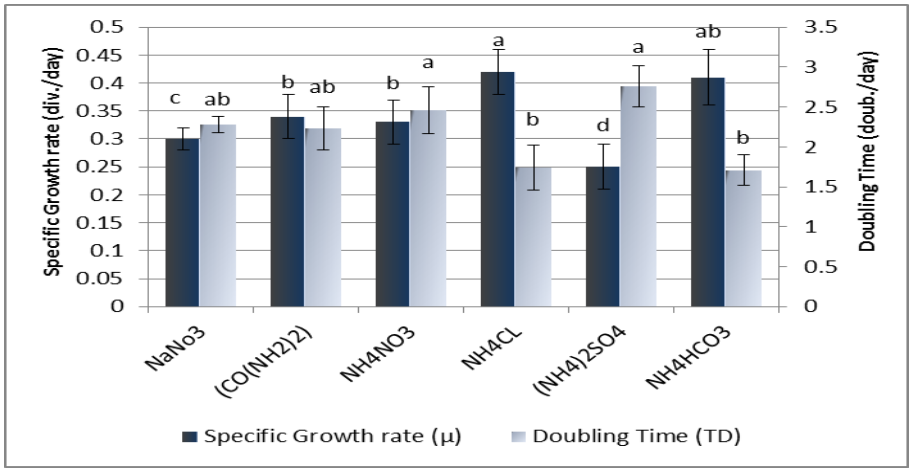

Fig. 3. Effect of different nitrogen sources on the specific growth rate (*u*) and doubling time (Td) at the maximum cell density at (day 9)

3.2. Biochemical composition of *N. oculata*

Nannochloropsis oculata was cultured under different nitrogen sources ($NaNO_3$, ($CO(NH_2)_2$), NH_4NO_3, NH_4CL, $(NH_4)_2SO_4$, NH_4HCO_3, where samples were harvested for analysis of biochemical composition after the late exponential phase (9 days). Results of total protein, carbohydrate, and lipid as a percentage of dry weight (% DW) are summarized in Table (4). Urea and $NaNO_3$ achieved the highest significant difference ($p \leq 0.05$) of cellular dry weight (CDW), while NH_4HCO_3 achieved the lowest dry weight content. The total protein content showed a remarkable increase of 38.28% and 34.37% in cultures enriched with ($CO(NH_2)_2$) and NH_4NO_3, respectively.

Carbohydrate content was significantly high in $NaNO_3$ culture, followed by NH_4HCO_3 culture with no significant difference ($p > 0.05$) with respect to other treatments. The lowest carbohydrate content (12.22%) was achieved in the culture enriched with urea. The highest total lipid content was exhibited by NH_4NO culture at 43.67%, followed by NaNO3 culture at 42.39%; however, the culture containing urea showed the lowest level (30.11%) compared to other treatments.

Table 4. The biochemical composition (% of dry weight) of *N. oculata* at different nitrogen sources harvested after 9 days of cultivation

Medium	Cell Dry Weight (CDW, g L^{-1})	Protein (%CDW)	Carbohydrate (%CDW)	Lipid (%CDW)
$NaNO_3$	0.74±0.030^{a}	28.83± 1.751^{cd}	19.09± 1.455^{a}	42.39±2.314^{a}
$(CO(NH_2)_2)$	0.83±0.031^{a}	38.28± 3.275^{a}	12.22± 2.665^{c}	30.11±1.986^{c}
NH_4NO_3	0.66±0.026^{b}	34.37± 1.458^{b}	15.78± 4.082^{abc}	43.67±3.395^{a}
NH_4CL	0.65±0.015^{b}	19.28± 1.275^{d}	13.19± 3.845^{bc}	37.48±0.640^{ab}
$(NH_4)_2SO_4$	0.57±0.026^{c}	32.78± 3.775^{bc}	15.31± 0.693^{abc}	35.75±4.325^{abc}
NH_4HCO_3	0.54±0.025^{d}	29.88± 2.466^{bc}	18.03± 1.285^{ab}	32.65±7.313^{bc}

Data are presented as mean ± standard deviation. Different superscripts within the same column indicate significant differences among treatments at level of $p \leq 0.05$, $a > b > c > d > e > f$).

3.3. Fatty acids profiles of *N. oculata*

As illustrated in Table (5), the fatty acids profiles of *N. oculata* varied among the various nitrogen sources. In the comparison of F/2 control media to the other mediums, palmitic acid (C16:0) was the most abundant saturated fatty acid, with a maximum value of 36.67% in NH_4Cl. Following the palmitic acid, was the stearic acid (C18:0), which recorded its highest value (10.12%) with NH_4HCO_3.

In addition, palmitoleic acid (C16:1) had nearly the same percentage values in all the used media, while NH4Cl had the lowest percentage value (1.27%). Linoleic acid (C18:2) was

the most abundant polyunsaturated fatty acid (PUFA) throughout all treatments, where the data showed the highest values of this fatty acid (6.95% and 4.19%) recorded with $(NH_4)_2SO_4$ and NH_4HCO_3, respectively. Cis-13.16-docosadienoic acid (C22:2) was the second polyunsaturated fatty acid, where its maximum percentage value (22.97%) was recorded with NH_4HCO_3 medium and arachidonic acid (C20:4 ω6), which recorded its highest value (18.24%) with urea. Similarly, docosahexaenoic acid (C22:6 ω3) DHA recorded its highest value (4.69%) with the same medium. Whereas, eicosapentaenoic acid (C20:5 ω3) achieved the highest value in $NaNo_3$ (9.53%) and NH_4HCO_3 (9.29%).

Table 5. Fatty acids profile (% of total fatty acids) of *N. oculata* cultured under different nitrogen sources after 9 days of cultivation

Fatty acid	$NaNO_3$	$(CO(NH_2)_2)$	NH_4NO_3	NH_4CL	$(NH_4)_2SO_4$	NH_4HCO_3
Saturated fatty acids (SFAs)						
C11:0	2.26 ±0.40 [a]	2.09±0.01 [c]	nd[*]	0.65 ±0.04 [e]	2.23 ±0.30 [b]	1.07 ±0.20 [d]
C12:0	0.86 ± 0.03 [a]	0.79±0.10 [b]	nd[*]	0.63 ± 0.20 [b]	nd[*]	nd[*]
C13:0	3.06 ± 0.40 [b]	3.36± 0.20 [a]	1.68 ± 0.20 [f]	2.03 ± 0.20 [e]	2.58 ± 0.30 [c]	2.42 ± 0.10[d]
C14:0	1.13±0.04 [d]	1.30± 0.20 [bc]	nd[*]	1.59 ± 0.02 [b]	1.76 ±0.03 [a]	1.22± 0.02 [c]
C15:0	8.59± 0.05 [a]	0.61± 0.04 [e]	1.01 ± 0.03 [b]	0.90 ± 0.02[c]	0.68± 0.02 [d]	nd[*]
C16:0	19.89±0.90 [c]	19.30± 0.20 [c]	11.52 ± 0.36 [d]	36.67 ± 0.02 [a]	31.15±0.30 [b]	20.78±1.3 [c]
C17:0	3.33±0.02 [b]	2.01± 0.20 [d]	2.09±0.03 [c]	1.76 ± 0.40 [e]	5.11± 0.08 [a]	nd
C18:0	4.06±0.05 [e]	4.80± 0.20 [d]	2.72 ± 0.03 [f]	7.34 ± 0.02 [b]	5.48 ±0.03 [c]	10.12±0.40[a]
C20:0	6.78±0.18 [b]	nd[*]	nd[*]	1.29 ±0.05 [c]	1.06 ±0.04 [d]	8.74 ±0.04 [a]
Σ (SFA)	**49.96**	**34.26**	**19.02**	**52.86**	**50.05**	**44.35**
Monounsaturated fatty acids (MUFAs)						
C14.1	3.30 ± 0.20 [a]	1.24± 0.03 [b]	nd[*]	0.69 ± 0.02 [c]	nd[*]	nd[*]
C15:1	0.94 ± 0.50 [d]	1.43± 0.50 [b]	5.34 ± 0.20 [a]	0.50 ± 0.02 [e]	1.17 ± 0.40 [c]	nd[*]
C16:1 ω7	1.64 ± 0.30 [d]	1.91± 0.20 [c]	1.93 ± 0.03 [c]	1.27 ± 0.40 [d]	2.84± 0.02 [ab]	2.90± 0.09 [a]
C17:1	5.57± 0.03 [c]	7.33± 0.05 [b]	3.56 ± 0.4 [e]	4.13 ± 0.02 [d]	3.06 ±0.02 [f]	17.71±0.04[a]
C18:1 ω9c	7.95 ±0.32 [c]	9.32± 0.04 [b]	7.95 ± 0.50 [c]	10.58 ± 0.02 [a]	7.31 ±0.02 [d]	2.27 ±0.2 [e]
Σ (MUFA)	**19.4**	**21.23**	**18.78**	**17.17**	**14.38**	**22.88**
Polyunsaturated fatty acids (PUFAs)						
C18:2	2.70 ±0.20 [e]	3.36±0.03 [c]	3.09 ±0.04 [d]	3.25 ±0.04 [c]	6.95 ±0.02 [a]	4.19 ±0.05 [b]
C 20:2	nd[*]	2.49 ±0.04 [a]	1.37 ±0.02 [b]	1.22 ±0.02 [c]	nd[*]	nd[*]
C18:3 ω6	nd[*]	0.62 ±0.02 [b]	nd[*]	0.60 ±0.02 [b]	2.17 ±0.03 [a]	nd[*]
C18:3 ω3	1.43±0.2 [b]	1.76 ±0.02 [a]	nd[*]	nd[*]	nd[*]	1.37±0.14 [c]
C20:3 ω6	3.18 ±0.02 [d]	4.43 ±0.02 [b]	3.76 ±0.02 [c]	5.57 ±0.02 [a]	4.10 ±0.16 [bc]	nd[*]
C20:3 ω3	nd[*]	1.26 ±0.02 [c]	11.12 ±0.3 [a]	9.80 ±1.90 [b]	Nd	nd[*]
C20:4 ω6	10.36±0.59 [c]	18.24 ±0.03 [a]	10.65±0.02 [bc]	nd[*]	11.52 ±0.03 [b]	nd[*]
C22:2	nd[*]	nd[*]	21.57 ±0.40 [b]	nd[*]	7.18 ±0.02 [c]	22.97±0.02[a]
C20:5 ω3	9.53 ±0.02 [a]	nd[*]	2.74 ±0.03 [c]	nd[*]	nd[*]	9.29±0.12 [b]
C22:6 ω3	1.99 ±0.02 [d]	4.69 ±0.24 [a]	nd[*]	2.28 ±0.18 [bc]	2.22 ±0.02 [c]	3.69±0.23 [b]
Σ (PUFA)	**29.19**	**36.85**	**54.27**	**22.72**	**34.14**	**41.51**

nd[*]: not detected. Data are presented as mean ± standard deviation. Different superscripts within the same row indicate significant differences among treatments *($p \leq 0.05$,* a > b > c > d*).*

DISCUSSION

Nitrogen is an indispensable macronutrient for microalgal growth, playing an essential role in protein, lipid and carbohydrate synthesis **(Sajjadi *et al*., 2018; Zarrinmehr *et al.*, 2020)**. The results in Table (3) show that all different nitrogen sources responded well to enhance the growth, but maximum growth was recorded for NH_4HCO_3, indicating the favorable nitrogen source for *N. oculata* growth. The present findings coincide with recent studies of **Mahdieh *et al.* (2019)** and **Abugrara *et al. (*2020)** who demonstrated that, ammonium bicarbonate was the most rapidly digested nitrogen source, which indicates that ammonium bicarbonate increased maximal cell density not only because of nitrogen but also because of bicarbonate as a carbon source. It is believed that *Nannochloropsis* sp. may absorb bicarbonate ions from the environment into the cytosol via the membrane of the plasma and eliminate CO_2 from HCO_3 using carbonic anhydrase **(Li *et al.*, 2018)**.

When $NaNO_3$, NH_4NO_3 and NH_4CL were used as nitrogen sources, the cell density of *N. oculata* was significantly better than that of CO $(NH_2)_2$. This indicates that *N. oculata* prefers inorganic nitrogen and nitrate as nitrogen sources, which may be associated with the mechanism of nitrogen metabolism of this alga. Furthermore, ammonium is a reduced form of nitrogen that can be directly assimilated into amino acids inside the cells, resulting in higher biomass during the growth **(Ruangsomboon, 2015)**. Supporting these findings, **El Khodary *et al*. (2021)** observed that the highest cell biomass was obtained in *N. salina* when cultivated on Max well water which contains high nutrient, especially nitrate concentration .This finding demonstrated that nitrate positively correlates with algal growth **(Elshobary *et al*., 2020)**. In contrast, the growth of *Chlorella* sp. GN1 with ammonium source was lower than that with urea **(Feng *et al.*, 2020)**. Moreover, **Nayak *et al.* (2019)** observed that some microalgal species grew in the medium containing urea faster than that containing nitrate and ammonium nitrogen source. **Campos *et al.* (2014)** found that the urea treatments had the highest cell densities of *N. salina*. This exhibits that nitrogen requirements vary depending on the species.

The present study revealed that urea treatment achieved the highest significant difference ($p \leq 0.05$) in total protein content otherwise; the lowest protein content was achieved by NH_4CL. The higher protein content achieved by urea could be attributed to its molecular structure, each urea molecule has two nitrogen atoms, whereas nitrate only contains one **(Danesi *et al.*, 2002)**, and urea contributes to a modest pH change **(Ho *et al.*, 2013)**, observed in the current study where pH in urea treatment had a minor change during the experimental days. In addition, this can be due to urea dissociating to form CO_2 and ammonium via the urease enzyme, then the absorption of ammonium is directly taking place into the cell and accumulates to synthesis amino acids **(Kim *et al.*, 2013)**. These results are compatible with that of **Fatini *et al.* (2021)** who found that, urea had the highest protein content while NH_4CL had the lowest for *Chlorococcum* sp. Similar observation was noted in terms of *Chlorella vulgaris*, *Hillea* sp., and *N. oculata* when

cultivated in urea as nitrogen source **(Lourenço *et al.*, 2002; Ho *et al.*, 2013)**. Furthermore, our findings revealed that there was no significant ($p > 0.05$) difference between NH_4NO_3, $(NH_4)_2SO_4$, NH_4HCO_3, and the protein content decreased, respectively. Comparable results were found in the work of **Simsek and Cetin (2019)** who showed that the protein concentration in NH_4NO_3, $(NH_4)_2SO_4$, and $NaNO_3$ media decreased over time.

The present results findings showed that $NaNO_3$ achieved the highest total carbohydrates then NH_4HCO_3 which may be exhibited by cumulative inorganic dissolved carbon concentration as additional sources of energy **(Yang & Gao, 2003)**. Similar findings have been found with *C. pyrenoidosa* and *S. obliquus* when exposed to increasing CO_2 in the culture conditions **(Srinivasan *et al.*, 2018)**. While, the lowest significant total carbohydrates were achieved by urea. However, urea achieved the highest total protein in this study which could be due to that the energy was used for protein synthesis rather than carbohydrate accumulation **(Lourenco *et al.*, 2002)**. $(NH_4)_2\ SO_4$ and NH_4CL had showed no significant differences ($P > 0.05$) in the carbohydrate content or lipid content as the decrease in the carbohydrate content parallels the increase in the lipid content. These findings concur with those of **Roopnarain *et al.* (2015)** who postulated that, decreasing carbohydrate content is associated with increased lipid content, implying that carbohydrates are converted to lipids via the pathway of pentose phosphate. This indicates that carbohydrates are the primary storage product, and lipids are the secondary storage product.

The total lipid content increased significantly with the addition of NH_4NO_3, followed by $NaNO_3$ ($P \leq 0.05$). The most conceivable reason for the better performance of ammonium nitrate over other nitrogen sources may be related to the presence of both the ammonium and nitrate in the same medium, which enhanced the lipid content due to the increase in the cellular storage lipids (triacylglycerides or TAGs) of microalgae when ammonium nitrate was used **(Pancha *et al.*, 2015)**. The microalgae cultivated in a medium containing multiple nitrogen forms (ammonium and nitrate) had higher lipid content than those growing in media containing only one nitrogen form **(Zhu *et al.*, 2019)**.

Those results comply with the findings of **Campos *et al.* (2014)** who recommended that NH_4NO_3 achieved the highest lipid content than urea, $NaNO_3$, and NH_4HCO_3. While, NH_4CL, $(NH_4)_2SO_4$, and NH_4HCO_3 exhibited a moderate effect on lipid content. On the contrary, urea had the lowest significant total lipid in the experiment. **Ruangsomboon (2015)** found that replacing nitrate with urea reduced lipid yield in *C. sorokiniana* and *I. galbana*, with a more significant negative influence on lipid productivity.

The chemical composition and fatty acids profile of microalgae are influenced by environmental conditions and nutrient medium **(Li *et al.*, 2020)**. Our findings showed that palmitic acid (C16:0) was the most abundant saturated fatty acid, which is consistent with **Abugrara *et al.* (2019)** who found that palmitic acid is the most plentiful component in the crude lipids of *N. oceanica*. In all treatments, oleic acid (C18:1) was the most abundant

monounsaturated fatty acid (MUFA), but its value was reduced to reach its minimum amount (2.27%) with NH_4HCO_3. This suggests that, when sodium bicarbonate was added to the culture medium, the oleic acid level was reduced, which is congruent with the study of **Abugrara *et al.* (2020)**. Additionally, NH_4HCO_3 achieved the highest percentages of MUFAs, which may be due to the presence of carbon affecting the desaturation of FA. Therefore, they proved that an increase in total polyunsaturated fatty acids (PUFAs) was associated with decreasing carbon availability **(Swarnalatha *et al.*, 2015)**. The highest proportion of PUFAs was achieved by NH_4NO_3 and urea, which elucidated that more PUFAs are associated with the increase in nitrogen supplementation in the media, exhibited with NH_4NO_3 and the urea structure as mentioned before **(Kaye *et al.*, 2015)**.

CONCLUSION

Overall, the current findings demonstrate that nitrogen sources can strongly influence growth and biochemical composition in *N.oculata* sp. The results concluded that ammonium bicarbonate has a great effect on cell density. Urea achieved the highest protein content 38.28%, ammonium nitrate showed the highest lipid content 43.67%, and ammonium bicarbonate recorded the highest carbohydrate content 18.03%. The highest PUFA content was recorded at ammonium nitrate. The upper- mentioned results may help in choosing the best nitrogen source in the cell yield and chemical characteristics of *N. oculata* sp., which represent potentially interesting microalgae for aquaculture purposes.

ACKNOWLEDGEMEMT

The authors would like to express their gratitude to Prof. Dr. Mostafa M. Elsheek, Faculty of Science, Tanta University, Tanta, Egypt for his assistance with manuscript revision and for his comments that improved the manuscript.

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