

UDC 582.273:546.17

THE EFFECT OF DIVERSE NITROGEN SOURCES IN THE NUTRIENT MEDIUM ON THE GROWTH OF THE GREEN MICROALGAE *CHROMOCHLORIS ZOFINGIENSIS* IN THE BATCH CULTURE

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Received by the Editor 27.05.2019; after revision 14.10.2019;
accepted for publication 25.12.2019; published online 30.12.2019.

The effect of three nitrogen (N) sources in the nutrient medium – sodium nitrate (NaNO₃), urea (CO(NH₂)₂), and ammonium chloride (NH₄Cl) – on the morphological and physiological characteristics of the green microalga *Chromochloris* (*Chlorella*) *zofingiensis*, a potential commercial producer of lipids and a ketocarotenoid astaxanthin, was studied. The alga was batch-cultivated in glass conical flasks from starting cell density (n) around $2.3 \cdot 10^6$ per mL and dry weight (DW) content of $0.06 \text{ g} \cdot \text{L}^{-1}$ in all variants at $120 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ PAR, $+20 \dots +21 \text{ }^\circ\text{C}$, and air bubbling at a rate of $0.3 \text{ L} \cdot \text{min}^{-1} \cdot \text{L}^{-1}$. The concentration of nitrogen sources (as elemental N) in the modified BBM nutrient medium was $8.83 \text{ mmol} \cdot \text{L}^{-1}$, the cultivation duration was 17 days. The dynamics of n and cell volumes, DW content, chlorophylls *a* and *b* (Chl*a* and Chl*b*), total carotenoids (Car), and lipids (Lip) in the cultures, concentration of N sources in the nutrient medium, and its pH were recorded. It was shown that the growth rate, size distribution of the cell populations, and the biomass chemical composition depended significantly on the nitrogen source in the nutrient medium. Using NH₄Cl as N source caused on the second day growth inhibition, cell swelling, aggregation, and discoloration; by the seventh day, it caused culture crash. *C. zofingiensis* cells took up NaNO₃ and CO(NH₂)₂ from the medium at a similar rate (0.626 and $0.631 \text{ mmol N} \cdot \text{L}^{-1} \cdot \text{day}^{-1}$, respectively), but the growth of the culture fed with CO(NH₂)₂ lagged; its cell volume and Chl*a*, Chl*b*, and total Car contents declined profoundly. The average dry matter productivity (P_{DW}) in the culture grown on CO(NH₂)₂ [$(0.086 \pm 0.004) \text{ g} \cdot \text{L}^{-1} \cdot \text{day}^{-1}$] was 32.6 % lower than in the culture grown on NaNO₃ [$(0.114 \pm 0.005) \text{ g} \cdot \text{L}^{-1} \cdot \text{day}^{-1}$]. At the same time, lipid productivity (P_{Lip}) of the urea-fed culture was comparable with that of the nitrate-fed culture (P_{Lip} of 28 and 26 $\text{mg} \cdot \text{L}^{-1} \cdot \text{day}^{-1}$, respectively). The lipid DW percentage of the former exceeded significantly that of the nitrate-fed culture (31.6 % vs 23.1 %, respectively). From the standpoint of profitability, the lag in biomass accumulation recorded in the urea-fed culture on P_{DW} is not critical since it is compensated by lowering the cost of nitrogen source for the nutrient medium (approximately by 230 %) and a higher biomass lipid content. *C. zofingiensis* grown in media with urea as the only N source deserves further investigation.

Keywords: *Chromochloris zofingiensis*, batch culture, nitrogen nutrition, growth, pigments, lipids

Chromochloris (*Chlorella*) *zofingiensis* (Dönnz) Fucíková and L. A. Lewis, 2012 (Chlorophyceae, Sphaeropleales), formerly *Chlorella zofingiensis* Dönnz, 1934 [12], belongs to a highly specialized ecological group of carotenogenic green microalgae capable of survival under extremely adverse environmental conditions by rapid transition of its vegetative cells to a resting state. This process is accompanied by a massive accumulation of neutral lipids and C40-ketocarotenoids (hereinafter kCar) in cytoplasmic lipid droplets.

These pigments serve as antioxidants protecting the key macromolecules of the cell under abiotic stress [18]. The relevance of ecological and physiological studies of such species is determined by (i) gaining further insight into the mechanisms of microalgae adaptation to anthropogenic disturbance of natural ecosystems and (ii) identifying new sources of bulk lipids and high-value kCar (astaxanthin and its closest metabolic precursors) widely used in the production of feed for aquaculture and poultry, dietary supplements, and cosmetics [18, 32]. The most prominent representative of this group of microalgae is *Haematococcus pluvialis* Flotow, 1844 (Chlamydomonadales) serving as a model in studies of the mechanisms and physiological significance of secondary carotenogenesis in Chlorophyceae. At the same time, *H. pluvialis* became the only industrially cultivated microalgal producer of natural astaxanthin (hereinafter Ast) [32].

In recent years, *C. zofingiensis* has been increasingly considered as a new commercially promising candidate producer of Ast that can compete with *H. pluvialis*. The main arguments in favor of *C. zofingiensis* are its (i) higher growth rate, (ii) wider environmental plasticity (especially with respect to temperature, salinity, and hydrodynamic effects), and (iii) a more complete extractability of pigments from the biomass due to less tough cell wall than in *H. pluvialis* [7, 16, 20, 23, 35]. A number of authors focus on the development of methods for its cultivation as a source of lipids for biodiesel. The capability of co-generation of lipids and a mixture of kCar from Ast group, a product, which is expensive and much sought after, constitutes a serious technological advantage of *C. zofingiensis* increasing profitability of both target products [11, 14, 23].

It is shown that *C. zofingiensis* can be successfully cultivated autotrophically, mixotrophically, and heterotrophically [7, 15, 20, 22, 23, 35]. Some authors prefer heterotrophic cultivation of algae on glucose as it yields the highest amount of biomass, lipids, and pigments [35]. The high cost of the industrial fermenters eliminates this advantage. A more cost-efficient method of growing the algae is their cultivation in mineral media in open ponds or simple flat panel photoreactor made from polyethylene film [29].

In autotrophic systems for lipids production from microalgae, nitrates (KNO_3 and NaNO_3) are usually used as a source of nitrogen (N). Moreover, in the cost structure of the nutrient media, nitrates are the second after CO_2 : production of 1 ton of biodiesel requires 250–500 kg of nitrate-N [24]. Accordingly, search for N sources bioavailable for *C. zofingiensis*, cheaper than nitrates, is crucial for optimization of its bulk cultivation. Alternative N sources for many Chlorophyceae species comprise ammonium salts and urea ($\text{CO}(\text{NH}_2)_2$). Ammonium (NH_4^+) is the most reduced form of N, it is directly involved in the synthesis of amino acids in chloroplasts, while the assimilation of nitrates and nitrites requires their reduction to NH_4^+ by reductases [30]. Urea is the most N-rich substrate. Its molecule contains 47 % of N and 20 % of carbon. It is readily absorbed by most green microalgae featuring pathways for efficient assimilation of urea [3, 8]. These observations are experimentally confirmed by successful cultivation of microalgae from the genera *Chlorella* and *Scenedesmus* with urea as the sole source of N in the medium [2, 8, 13]. However, the genera *Chlorella* and *Chromochloris* were recently shown to be evolutionary remote [12]. There are a lot of data sets published about this cultivation. However, there is no investigation of its ability to be cultivated with urea.

The present study aimed at the experimental verification of this hypothesis under conditions of autotrophic batch culture. The key goals were to assess the effect of three N sources in the nutrient medium – sodium nitrate (NaNO_3), urea ($\text{CO}(\text{NH}_2)_2$), and ammonium chloride (NH_4Cl) – on the main morphophysiological characteristics of *C. zofingiensis*: its growth rate, cell size, nutrient uptake rate, dry weight (hereinafter DW), and pigment and lipid content expressed per cell, per unit culture volume, or DW.

MATERIAL AND METHODS

The strain *C. zofingiensis* CALU-190 (CCAP-211/14 = UTEX 32 = SAG 211-14 = ATTC 30412) obtained in 2006 from the Centre for Culture Collection of Microorganisms of St. Petersburg State University served as the object for this study. The strain was maintained on BBM medium [5] solidified with 1.5 % agar at the temperature round +15 °C and 35 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PAR with subculturing every 45 days.

To obtain the inoculate, the cells were transferred from the solid medium to the liquid BBM medium, diluted 2 times, and grown for 7 days in glass conical flasks at natural ambient light, room temperature +20...+22 °C, and bubbling with sterile air (0.3 L·min⁻¹·L⁻¹). Then the culture was sedimented by centrifugation (1000 g, 5 min); the cell pellet was transferred to complete BBM medium and cultivated for another 4 days at 120 μmol photons·m⁻²·s⁻¹ PAR from “Feron” DL 20W 6400K lamps and a photoperiod 15 h : 9 h light : darkness. The temperature and bubbling rate were the same as in the previous stage. The resulting culture consisting of young rapidly dividing cells was used as an inoculate.

In the experimental variant no. 1 (NaNO₃-fed cultures), *C. zoofingiensis* was grown on the BBM 3N medium of the following composition (g·L⁻¹ distilled water): NaNO₃ – 0.75; MgSO₄·7H₂O – 0.075; NaCl – 0.03; K₂HPO₄·3H₂O – 0.04; KH₂PO₄ – 0.1; Na₂EDTA – 0.05; KOH – 0.03; H₃VO₃ – 0.011; ZnSO₄·7H₂O – 0.0094; MnCl₂·4H₂O – 0.0014; Na₂MoO₄·2H₂O – 0.0024; CuSO₄·5H₂O – 0.0016; Co(NO₃)₂·6H₂O – 0.0005; FeSO₄·7H₂O – 0.005; CaCl₂ – 0.019; H₂SO₄ conc. – 1 μL.

In the nutrient media for the experimental variants no. 2 and no. 3, sodium nitrate was replaced by equimolar, as calculated per elemental nitrogen (8.83 mmol·L⁻¹), amount of urea (CO(NH₂)₂ – 0.265 g·L⁻¹), or ammonium chloride (NH₄Cl – 0.472 g·L⁻¹). The cultivation conditions were otherwise the same for all of the experimental variants: the starting cell density (*n*) about 2.3·10⁶ per mL (0.06 g DW·L⁻¹), 120 μmol photons·m⁻²·s⁻¹ PAR, 0.65 L of starting culture in 1-L conical flasks, (20 ± 1) °C, bubbling at 0.3 L·min⁻¹·L⁻¹.

The changes in cell density of the cultures (mL⁻¹) were recorded using a hemocytometer (4-grid Goryaev chamber; MiniMed, Russia) [37].

Dry weight content (g·L⁻¹) was determined gravimetrically using Whatman GF/C glass-fiber filters with a pore size of 1.2 μm [37].

Cell length (L) and width (W) were measured on photomicrographs acquired with the Leica DM-1000 photomicroscope (Leica Microsystems AG, Germany) and ImageJ software (National Institute of Health, USA). The volume of cells (V) was calculated by the formula for elongated spheroid or sphere. For the analysis of cell population size distribution, 100 cells were sampled.

Concentration of N sources in the culture medium was controlled potentiometrically or spectrophotometrically. Nitrate concentration was measured using a nitrate-selective electrode ELIS-121NO₃ (JSC “Prompribor”, Moscow, Russia). Urea was quantified by the color reaction with diacetylmonooxime [17]. Nitrite was assayed by Bendschneider – Robinson method [29].

Contents of chlorophylls (Chl*a*, Chl*b*) and total carotenoids (Car) in cultures was determined spectrophotometrically on SF-2000 spectrophotometer (MTPK-LOMO, Russia) after extraction of pigments from crude biomass by dimethyl sulfoxide (DMSO) [38].

Lipids were extracted according to Bligh and Dyer and assayed gravimetrically [4].

Maximum specific growth rates (μ_{max}, day⁻¹), average productivity in terms of cell density, DW, and total lipids (P_{Lip}) (mg·L⁻¹·day⁻¹) were calculated [40].

All measurements were performed in triplicate (biological replications) with 3 analytical replications for each biological replication. Averages and the corresponding standard deviations are shown in the text and figures. The significance of average differences was determined by Student’s *t*-test at the significance level *p* < 0.05.

RESULTS AND DISCUSSION

For this work, we opted for BBM, the most popular medium for laboratory cultivation of green microalgae with sodium nitrate as a N source [5]. As before for screening new commercially promising astaxanthin producers [6, 25, 36], we modified this medium by increasing its N content three-fold (BBM 3N). In this study, the BBM 3N medium served as a control. In the experimental variants, NaNO₃ was replaced by CO(NH₂)₂ and NH₄Cl. Moreover, urea, a preferable N source for certain biotechnologically significant

species of Chlorophyceae [2, 8, 9], was the most interesting N source considering the goal of minimizing the cost of nutrient media for *C. zofingiensis*. NH_4Cl was chosen among ammonium salts also for the cost optimization reasons; it is also one of the least tested substrates for the cultivation of this species.

Changes in cell density and DW in the batch cultures of *C. zofingiensis* (Fig. 1) showed that of the three N sources tested, only NaNO_3 and $\text{CO}(\text{NH}_2)_2$ provided for the algae rapid growth.

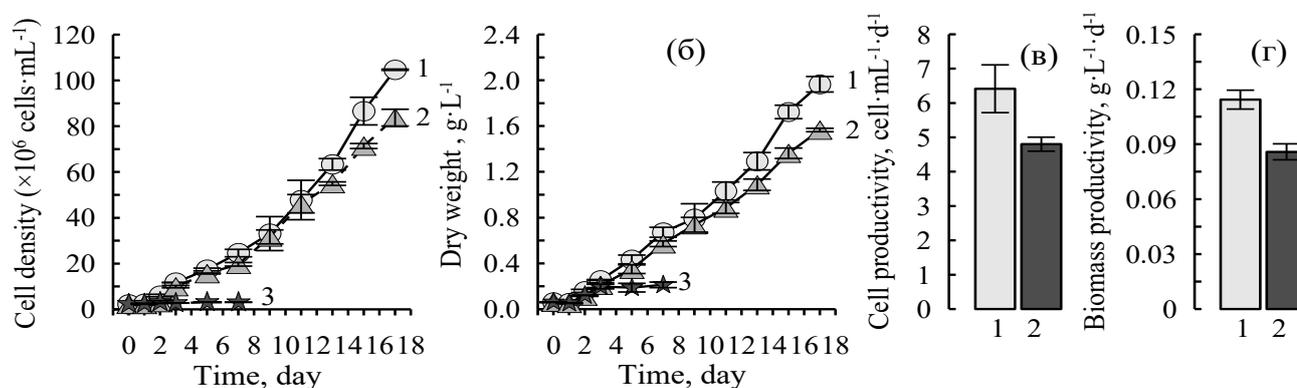


Fig. 1. Growth parameters of the *Chromochloris zofingiensis* batch cultures as a function of the nitrogen source in the nutrient medium. 1 – NaNO_3 ; 2 – $\text{CO}(\text{NH}_2)_2$; 3 – NH_4Cl

Рис. 1. Ростовые характеристики накопительных культур *Chromochloris zofingiensis* в зависимости от источника азота в питательной среде. 1 — NaNO_3 ; 2 — $\text{CO}(\text{NH}_2)_2$; 3 — NH_4Cl

The NH_4Cl -fed culture displayed a turbidity and cessation of the cell division by the second day of the experiment. The algae cells in this variant swelled rapidly (their average volume increased 1.6 times during the first day; Fig. 2a). Starting from the third day, Chl content per cell (Fig. 3a) and per unit cell volume (Fig. 3b) declined sharply. The buildup of stress was also evidenced by a significant increase in the Car/Chl ratio index (Fig. 3c). On the seventh day, the vast majority of cells in this culture looked colorless under a light microscope and aggregated into large, loose clumps held together by the products of dead cell destruction by bacteria. The cell death in the NH_4Cl -fed culture was likely caused by an excess of the ammonium salt, which acidified the culture medium as a result of the salt hydrolysis yielding a strong acid and a weak base: $\text{NH}_4^+ + \text{Cl}^- + \text{HOH} \leftrightarrow \text{NH}_4\text{OH} + \text{Cl}^- + \text{H}^+$ (Table 1).

Table 1. The effect of N species in the medium on its pH

Таблица 1. Влияние химической формы азота на pH питательных сред

Day	Time of the day	pH		
		NaNO_3	$\text{CO}(\text{NH}_2)_2$	NH_4Cl
5	16:15	8.53	7.07	4.45
7	14:45	8.90	7.28	4.41
13	14:30	9.10	7.70	–
15	14:30	9.00	7.60	–

Although the $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ molar ratio of the BBM 3N medium corresponds to that of a potassium-phosphate buffer (1:4), the buffer capacity of the medium is still insufficient to maintain pH in an optimum range for this species (5.5–8.5) [23]. A similar problem with cultivation of other chlorophytes (*Chlorella sorokiniana*, *Scenedesmus* sp., *Monoraphidium* sp.) on ammonium salts (NH_4Cl and NH_4NO_3) was reported [10, 21]. According to [28, 39], the main cause of cell death in such cases is inhibition of ATP synthesis in chloroplasts. Possible solution to the problem of growth inhibition by acidification of the medium during *C. zofingiensis* cultivation on ammonium salts as N source comprises cultivation

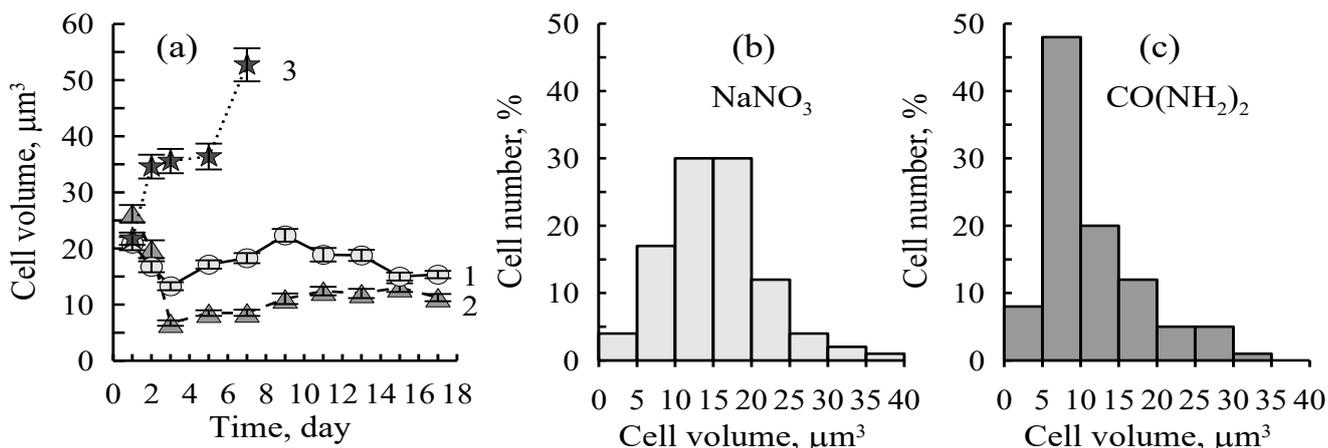


Fig. 2. Effect of the N source in the nutrient medium on (a) the dynamics of average cell volume and cell size distribution of cultures fed with (b) NaNO_3 or (c) $\text{CO}(\text{NH}_2)_2$ on the 17th day of *C. zofingiensis* cultivation. 1 – NaNO_3 ; 2 – $\text{CO}(\text{NH}_2)_2$; 3 – NH_4Cl

Рис. 2. Влияние источника азота в питательной среде на (a) динамику средних объёмов клеток и размерную структуру популяций клеток в вариантах (b) NaNO_3 и (c) $\text{CO}(\text{NH}_2)_2$ на 17-е сутки культивирования *C. zofingiensis*. 1 — NaNO_3 ; 2 — $\text{CO}(\text{NH}_2)_2$; 3 — NH_4Cl

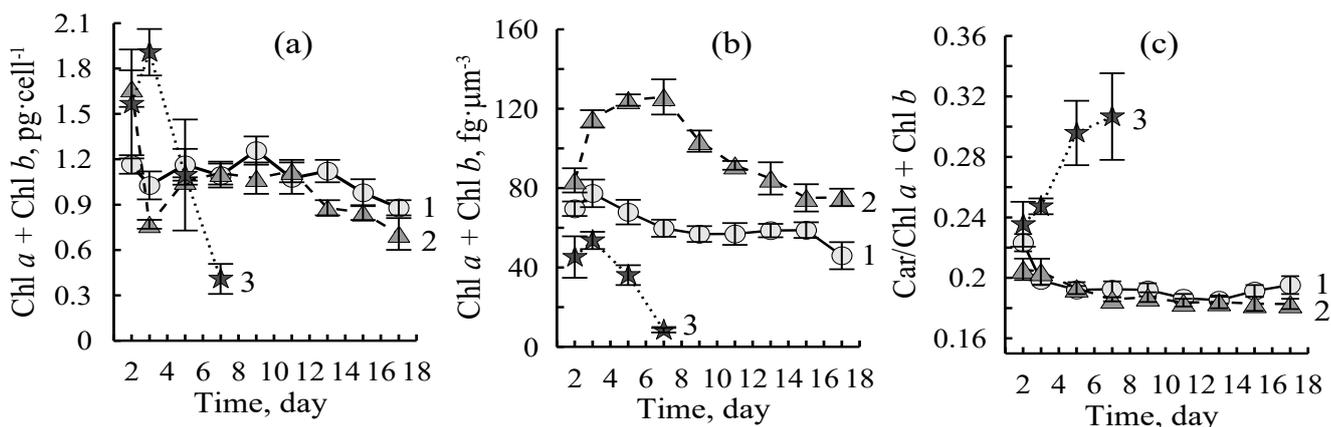


Fig. 3. Time-course of changes in chlorophylls content (a, b) and Car/Chl index (c) in *C. zofingiensis* cells as affected by the nitrogen source in the nutrient medium. 1 – NaNO_3 ; 2 – $\text{CO}(\text{NH}_2)_2$; 3 – NH_4Cl

Рис. 3. Динамика содержания хлорофиллов (a, b) и индекса Кар/Хл (c) в клетках *C. zofingiensis* в зависимости от источника азота в питательной среде. 1 — NaNO_3 ; 2 — $\text{CO}(\text{NH}_2)_2$; 3 — NH_4Cl

in chemostat mode with pH adjustment by daily application of alkali solution (tested with *Scenedesmus* sp. and *Monoraphidium* sp. [10]). A suitable alternative would be increasing the buffer capacity of the nutrient medium by addition of an organic buffer (HEPES, TAPS, Bicine, MES, etc.) [34]. Both options require further research. At this stage, one can only argue that NH_4Cl is not a suitable N source for cultivation of *C. zofingiensis* according to the scheme employed (unbuffered BBM 3N medium; starting cell density of $2 \cdot 10^6$ per mL; $8.82 \text{ mmol} \cdot \text{L}^{-1}$ of ammonium N).

In the cultures fed with NaNO_3 or $\text{CO}(\text{NH}_2)_2$, cells divided vigorously after a short (1–2 days) lag phase; vigorous cell division was observed throughout the observation period (Fig. 1). The duration of the exponential growth phase in the NaNO_3 -fed cultures was 2 days vs 1 day in the $\text{CO}(\text{NH}_2)_2$ -fed cultures. In the $\text{CO}(\text{NH}_2)_2$ -fed cultures, the maximum specific growth rate (1.21 day^{-1}) was 57 % higher than in the NaNO_3 -fed cultures (0.77 day^{-1}), but the average productivity in terms of cell density and biomass accumulation of the urea-fed cultures was significantly lower than of the nitrate-fed cultures (by 34 and 32 %, respectively).

In both variants, the culture growth curves (monitored via either cell density or DW) of *C. zofingiensis* did not reach stationary phase for quite a long time. By contrast, the previously studied carotenogenic species (*Haematococcus pluvialis*, *Coelastrella rubescens*, etc.) reached stationary phase by the 8th–9th day of cultivation under similar conditions in the BBM 3N medium [6, 25]. Such differences are probably due to the fact that these species have much (1–2 orders of magnitude) larger cells than *C. zofingiensis*. Thus, the average cell volume (hereinafter ACV) of *H. pluvialis* IMBR-2 featuring the smallest cells among the strains in the collection of A. O. Kovalevsky Institute of Biology of the Southern Seas of RAS varied, under similar external conditions, in the range of 1000–2500 μm^3 depending on the growth stage; in *C. rubescens* ACV varied in the range of 60–130 μm^3 , and in *Pseudospongiococcum protococcoides* – in the range of 150–200 μm^3 [6, 25]. After the end of lag phase, the ACV in the NaNO_3 -fed cultures of *C. zofingiensis* was just 13–22 μm^3 ; the urea-fed cultures were characterized by even smaller ACV (6–13 μm^3 ; Fig. 2a). Obviously, the smaller the cells are, the later the slow-down of culture growth due to nutrient starvation will occur (provided that the cultures were initiated at the same cell density and biogenic element concentration in the medium); it was the case in this work. Only after 15–17 days of cultivation, both cultures of *C. zofingiensis* showed the signs of growth slowdown, although the concentration of NaNO_3 and $\text{CO}(\text{NH}_2)_2$ fell below determination limit already on the 13th day. The corresponding average uptake rates were close (0.626 and 0.631 $\text{mmol N}\cdot\text{L}^{-1}\cdot\text{day}^{-1}$, respectively; Fig. 4a). In the NaNO_3 -fed culture, the N source including nitrite accumulated in the medium at an average rate of 0.001 $\text{mmol N}\cdot\text{L}^{-1}\cdot\text{day}^{-1}$ as a result of incomplete reduction of NO_3^- to NH_4^+ [30] (Fig. 4b). A similar short-term maintenance of cell division upon depletion of nitrate nitrogen N in the medium was previously observed in *H. pluvialis* batch cultures [40].

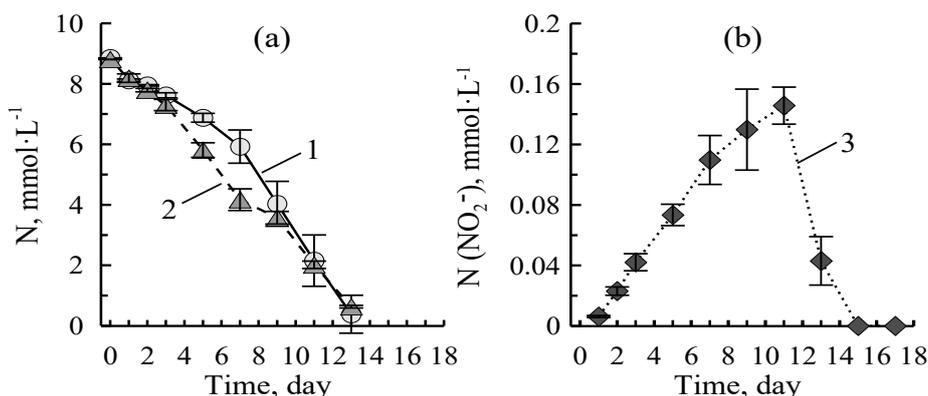


Fig. 4. Dynamics of nitrogen species content in the nutrient medium (a – absorbed N; b – released N). 1 – NaNO_3 ; 2 – $\text{CO}(\text{NH}_2)_2$; 3 – NaNO_2

Рис. 4. Динамика содержания различных форм азота в питательной среде (а — поглощаемый азот; б — выделяемый азот). 1 — NaNO_3 ; 2 — $\text{CO}(\text{NH}_2)_2$; 3 — NaNO_2

This phenomenon stems likely from the ability of microalgae to maintain intracellular N pool even under shortage of exogenic N via increasing the active transmembrane transport of the biogenic element by (i) phosphorylation of specific transporters and increasing their affinity to (NO_3^-) (ions) and urea and (ii) up-regulating genes responsible for assimilation of N sources [26, 30].

Mobilization of intracellular N reserves also plays a role in the regulation of intracellular nitrogen pool under N starvation. This suggestion is in line with the observed kinetics of the photosynthetic pigment content of *C. zofingiensis* expressed per cell, unit culture volume, or biomass (Fig. 3b and c; Fig. 5). It reflects a decline in the pigment (mainly chlorophyll) content under nitrogen shortage and/or decomposition of the pigment-protein complexes to support the subsistence pool of key N-containing biomolecules (enzymes, macroergic compounds, nucleic acids, etc.) [19].

It is believed that for many chlorophyte species urea is the most preferred N source providing for higher growth rates as compared with cultivation in nitrate- and ammonium-containing medium since molecule of urea contains more N than inorganic N species, in addition to the bioavailable carbon [2, 8, 13, 33].

However, growing *C. zofingiensis* under conditions employed in this work did not demonstrate the advantages of urea. The urea-fed culture lagged, although slightly, behind the nitrate-fed culture considering cell division rate, DW accumulation, and photosynthetic pigment content. At the same time, it was commensurate to the NaNO_3 -fed culture in terms of total lipid productivity and even exceeded it by 36.4 % considering the DW percentage of lipids (31.65 % vs 23.21 %; $p < 0.05$; Fig. 6).

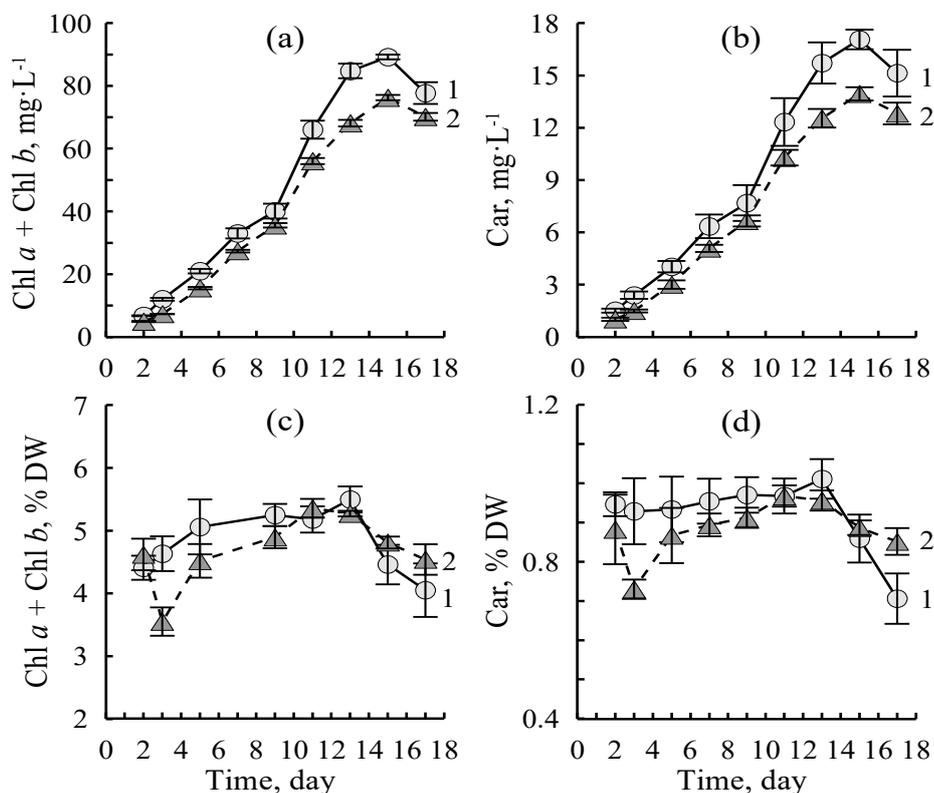


Fig. 5. The changes in chlorophylls and total carotenoids content in cultures (a, b) and dry weight (c, d) of *C. zofingiensis* depending on the nitrogen source in the nutrient medium. 1 – NaNO_3 ; 2 – $\text{CO}(\text{NH}_2)_2$

Рис. 5. Динамика содержания хлорофиллов и суммарных каротиноидов в культурах (a, b) и сухой биомассе (c, d) *C. zofingiensis* в зависимости от источника азота в питательной среде. 1 — NaNO_3 ; 2 — $\text{CO}(\text{NH}_2)_2$

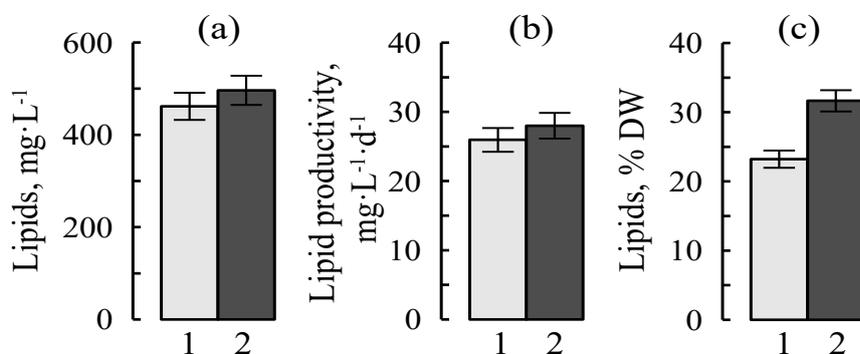


Fig. 6. The total lipid content in the cultures on the 17th day (a), the average lipid productivity of the cultures (b), and the lipid DW percentage (c) of *C. zofingiensis* depending on the nitrogen source in the nutrient medium. 1 – NaNO_3 ; 2 – $\text{CO}(\text{NH}_2)_2$

Рис. 6. Содержание суммарных липидов в культурах на 17-е сутки (a), средняя продуктивность культур по липидам (b) и массовая доля липидов в сухом веществе (c) *C. zofingiensis* в зависимости от источника азота в питательной среде. 1 — NaNO_3 ; 2 — $\text{CO}(\text{NH}_2)_2$

At the same time, cost analysis suggests that a lag of the urea-fed culture as high as even 30 % considering biomass productivity is not critical since it will be compensated by savings (approximately 230 %) on the cost of reagents for the nutrient medium (Table 2).

Table 2. Comparative characteristics of some technological parameters and price of urea and sodium nitrate as nitrogen sources in nutrient media for *C. zofingiensis*

Таблица 2. Сравнительная характеристика некоторых технологических показателей и стоимости карбамида и нитрата натрия как источников азота в питательных средах для *C. zofingiensis*

N source	Russian standard (GOST)	N, mass %	Consumption, kg per m ³ of medium	Wholesale price, RUR per kg*	N costs, RUR per m ³ of medium
Urea	2081-92	46.6	0.265	26	6.89
Sodium nitrate	828-77	16.5	0.750	30	22.50

* Mineral fertilizer wholesale prices (<https://agrosserver.ru>, accessed on April 03, 2019)

As a rule, this is the decisive advantage of urea as N source for mass cultivation of microalgae for lipids [1, 8, 13]. Analysis of the reports published on this problem shows that the potential of urea as an N-rich carbon-containing nutrient that accelerates the microalgae growth is not always implemented in practice. It is manifested mainly in the cultivation of representatives of the genus *Chlorella* [2, 13, 33]. Many other species of Chlorophyta (e. g. *Chlamydomonas reinhardtii*, *Scenedesmus bijugatus*, *Haematococcus pluvialis*) also take up urea efficiently, but their biomass productivity on this substrate is slightly lower than on nitrates [1, 30, 32]. At the same time, a slightly lower biomass yield of the urea-fed cultures is normally compensated by a higher lipid content of the biomass and a better suitability of the fatty acid composition of the lipids for biodiesel [10, 28].

Notably, it is very difficult to compare the published data on N source preference for different microalgal taxa due to disparate cultivation conditions (illumination, temperature, availability of phosphorus, CO₂, and trace elements, peculiarities of inocula preparation, etc.) which modulate profoundly the effect of N sources on the microalgae growth.

Thus, in *Chlorella vulgaris*, the advantages of urea over other nitrogen sources were realized only after acclimation of the inoculate to the new chemical form of nitrogen, i. e. only on the second cycle of cultivation, according to which the growth rate on urea was 1.5 times higher than on nitrates [27]. This fact is directly related to the results obtained in this work, since the transfer of *C. zofingiensis* inoculate grown on nitrates to the nutrient medium on urea could lead to a slowdown in growth and to a noticeable grinding of the culture (Figs 1, 2), which, in turn, affected the productivity of the crop for dry matter and pigments.

Another plausible reason for the lag in the growth of urea-fed culture could be excess of urea in the nutrient medium for a given starting cell density. Imbalance of urea uptake and assimilation could lead to an unfavorable buildup of ammonium ions in the cell during enzymatic hydrolysis of urea [3] and, as mentioned above, to inhibition of ATP biosynthesis in chloroplasts [39].

Conclusion. We studied the morphological and physiological characteristics of a green microalga *Chromochloris zofingiensis* enrichment culture in the BBM 3N medium supplemented with sodium nitrate, ammonium chloride, or urea equalized by nitrogen (8.8 mmol·L⁻¹) and starting cell density of 2·10⁶ per mL. Growth rate and chemical composition of the biomass were affected significantly by chemical nature of N source. NH₄Cl inhibited cell division by the second day of cultivation, caused cell swelling, aggregation, and discoloration leading to the culture crash by the seventh day. Our results show that NH₄Cl is not a suitable N source for cultivation of *C. zofingiensis* under our experimental conditions. The cells of *C. zofingiensis* efficiently took up urea at a rate similar to the rate of NaNO₃ uptake (0.631 and 0.626 mmol N·L⁻¹·day⁻¹). Nevertheless, the CO(NH₂)₂-fed cultures were characterized by a slight growth slowdown as well as a significant decrease in the cell volume and photosynthetic pigment content

expressed per cell, unit cell volume (μm^3), and culture volume. The average DW productivity in this experimental variant $[(0.086 \pm 0.004) \text{ g}\cdot\text{L}^{-1}\cdot\text{day}^{-1}]$ was 32.6 % lower than that of the NaNO_3 -fed culture $[(0.114 \pm 0.005) \text{ g}\cdot\text{L}^{-1}\cdot\text{day}^{-1}]$. At the same time, both cultures exhibited a similar lipid productivity (26 and 28 $\text{mg}\cdot\text{L}^{-1}\cdot\text{day}^{-1}$; $p > 0.05$). Mass fraction of lipids in DW of the urea-fed culture was significantly higher than in the nitrate-fed culture (31.6 and 23.1 % DW, respectively). From economic standpoint, the lag in biomass productivity (32 %) exhibited by the urea-fed culture is of secondary importance since it is more than covered by a large (about 230 %) savings on the N source cost. To conclude, our results lay a foundation for further research on the optimization of *C. zofingiensis* cultivation using urea as the sole N source in the nutrient medium.

This work was carried out within the framework of government research assignment of IBSS "Investigation of the regulation mechanisms of biotechnological system productivity for developing if scientific foundation for production of biologically active substances and bulk products technical from marine source" (no. AAAA-A18-118021350003-6).

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ВЛИЯНИЕ РАЗЛИЧНЫХ ИСТОЧНИКОВ АЗОТА В ПИТАТЕЛЬНОЙ СРЕДЕ НА РОСТ ЗЕЛЁНОЙ МИКРОВОДОРОСЛИ *CHROMOCHLORIS ZOFINGIENSIS* В НАКОПИТЕЛЬНОЙ КУЛЬТУРЕ

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Исследовали влияние трёх источников азота в питательной среде — нитрата натрия (NaNO₃), мочевины (CO(NH₂)₂) и хлорида аммония (NH₄Cl) — на морфофизиологические характеристики зелёной микроводоросли *Chromochloris (Chlorella) zofingiensis* как потенциального промышленного источника липидов и кетокаротиноида астаксантина. Водоросль выращивали методом накопительной культуры в стеклянных конических колбах при начальной численности клеток (*n*) во всех вариантах около 2,3·10⁶ кл·мл⁻¹, содержания сухого вещества (СВ) 0,06 г·л⁻¹, интенсивности ФАР 120 мкмоль фотонов·м⁻²·с⁻¹, температуре +20...+21 °С и скорости продувки культур воздуха 0,3 л·мин⁻¹·л⁻¹. Концентрация всех источников азота в модифицированной питательной среде ВВМ в пересчёте на атомарный азот (N) составляла 8,83 ммоль·л⁻¹, продолжительность культивирования — 17 суток. Регистрировали динамику *n* и объёмов клеток, содержания в культурах СВ, хлорофиллов (Хла и Хлб), суммарных каротиноидов и липидов, концентрации N в среде и её pH.

Показано, что скорость роста, размерная структура клеточных популяций и химический состав биомассы существенно зависели от химической формы азота в питательной среде. NH_4Cl уже на 2-е сутки вызвал ингибирование деления, разбухание, агрегацию и обесцвечивание клеток, а на 7-е сутки — гибель культуры. Клетки *C. zofingiensis* поглощали NaNO_3 и $\text{CO}(\text{NH}_2)_2$ из среды со сходной скоростью ($0,626$ и $0,631$ ммоль $\text{N}\cdot\text{л}^{-1}\cdot\text{сут}^{-1}$ соответственно), однако культура, выращиваемая на $\text{CO}(\text{NH}_2)_2$, отставала в росте и отличалась существенным уменьшением объёмов клеток и снижением в них содержания пигментов. Средняя продуктивность по сухому веществу ($P_{\text{СВ}}$) при росте на $\text{CO}(\text{NH}_2)_2$ [$(0,086 \pm 0,004)$ г·л⁻¹·сут⁻¹] была на 32,6 % ниже, чем при росте на NaNO_3 [$(0,114 \pm 0,005)$ г·л⁻¹·сут⁻¹]. В то же время по продуктивности по липидам ($P_{\text{лип}}$) культуры не различались (28 и 26 мг·л⁻¹·сут⁻¹ соответственно), а массовая доля липидов в СВ была достоверно выше в варианте $\text{CO}(\text{NH}_2)_2$ — 31,6 % против 23,1 % в варианте NaNO_3 . В экономическом аспекте отставание карбамидной культуры по $P_{\text{СВ}}$ не является критичным, так как компенсируется снижением затрат на реагенты для питательной среды (примерно на 230 %) и более высоким содержанием липидов в биомассе. Это обстоятельство служит основанием для продолжения исследований *C. zofingiensis* как потенциального источника липидов с использованием мочевины в качестве единственной формы азота в питательной среде.

Ключевые слова: *Chromochloris zofingiensis*, периодическая культура, азотное питание, рост, пигменты, липиды