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# Enhancement of Pigments Production by *Nannochloropsis oculata* Cells in Response to Bicarbonate Supply

**Giovanna Salbitani [1,*], Francesco Del Prete [1], Simona Carfagna [1], Giovanni Sansone [1] and Carmela M. A. Barone [2,*]**

[1] Dipartimento di Biologia, Università di Napoli Federico II, Via Cinthia 21, 80126 Napoli, Italy; francesco.delprete@unina.it (F.D.P.); simona.carfagna@unina.it (S.C.); giovanni.sansone@unina.it (G.S.)
[2] Dipartimento di Agraria, Sezione Scienze Delle Produzioni Animali, Università Degli Studi di Napoli Federico II, Via Università 100, 80055 Portici, Italy
* Correspondence: giovanna.salbitani@unina.it (G.S.); carmela.barone@unina.it (C.M.A.B.)

**Abstract:** In this study, the effects of bicarbonate addition on growth and pigment contents of the unicellular microalga *Nannochloropsis oculata*, were evaluated. *N. oculata* represents an interesting source of biomolecules widely used for food supplements and nutraceuticals. The bicarbonate was supplemented to microalgae cultures at concentrations of 0, 6, 18, 30, 42 and 60 mM. The cultures supplemented with salt at highest concentrations (42 and 60 mM) showed a significant increase in algal growth, demonstrated by the optical density spread. The intracellular content of pigments such as chlorophyll *a* and total carotenoids reached the highest values in cells from cultures supplied with bicarbonate. In fact, concentrations of bicarbonate from 30 to 60 mM strongly improved, for a short period of only 72 h, the cellular levels of chlorophylls and carotenoids. These are interesting pigments with commercial applications. The utilization of bicarbonate could represent an interesting sustainable opportunity to improve microalgae cultivation for cellular growth and pigment contents.

**Keywords:** bicarbonate; carotenoids; chlorophyll; microalgae; *Nannochloropsis oculata*

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## 1. Introduction

In recent years, a marked interest has grown around microalgae cultivation because of its multiple biotechnological applications. As of today, microalgae farming aims to produce high market value molecules and technologies, such as pigments, proteins, lipids, carbohydrates, minerals, vitamins and other elements that can be employed as sources of pharmaceuticals, cosmetics, health and dietary supplements [1–5]. In addition, microalgae has generated interest for producing eco-sustainable biofuels [6,7], also known as 3rd generation fuels.

Plants are excellent sources of pigments, such as chlorophylls, carotenoids, betacyanin, etc. [8]. Microalgae, like plants, synthetize a lot of pigments whose composition and quantity can differ according to species and growing conditions. Their cultivation, when compared to plants, exhibits many advantages such as faster growth, higher biomass productivity and smaller land area for cultivation [9]. For this reason, microalgae are an interesting alternative platform for pigment production when compared to the traditional sources [9].

Microalgae are unicellular organisms considered the greatest primary producers of any aquatic habitat; they have high growth rates, requiring only water, nutrients and carbon dioxide ($CO_2$) for their survival [10,11].

In plant cell, the right supply of inorganic carbon is crucial for photosynthetic activity and then for a performant Calvin-Benson cycle [11,12]. The main additional inorganic carbon (C) source for microalgae cultivation systems is the pure gaseous $CO_2$. However, insufflation of carbon dioxide could prove expensive (~35–50% of the total costs) and inefficient with much of $CO_2$ lost to the atmosphere [6,13,14]. The use of bicarbonate, as

feedstock for algae, could represent a valid alternative in aqueous solution being cheaper and showing a better solubility than $CO_2$ (90 g $L^{-1}$ for $NaHCO_3$ and 1.5 g $L^{-1}$ for $CO_2$ at 25 °C) [15]. In addition, using innovative technology, atmospheric $CO_2$ can be captured and converted into bicarbonate [7,16]. In this case, bicarbonate can also represent an eco-sustainable alternative to $CO_2$.

In marine aquatic environments (pH ~8.2), the majority (>90%) of dissolved inorganic carbon (DIC) is bicarbonate ($HCO_3^-$) and the percentage of spontaneous conversion in $CO_2$ is quite low [17]. In fact, according to Mondal et al. [18], between pH ranging from 6.4 to 10.3 most of the $CO_2$ available in water is in the bicarbonate form. Many microalgal species can actively carry $HCO_3^-$, through membrane pumps, from the external environment into the cell [18,19]. Once into the cytosol, bicarbonate is then converted by carbonic anhydrase (EC: 4.2.1.1) to $CO_2$, usable in chloroplasts by Rubisco (EC: 2.1.1.127) [12,17,18]. Most of the microalgae have a well-developed carbon concentrating mechanism (CCM) that pumps extracellular bicarbonate ions into the cells and raises the intracellular levels of $CO_2$, promoting the carboxylation reaction and suppressing the oxygenase reaction of Rubisco and then the photorespiration [18,20,21].

In this study, microalgae cultures were grown in a medium that was enriched with a bicarbonate solution as an inorganic carbon source alternative to pure $CO_2$-gas.

The microalga used for this research is *Nannochloropsis oculata* (Eustigmatophyceae), a unicellular green alga, found in both marine and freshwater environments. This contains a plentiful number of proteins, pigments, and polyunsaturated fatty acids [22]. *Nannochloropsis* sp. is one such industrially significant species due to its elevated photosynthetic efficiency and lipid productivity [14,23,24]. The total lipid content in *Nannochloropsis* ranges between 37 to 60% of dry weight. In addition, high yields in the omega-3 (ω-3), in particular eicosapentaenoic acid (EPA), make *Nannochloropsis* sp. one of the most industrially promising microalgae [23]. In photoautotrophic conditions *Nannochloropsis* sp. produces valuable carotenoids, which not only perform important functions during photosynthesis, but their antioxidant ability gives them potential commercial applications for human consumption [25]. However, although there is industrial interest in carotenoids applications, their yield by microalgae is crucial in commercial feasibility. Important developments in pigment production could lead to an interesting effect on commercial application.

The aim of the present research is to evaluate the short-term effects (72 h) of different concentrations of bicarbonate-solution (from 0 to 60 mM in the culture) on cell growth and pigment contents of *N. oculata*.

## 2. Materials and Methods

### *2.1. Algal Strain and Cultivation Conditions*

*Nannochloropsis oculata* (strain 38.85) was obtained from the algae collection of Goettingen University (SAG). The strain was cultivated in f/2 medium [26] prepared with natural seawater collected from the Gulf of Naples. Seawater was filtered through a 0.45 µm pore size membrane and put in a stove at 80 °C overnight. The salinity of seawater dropped to 20 ppt (parts per thousand).

The experiments were conducted in a polycarbonate annular photo-bioreactors (external column: 21 cm in diameter, 100 cm in length; internal column: 15 cm in diameter, 100 cm in length) with a working volume of 15 L, illuminated internally with fluorescent lamps (Philips TLD 30 W/55), equipped with an air dispensing system for homogenizing the cultures. The experiments were carried out under controlled conditions: temperature 26 ± 1 °C; light intensity of continuous illumination was set to 120–135 µmol $s^{-1}$ $m^{-2}$; continuously bubbling of filtered air at 80–100 L $h^{-1}$. The initial pH was adjusted to 8.1. Culture purity and culture growth were monitored by optical microscopy.

Cultures of *N. oculata* were supplied with different concentration of bicarbonate-solution. The media were enriched with the solution until reaching $NaHCO_3$ concentrations

of 0, 6, 18, 30, 42 and 60 mM (corresponding to $NaHCO_3$ 0, 0.5, 1.5, 2.5, 3.5, 5 g $L^{-1}$, respectively).

*N. oculata* cultures were daily monitored and the growth was spectrophotometrically evaluated by measuring the optical density (OD) at 600 nm.

The cells harvesting was performed by centrifugation at 15,000 rpm for 10 min. the collected biomass was lyophilized and stored at −20 °C until further processing.

### 2.2. Chlorophyll a and Total Carotenoid Contents

The chlorophyll *a* (Chl-*a*) and total carotenoid (Car) contents were estimated spectrophotometrically. An aliquot of 2 mL of culture was centrifuged at 4000× *g* for 10 min. The pellet was resuspended with 2 mL of *N*,*N*-dimethylformamide (1:1 ratio) and transferred into a glass tube. Pigments were extracted in the dark at 4 °C for about 24 h. The absorbance of the samples was measured, using glass cuvettes, at 664, 647 and 470 nm. The Chl-*a* and Car were calculated according to Inskeep and Bloom and Wellburn formula, respectively [27,28]. Equations to calculate Chl-*a* and Car are the following:

$$\text{Chl-}a = (12.70 \cdot A_{664}) - (2.79 \cdot A_{647})$$

$$\text{Chl-}tot = (17.90 \cdot A_{647}) + (8.08 \cdot A_{664})$$

$$\text{Car} = (1000 \cdot A_{470}) - (1.12 \cdot \text{Chl-}a) - (34.07 \cdot (\text{Chl-}tot\text{-Chl-}a))/245$$

$A_{647}$ = absorbance at 647 nm (maximum for Chl-*b*); $A_{664}$ = absorbance at 664 nm (maximum for Chl-*a*); $A_{470}$ = absorbance at 470 (maximum for Car).

Values from these equations are expressed as mg $L^{-1}$. To express the pigment as ng $cell^{-1}$, the number of *Nannochloropsis* cells *per* mL of culture was determined by Bürker chamber. Aliquots (10 µL) of algal culture were used. Direct counting of algae number was made by optical microscope. The counting was performed in triplicate.

### 2.3. Statistical Analysis

The data was analysed by two-way analysis of variance (ANOVA) followed by the Tukey's test (*post-hoc*) for growth parameters and one-way ANOVA for biomass characteristics by using SAS (Version 9.1.3 Institute Inc., Cary, NC, USA). The level of significance was $p \leq 0.05$.

## 3. Results

### 3.1. Culture Growth and pH

The effect of bicarbonate supply on *Nannochloropsis oculata* growth was monitored as changes in optical density (OD) over the time period (72 h). The addition of the $HCO_3^-$ solution (0, 6, 18, 30, 42 and 60 mM medium concentration) occurred in the culture medium as a single administration when the cells number reached ~$1.2 \times 10^6$ cell $mL^{-1}$. During the first 24 h a slightly decrease in the optical density was observed in the experimental cultures. After 24 h from the start of the treatment, the supplemented cultures show a slight slowdown in optical density with respect to the samples without $HCO_3^-$ addition (control or 0 mM condition). At the end of the experiments (72 h), values of growth rate were higher in the cultures supplemented with 60 mM bicarbonate-solution, than in the control (Figure 1). The growth rate in the control cultures was 0.330 ± 0.02 $d^{-1}$. At the highest concentration of bicarbonate, the cellular growth rate reached a value of 0.50 ± 0.230 $d^{-1}$, although the maximum value (0.72 ± 0.259 $d^{-1}$) was found in culture supplemented with 30 mM of bicarbonate.

Figure 2 shows the pH changes over the time period. After 24 h from the bicarbonate addition, the cultures pH was comparable to each other, while after 72 h the pH of the cultures supplemented with 18, 30, 42 and 60 mM of bicarbonate-solution highlighted an increase with respect to cultures enriched with 0 and 6 mM of bicarbonate.

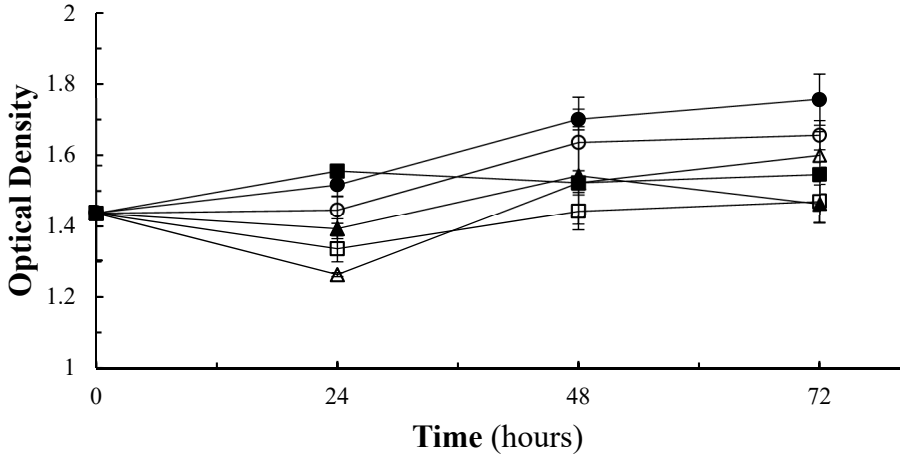


**Figure 1.** *Nannochloropsis oculata* optical density ($OD_{600}$) ± SD ($n$ = 3) at six different concentrations of bicarbonate: 0 (■), 6 (□), 18 (○), 30 (Δ), 42 (▲) and 60 (●) mM.

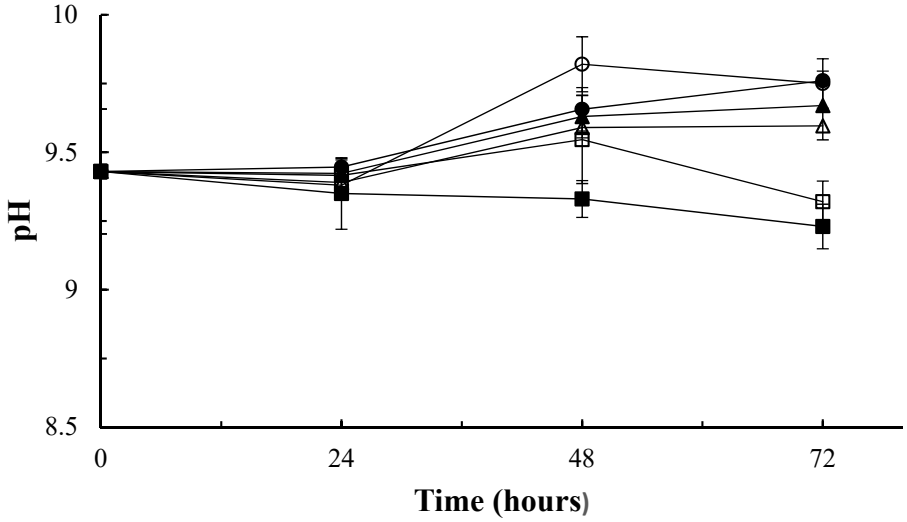


**Figure 2.** PH curves from 0 to 72 h for *Nannochloropsis oculata* cultures supplemented with bicarbonate 0 (■), 6 (□), 18 (○), 30 (Δ), 42 (▲) and 60 (●) mM. Error bars represent SD ($n$ = 3).

### *3.2. Chlorophyll a and Total Carotenoid Contents*

Chlorophyll *a* (Chl-*a*) and total carotenoids measured in *N. oculata* after 72 h were shown in Figures 3 and 4, respectively. Cells from cultures supplemented with 6 mM of bicarbonate did not display variations in Chl-*a* contents compared to control conditions (1.33 ± 10.03 and 1.23 ± 0.01 ng cell$^{-1}$, respectively). For the other treatments, a significant increase in Chl-*a* was observed ($p < 0.05$) and maximum value was reached with 60 mM of bicarbonate (5.3 ± 0.20 ng cell$^{-1}$).

With regards to total carotenoid contents, no significant differences were recorded among the control (0.29 ± 0.06 ng cell$^{-1}$) and cultures supplemented with the solution, until 30 mM. The highest carotenoids value was recorded for the treatment with 60 mM of bicarbonate, about 4-fold more than control (1.46 ± 0.08 ng cell$^{-1}$, $p < 0.05$).

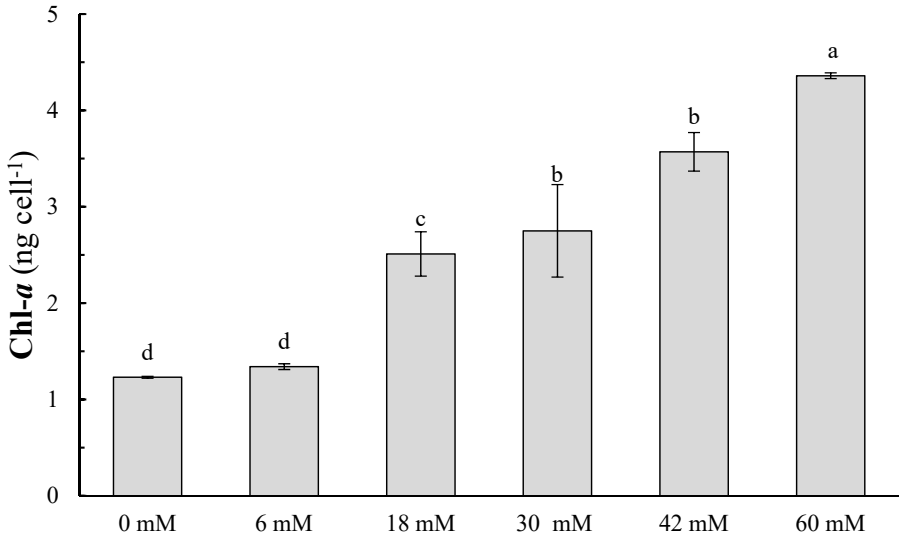


**Figure 3.** Chlorophyll *a* content in *Nannochloropsis oculata* cells grown for 72 h under different bicarbonate supplementation (0, 6, 18, 30, 42 and 60 mM). Error bars represent SD ($n$ = 3). Different letters above each bar indicate significant differences ($p \leq 0.05$).

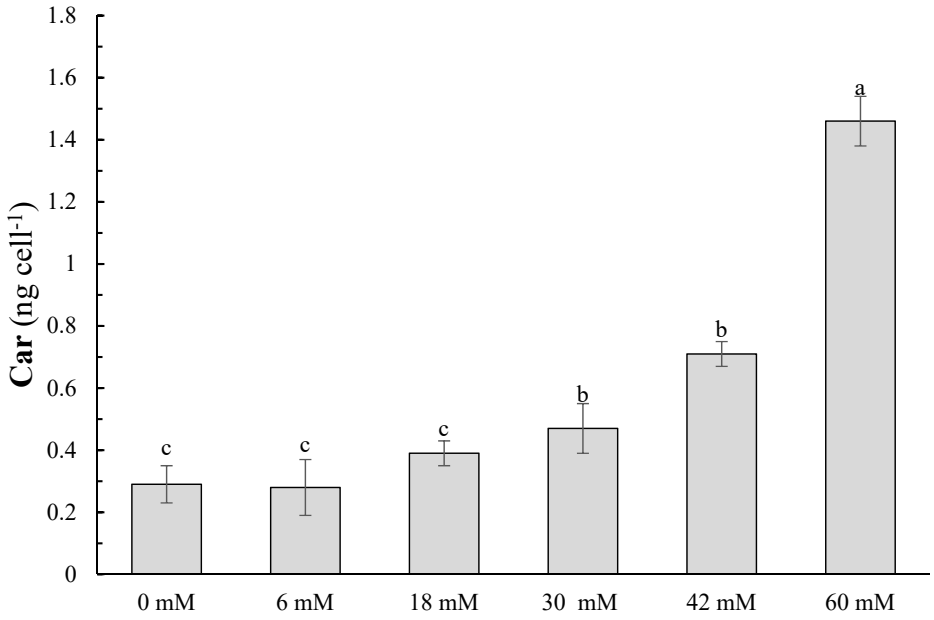


**Figure 4.** Total carotenoids contents in *Nannochloropsis oculata* cells grown for 72 h under different bicarbonate supplementations (0, 6, 18, 30, 42 and 60 mM). Error bars represent SD ($n$ = 3). Different letters above each bar indicate significant differences ($p \leq 0.05$).

## 4. Discussion

Microalgae, as well as higher plants, besides light and water, need inorganic carbon to perform photosynthesis. As previously demonstrated, although microalgae can grow only using environmental $CO_2$, their cellular growth and biomass can be improved by supplementing non-gaseous inorganic carbon (bicarbonate) to cultures [14,29,30].

In the present study, cultures of *Nannochloropsis oculata* were grown in a medium supplemented with bicarbonate to explore the short-term (72 h) changes occurring in the growth and pigment contents of the cells. *Nannochloropsis* sp. represent a very interesting microalgae known for their numerous commercial applications.

The utilization of bicarbonate, as an alternative to pure $CO_2$-gas for algal cultivation, could have sustainable and economic relevance when considering large-scale microalgae

productions. According to previous studies, an adequate concentration of bicarbonate in the culture medium improves the biomass productions and inhibits protozoa and bacteria contaminations [11,31].

In this study, the addition of bicarbonate to *Nannochloropsis oculata* cultures resulted in a significantly greater optical density, especially in cultures supplemented with the higher bicarbonate concentration (60 mM), demonstrating a positive effect on cellular growth. However, during the first 24 h a slight decrease in optical density was measured. According to literature about some microalgae, bicarbonate addition induced a change in metabolism shifting the cell from a growth state to a carbon-containing-product formation [32]. This results in a slowdown in growth as observed during the first 24 h. It was clearly shown that cellular replication considerably stopped at the time of bicarbonate addition in *Chlorella vulgaris* [29]. Some studies proved that algal growth can be promoted by $HCO_3^-$ supply even though, at high concentration, it can have deleterious effects on cells [33]. In fact, according to de Farias Silva et al. [15], in *Synechoccus* sp. an excess of $NaHCO_3$ caused salt stress, ROS (reactive oxygen species) production and a decline in photosynthetic efficiency due to a PSII complex damage. In general, in plant cell, salinity creates several physiological and molecular changes, such osmotic stress, oxidative damage and a decrease of photosynthetic activities [33,34].

In microalgae, the threshold for bicarbonate tolerance is variable and depends not only on species-to-species but also on strain-to-strain. Indeed, *Chlorella vulgaris* showed an optimum tolerance of bicarbonate at ~12 mM [35], while the marine microalgae *Nannochloropsis salina* and *Tetraselmis suecica* showed an increase of cell density and growth rates in cultures supplemented with $NaHCO_3$ 60 and 12 mM, respectively [14,17]. This research reveals that *Nannochloropsis oculata* tolerates sodium bicarbonate at least until 60 mM concentration; it is well demonstrated by the rise of growth rate and cell density.

In the current study, the cellular growth and the bicarbonate concentrations correlated with the pH of the culture medium. In culture supplemented with the highest bicarbonate concentration the pH reaches the value of 9.8. It has been demonstrated in *Chlamydomonas reinhardtii*, *Dunaliella parva*, and *Anabaena variabilis* that the removal of inorganic C from culture medium, for its utilization in photosynthesis and the consequent $O_2$ evolution, leads to an increase in extracellular pH [36]. The pH of the culture medium can influence the microalgal growth and the culture densities. Here we are able to argue that pH until 9.8 did not negatively impact the growth of *N. oculata* cells.

Microalgae are commercial sources of natural pigments such as chlorophylls and carotenoids that can be produced in a variable quantity depending on microalgal growth conditions. These molecules are of great interest for their utilizations as food additives, nutraceuticals and pharmaceuticals [12]. The addition of bicarbonate leads to an increase in chlorophyll *a* and total carotenoids, with the highest values in cells supplemented with a bicarbonate concentration of 60 mM. The rise in Chl-*a* and Car in algal cells may be due to the greater availability of inorganic carbon in the culture medium that supports the synthesis of new pigments. It is well known that the physiological condition of growth can influence the pigment constituents of microalgae. Chl-*a* represents an essential pigment for plant cell and its intracellular level is an indicator of photosynthetic capacity of the organism. In fact, the increase of photosynthetic pigments improves the ability of microalgae to capture the light [37]. In *Chlorella sorokiniana*, the addition of bicarbonate induces an increase in pigments and in Fv/Fm values, up to 95% higher than in the control culture indicating a strong photosynthetic performance [11,12].

## 5. Conclusions

In conclusion, in *Nannochloropsis oculata* low concentrations of bicarbonate (6 and 18 mM) did not show significant changes on growth rates and pigment contents of the cells. However, concentrations of bicarbonate from 30 to 60 mM, strongly improved, in only 72 h, the cellular levels of chlorophylls and carotenoids, which are important photosynthetic pigments with wide commercial applications.

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