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Quantity and Quality of Light on Growth and Pigment Content of *Dunaliella* sp. and *Anabaena* sp. Cultures and the Use of Their Absorption Spectra as a Proxy Method for Assessment

George N. Hotos

Plankton Culture Laboratory, Department of Fisheries and Aquaculture, University of Patras, 30200 Messolonghi, Greece; ghotos@upatras.gr; Tel.: +30-26310-58252

Abstract: (1) Background: Microalgae cultures are greatly facilitated if growers can easily and economically ascertain the quantitative and qualitative status of the culture continuously with satisfactory accuracy. (2) Methods: The locally isolated microalgae *Dunaliella* sp. and *Anabaena* sp. were cultured in small volumes with 2 intensities of white light (2000 and 8000 lux) and with green, blue and red light and the increase of their biomass and pigments was studied. Pigment analyses, continuous recordings of absorption spectra and calibration curves were performed. (3) Results: The intensity of 8000 lux white light yielded the highest increase in biomass, chlorophylls and carotenoids in *Dunaliella* sp. while 2000 lux and green light caused the greatest increase in biomass and phycocyanin in *Anabaena* sp. From the examination of the absorption spectra, the evolution of the pigment content can be estimated and both pigments and biomass are correlated very satisfactorily with the wavelength of 750 nm. (4) Conclusions: The use of absorption spectra as an easy, fast and economical method can be a useful tool for a good approximation of the state of the microalgae culture. This is clearly shown when the spectra of the culture under different lighting colors are compared which have a catalytic effect on the level of the pigments leading to the increase in carotenoids and phycocyanin of the green light.

Keywords: microalgae culture; pigments; absorption spectrum; *Dunaliella*; *Anabaena*

1. Introduction

Microalgae are now a widespread and rapidly growing branch of primary production, as they offer many and important advantages in terms of biomass production [1–4]. From an ecological point of view, they can contribute significantly to curbing anthropogenic carbon emissions, becoming a useful tool in the arsenal of climate change mitigation [5,6]. In particular, cultivation of marine species compares favorably with both freshwater species and grass crops, both because valuable irrigation resources are not lost and because it is possible to use coastal or degraded saline soils for cultivation [7]. In many countries of the world, the cultivation of marine microalgae serves productive purposes in a wide range of applications, from live food in fish hatcheries, feed production, biofuel production, soil remediation and fertilization, water decontamination, chemical production, pharmaceutical and cosmetic substances, to direct human consumption and the production of valuable antioxidant pigments [8–12].

In particular, for these pigments that make up the photosynthetic machinery of algae (chlorophylls, carotenoids, phycobiliproteins), a thriving industry has developed, producing ever-increasing quantities of pigments that have been shown to have beneficial effects on human health [13–15]. For example, the carotenes and xanthophylls found in all microalgae (eukaryotes and cyanobacteria), as well as the phycocyanin of cyanobacteria, have potent antioxidant and possibly anticancer effects [16]. The content of photosynthetic units of cells in relation to their pigment composition differs between different species of microalgae and for this reason breeders have selected certain species known to overproduce some of them [15]. Well-known cases are the species

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of the genus *Dunaliella* for the production of β-carotene [17,18] and *Arthrospira* (*Spirulina*) for phycocyanin [19,20].

However, even for species selected for cultivation, there is much scope for optimizing and maximizing their pigment content, depending on how some critical conditions for cultivation are modified. Much work has examined the effects of conditions (temperature, salinity, pH, nutrients, light) on the growth rate of the culture, the amount of phyco-mass produced, and the change in cellular constituents in various microalgae. If the goal of cultivation is to maximize production of either the final biomass or selected constituents, or (what makes most sense) to achieve the ideal combination of both, this should be done simultaneously at the lowest possible cost and, of course, under the desired growth conditions. Undoubtedly, according to many researchers, the quantity and quality of light have a catalytic effect on this process. The intensity of white light has the greatest influence on cell growth and pigment content [21]. Too low an intensity results in a slow increase, while too high an intensity has an inhibitory effect. At the same time, the content of cells in terms of the proportions of photosynthetic pigments changes drastically depending on the intensity of illumination. In addition to light intensity, color has also been found to have a catalytic effect, especially in the synthesis of the pigments of the photosynthetic apparatus of microalgae [22]. More and more studies are focusing on the effect of colored light on changing photosynthetic pigment ratios. Therefore, a production scheme with a suitable arrangement of white and colored light is of utmost importance to produce the maximum algal biomass with the maximum yield of desired pigments in the shortest time and with the lowest financial cost. In this sense, any monitoring technique that allows growers to assess the condition of the developing phyco-culture can be a useful tool with predictive value. The absorbance spectrum of a live culture sample can provide valuable information about both the density of the culture and the pigments it contains. However, in the literature, the absorption spectrum has received little attention for this purpose, whereas in reality and under production conditions, it can provide valuable information about the current condition of the culture in a simple and inexpensive way, if not with the precision required for laboratory analysis, at least with an approach that meets the general needs of the grower who wants to know what condition his culture is in. In the present work we try to combine the evaluation of the use of the absorption spectrum as a predictive tool for the density of the culture and the content of the cell in photosynthetic pigments and at the same time to study the effect of the intensity and color of the light on the increase of the biomass and of cellular pigment composition. For this purpose, locally isolated marine microalgae [23,24], the chlorophyte *Dunaliella* sp. and the filamentous cyanobacterium *Anabaena* sp., were chosen for the experiments. Because these species are either established species in algae cultures (*Dunaliella*), or species with promising cultivation potential (*Anabaena*) in the way the well-known cyanobacterium *Spirulina* is cultivated [25], I attempt in the present work to study their absorption spectra in the various lighting conditions in order to give growers a quick and efficient way to assess crop condition and predict pigment content with reasonable accuracy.

2. Materials and Methods

The chlorophyte *Dunaliella* sp., and the cyanobacterium *Anabaena* sp. were the marine microalgae species utilized. Both species were isolated during a survey in the lagoonal waters of Messolonghi in Western Greece (38°20′05.16″ N, 21°25′28.51″ E) and were maintained as monocultures in the lab. According to an experimentation protocol, all were batch-cultured indoors (Figure 1) in air-conditioned room at 19–20 °C, at salinity of 35 ppt, and at white light intensities of 2000 and 8000 lux. The *Anabaena* culture vessels consisted of 1.5 L inverted plastic bottles and for *Dunaliella* 1-L conical glass Erlenmeyer flasks were used, filled to the 1L mark with previously sterilized sea water enriched with Walne's medium. The three final stock solutions A, B, and C that make up Walne's medium formula were each used at a ratio of 1 mL per liter of culture water. For essential nutrients N, P, and K, 300 grams of $NaNO_3$, 20 grams of NH_4Cl, and 30 grams of KH_2PO_4 (Merck, Germany) were diluted in 1 liter of distilled water to create solution A. $ZnSO_4.H_2O$ (30 g), $CuSO_4.5H_2O$ (25 g), $CoSO_4.7H_2O$ (30 g), and $MnSO_4.H_2O$ (20 g) are trace elements (Merck, Germany)

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dissolved in 1 L of distilled water to make solution B. Solution C contains 100 mg of vitamin B12, 100 mg of biotin and 10 mg of thiamine (Merck, Germany) diluted in 1 L of distilled water.

White light emitted by a series of 20 watt 1600 lm LED lamps was used. Intensities of 40 and 160 μmol photons/m²/s (2000 and 8000 lux, respectively) were obtained by placing the vessels at the correct distance from the lamps. Intensity was measured at the center of the outer surface of the vessels using a luminometer (BIOBLOCK LX -101, Panasonic). The illumination period of 16hL:8hD was controlled by an electric timer that switched the lamps on and off accordingly. For the experimentation using colored light, the vessels were covered with different color filter wrappers (green, blue and red) and placed at the appropriate distance of white LED light receiving 8000 lux. Cultures were maintained in suspension by injecting air (with its natural CO_2 content) through 2-mL glass pipettes (one in each vessel) at a rate of half the culture volume per minute. The pipettes were connected via sterilized plastic tubing to the 0.45 μm filtered central air supply system, which was fed by a blower.

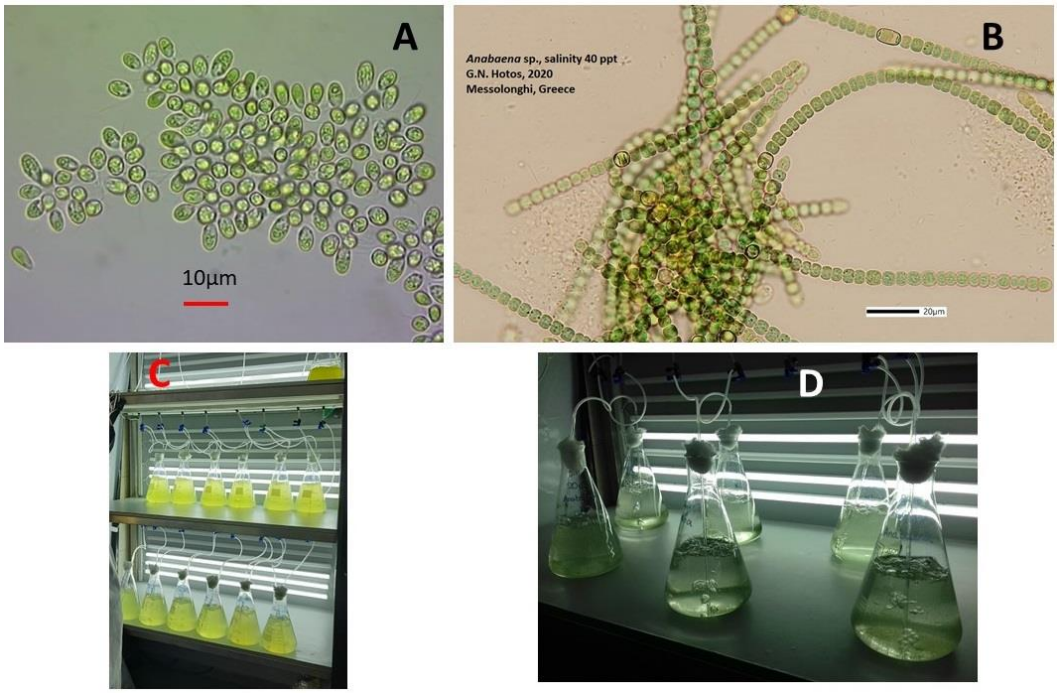

Figure 1. A: *Dunaliella* sp. B: *Anabaena* sp. C: Bottles with *Dunaliella* on 5th day of culture at two different distances from light sources so as to create L-light (2000 lux) and XL-light (8000 lux). D: Bottles with *Anabaena* on 2nd day of culture at two different distances from light sources.

Daily cell counts using a Fuchs-Rosenthal hematocytometer for unicellular species and drying and weighing a sample of the filamentous cyanobacteria filtrate every 2-4 days were used to record algal growth, which was measured either as cells/mL for unicellular microalgae or as dry weight (g/L) for the filamentous cyanobacterium. Simultaneously, optical density was recorded at 750 nm in the spectrophotometer (for all species). Dry weight was calculated by filtering a known amount of culture through 0.45 μm GF/C filters in a vacuum pump (Heto-SUE-3Q). The filters were next washed with ammonium formate for removing salts and then placed in an oven at 100 °C for 2 h.

Untreated culture samples were used to measure the absorbance spectra of the cultures at different stages of maturation. The spectra were recorded in a Shimadzu UV-1800 (Kyoto, Japan) spectrophotometer and the data were transformed using its UVprobe 3.2 software.3.

Chlorophyll-a and total carotenoids in the case of the cyanobacteria. were extracted with absolute methanol from centrifuged culture samples, and their concentrations (μg/mL) were determined spectrophotometrically in a Shimadzu Uvmini-1240 UV-visible (Kyoto, Japan) spectrophotometer using the following equations:

chl-a =12.9447 (A665-A720)

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$$\text{Total carot.} = \frac{1000(\text{A470} - \text{A720}) - 2.86\text{chlor.} - \text{a}}{221}$$

where: (A) stands for the absorbance (or optical density-OD) of the processed sample at the indicated wave length.

In the case of the chlorophyte *Dunaliella* sp. chlorophyll-a, chlorophyll-b, and total carotenoids were extracted with DMSO from centrifuged culture samples, and their concentrations (μg/mL) were calculated spectrophotometrically using the following equations

$$\text{chl-a} = 12.47(\text{A665}) - 3{,}62(\text{A649})$$

$$\text{chl-b} = 25.06(\text{A649}) - 6.5(\text{A665})$$

$$\text{Total carot.} = \frac{1000(\text{A480}) - 1.29(\text{chlor.} - \text{a}) - 53.78(\text{chlor.} - \text{b})}{220}$$

The estimation of β-carotene was done according to [26]. Briefly, 1 mL of algal cells were harvested, centrifuged for 10 min at 3000 rpm to remove the pellet, and then 3 mL of a hexane/ethanol (1:2) mixture was added to the vortex. After that, the mixture was centrifuged at 3000 rpm for 10 minutes. After two phases had formed, the upper phase which contained the β-carotene was collected and its optical densities at 450 nm was assessed The following formula was used to determine the concentration of β-carotene as μg/mL:

$$\text{β-carotene (μg/mL)} = 25 \text{ x A450}$$

By freezing (-20 °C) for 24 hours a concentrated known amount of cyanobacterial culture in 0.1 M sodium phosphate buffer (pH 7.1) as solvent at a ratio of 1:10 (algal mass: solvent), and then thawing at 4 °C in the dark, the phytocyanin (PC) content was extracted. Over the course of two days, the process of freezing and thawing was repeated. The slurry of the sample was then centrifuged at 3000 rpm for 5 minutes to determine the concentration of phycocyanin (PC, measured in mg/mL) in the supernatant using spectrophotometry and the equation:

$$\text{PC} = \frac{\text{A}_{615} - 0.474\ \text{A}_{652}}{5.34}$$

From the above equation the yield in phycocyanin (PC) in mg PC/g dry weight was calculated using the equation:

$$\text{PCyield} = \frac{\text{PC}\left(\frac{\text{mg}}{\text{mL}}\right) \text{ x V (mL)}}{\text{D.W. (g)}}$$

where: PCyield = mg of phycocyanin per g algal dry weight

V = volume of solvent used (mL)

D.W. = grams of dry weight of the algal mass used

Using the free PAST3 program, the various variables were statistically treated using ANOVA and Tukey's test for comparison of means at the 0.05 level of significance. Statistical calculations of regression lines, Pearson's correlation coefficient, standard deviation (SD) and standard error (SE) were made with Excel software (Microsoft, USA).

3. Results

3.1. Dunaliella sp.

3.1.1. Effect of low (2000 lux) and high (8000 lux) white light illumination

The cultures lasted 17 days and their absorption spectra were taken every second day from the 3rd day onwards. As seen in Figure 2, on day 3, the cell density in the condition of low light (to be named hereafter as L-light) (Figure 2A) the cell density of the culture was 850,000 ± 35,117 (SE) cells/mL while in the condition of high light (to be named hereafter as XL-light) (Figure 2B) on the same day reached 4,600,000 ± 149,332 cells/mL, an indication of rapid growth since the beginning in XL as contrasted to L, where an initial lag phase was much pronounced approaching a similar cell density only around the 7th-8th day.

Maximum cell density on day 18 in XL reached 25,150,000 ± 524,309 cells/mL significantly higher ($P<0.05$) than the relevant one of the same day in L (18,529,000 ± 265,016 cells/mL). Comparing also

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the cell densities in previous days (5th-7th-9th-11th-13th-15th), it was found that in XL, the densities of each day were higher ($P<0.05$) than their counterparts in L.

In L-light (Figure 2A) till the 11th day the spectra exhibited weak peaks with only a slight elevation at the wave length of 680 nm which corresponds to chlorophyll-a. On the 13th day and afterwards (15th and 17th day) when cell densities exceeded 11,000,000 cells/mL all characteristics peaks for chlorophyll-a (680 and 440 nm), chlorophyll-b (650 nm) and total carotenoids (484 nm) became very evident. The situation in XL-light spectra was totally different (Figure 2B) as the above mentioned peaks became clearly evident as early as from the 7th day (at 7,700,000 cells/mL) and onwards, reaching much higher peaks than the corresponding ones of L-light at all the characteristic of each pigment wave lengths. In the highest cell density of 25,150,000 cells/mL of the 17th day the peak for total carotenoids (484 nm) was much pronounced.

Using the values of absorbance (OD) at 750 nm from the spectra of Figure 3 the regression lines of cell density vs absorbance in both light regimes (L and XL) resulted in perfect fitness with equations of: cells/mL = (2 x 10^7 x OD 750nm) – 4 x 10^6 with $R^2 = 0.9951$ for L-light and cells/mL = (2 x 10^7 x OD 750nm) – 9 x 10^6 with $R^2 = 0.9971$ for XL-light (Figure 3A and 3D respectively).

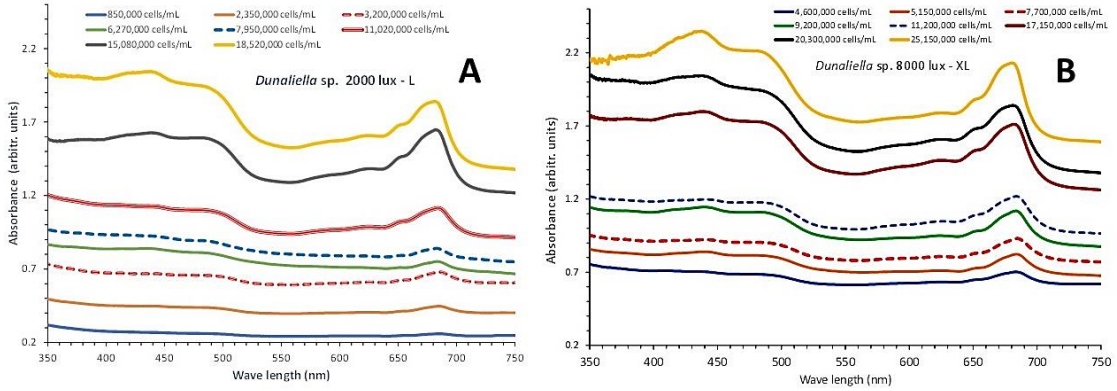

Figure 2. The absorption spectra at 8 consecutive days (3rd to 17th) of the cultures of Dunaliella sp. exposed to low 2000 lux (A) and high illumination of 8000 lux (B).

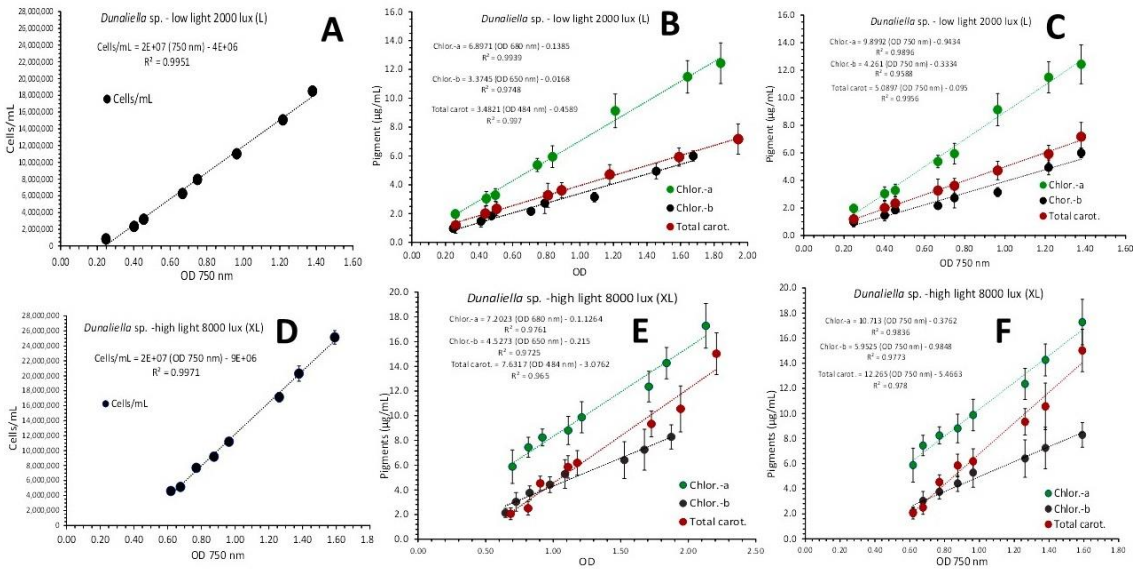

Figure 3. A and D: Regression lines of cell density vs optical density (OD) at 750 nm for L-light and XL-light respectively. B and C: Regression lines of chlorophyll-a, chlorophyll-b and total carotenoids concentration as µg/mL ± SD vs OD at each wavelength exhibiting the maximum absorbance for each pigment (B) and versus the OD at the wave length of 750 nm (C) in Dunaliella culture under L-light. E and F: The same as in B and C correspondingly in Dunaliella culture under XL-light.

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Before using as above the corresponding values of OD at 750 nm for examining the correlation between the content of various pigments with their spectrum's value of OD at 750 nm, their OD values corresponding to the peak at the spectrum for each particular pigment were examined. In L-light (Figure 3B) using the spectral OD values of 680 nm for chlorophyll-a, 650 nm for chlorophyll-b and 484 nm for total carotenoids, the resulted equations were: Chlor.-a (μg/mL) = (6.8971 x OD 680 nm) - 0.1385 with R^2 = 0.9939, Chlor.-b (μg/mL) = (3.3745 x OD 650 nm) - 0.0168 with R^2 = 0.9748 and Total carot. (μg/mL) = (3.4821 x OD 484 nm) - 0.4589 with R^2 = 0.997. In XL-light (Figure 3E) the relevant equations were: Chlor.-a (μg/mL) = (7.2023 x OD 680 nm) - 0.1264 with R^2 = 0.9761, Chlor.-b (μg/mL) = (4.5273 x OD 650 nm) - 0.215 with R^2 = 0.9725 and Total carot. (μg/mL) = (7.6317 x OD 484 nm) - 3.0762 with R^2 = 0.965. After the ascertainment of the strong relation between the pigment content and the relevant OD of maximum absorbance as were exhibited above, the use of OD at 750 nm was applied to all (Figure 3C and 3F). In L-light (Figure 3C) the resulted equations were:

Chlor.-a (μg/mL) = (9.8992 x OD 750 nm) - 0.9434 with R^2 = 0.9896, Chlor.-b (μg/mL) = (4.261 x OD 750 nm) - 0.3334 with R^2 = 0.9588 and Total carot. (μg/mL) = (5.0897 x OD 750 nm) - 0.095 with R^2 = 0.9956. In XL-light (Figure 3F) the relevant equations were: Chlor.-a (μg/mL) = (10.713 x OD 750 nm) - 0.3762 with R^2 = 0.9836, Chlor.-b (μg/mL) = (5.9525 x OD 750 nm) - 0.9848 with R^2 = 0.9773 and Total carot. (μg/mL) = (12.265 x OD 750 nm) - 5.4663 with R^2 = 0.978.

Starting the measurement of pigments on the 3rd day of the cultures, it was found that in XL-light (Figure 4B) at all days (3rd-5th-7th-9th-11th-13th-15th-17th) all pigment concentrations were significantly higher ($P<0.05$) than their counterparts in L-light (Figure 4A). Maxima of all pigments (in μg/mL ± SE) were recorded on the 17th day for both light regimes. In L-light chlor.-a: 12.43 ± 0.815, chlor.-b: 5.997 ± 0.204, total carot.: 7.173 ± 0.606 and β-carot: 3.113 ± 0.126. In XL-light chlor.-a: 17.283 ± 1.046,, chlor.-b: 8.290 ± 0.569, total carot.: 15.03 ± 0.977 and β-carot: 7.152 ± 0.318.

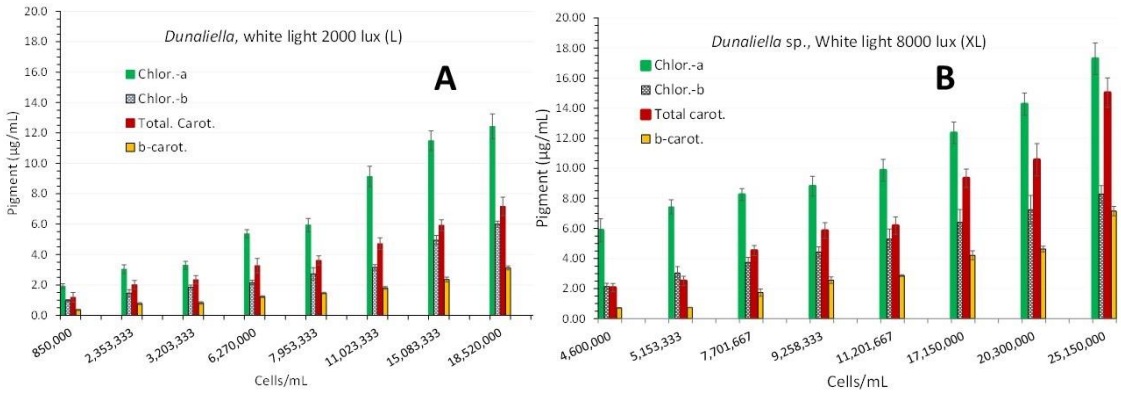

Figure 4. Pigment content in μg/mL ± SE for chlorophyll-a, chlorophyll-b, total carotenoids and b-carotene at 8 different culture densities of Dunaliella sp. in L-light (A) and XL-light (B). Each successive culture density starting from the 3rd day of culture, represents measurements taken every 2 days.

3.1.2. Effect of colored (green, blue and red) light illumination

The cultures lasted 18 days and their absorption spectra were taken on 2nd, 4th, 6th, 12th, 15th and 18th days. As seen in Figure 5 the spectra of green (Figure 5A) and blue light (Figure 5C) were prominently much more elevated in terms of absorption values as compared to red light (Figure 5B) in which a collapse of the culture occurred on 18th day with its spectrum plummeting. In fact, the signs of collapse in the red light became evident from the 15th day's spectrum which became almost indistinguishable from the spectrum of the 12th day. The uppermost spectra correspond to the measured highest cell densities of the 18th day which were 18,850,000 ± 287,924 (SE) cells/mL for green light, 20,150,000 ± 504,477 cells/mL for blue light and 11,776,600 ± 103,494 cells/mL for red light (on the 15th day). The density in blue light is significantly higher than that in green light and both are higher ($P<0.05$) than the relevant one in red light. In general, all cell densities of every day in green and blue light are significantly higher than their counterparts in red light. Using the values of

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absorbance (OD) at 750 nm from the spectra of Figure 5A, the regression line of cell density vs absorbance using data from all colors resulted in perfect fitness with equation of: cells/mL = (2 x 10^7 x OD 750nm) – 5 x 10^6 with R^2 = 0.9867.

Before using as above the corresponding values of OD at 750 nm for examining the correlation between the content of various pigments with their spectrum's value of OD at 750 nm, their OD values corresponding to the peak at the spectrum for each particular pigment were examined. First this procedure was made using the sum of data from all colors for each pigment (Figure 6B), using the spectral OD values of 680 nm for chlorophyll-a, 650 nm for chlorophyll-b and 484 nm for total carotenoids. The resulted equations were: Chlor.-a (μg/mL) = (7.6573 x OD 684nm) + 0.6695 with R^2 = 0.97, Chlor.-b (μg/mL) = (4.5815 x OD 650nm) + 0.1873 with R^2 = 0.9178 and Total carot. (μg/mL) = (4.0644 x OD 484nm) + 0.6199 with R^2 = 0.9276. After the ascertainment of the strong relation between the pigment content and the relevant OD of maximum absorbance as were exhibited above, the use of OD at 750 nm was applied to all (Figure 6C). The resulted equations were: Chlor.-a (μg/mL) = (12.15 x OD 750nm) - 1.2231 with R^2 = 0.9454, Chlor.-b (μg/mL) = (6.2648 x OD 750nm) - 1.007 with R^2 = 0.8709 an Total carot. (μg/mL) = (6.6353 x OD 750nm) - 0.4964 with R^2 = 0.931.

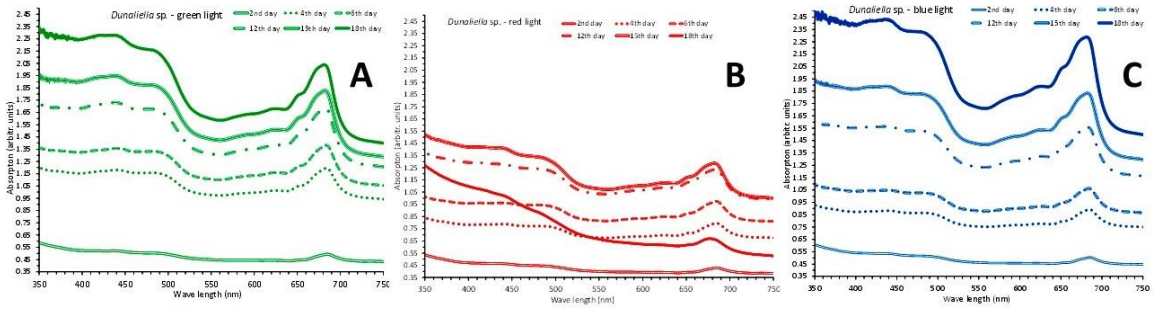

Figure 5. The absorption spectra at 6 different days (2nd – 4th – 6th – 12th -15th -18th) of the cultures of Dunaliella sp. exposed to green (A), red (B) and blue (C) light.

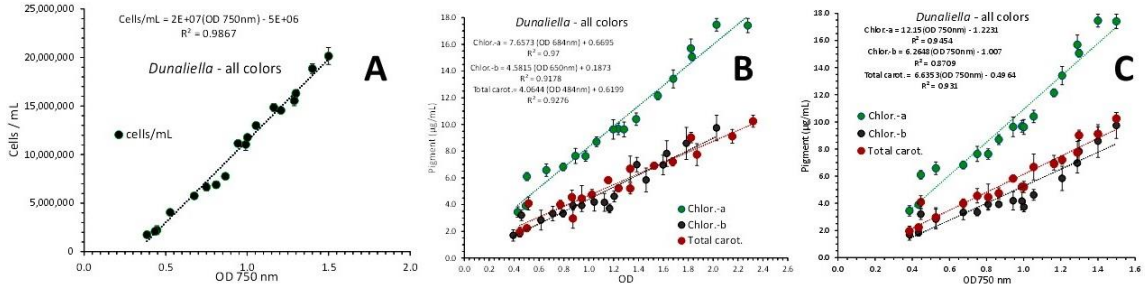

Figure 6. A: Regression line of cell density (in cells/mL ± SD) vs optical density (OD) at 750 nm for all colors. B and C: Regression lines of chlorophyll-a, chlorophyll-b and total carotenoids concentration as μg/mL ± SD vs OD at each wavelength exhibiting the maximum absorbance for each pigment (B) and versus the OD at the wave length of 750 nm (C), in Dunaliella cultures, using data from all colors.

From Figure 7 where the above described regression equations were applied to each color separately, it was found that even better perfect fits were produced for each pigment with values of R^2 above 0.9.

Pigment content was clearly affected by the light color used (Figure 8). All kind of pigments (chlor.-a, chlor.-b, total carot., b-carot.) were much higher ($P<0.05$) in green and blue color compared to red at all relevant days (except the first one between green and red). Although for the first 4 measurements (except for the first on 3rd day) chlorophylls, total carotenoids and b-carotene were significantly higher in green light compared with the respective values in blue light ($P<0.05$), in the last 2 measurements on 15th and 18th day (15.5 x 10_6 and 18.85 x 10^6 cells/mL for green light and 16.3 x 10^6 and 20.15 x 10^6 cells/mL for blue light respectively), chlorophyll-a (15.69 ± 0.410 and 17.47 ± 0.274 in green and 15.06 ± 0.163 and 17.41 ± 0.308 in blue light, respectively) and chlorophyll-b (6.97 ± 0.422 and 8.58 ± 0.687 in green and 7.83 ± 0.557 and 9.74.41 ± 0.533 in blue light, respectively) values were

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similar (P>0.05). On the same last two days, total carotenoids (7.74 ± 0.475 and 9.11 ± 0.297 in green and 9.02 ± 0.220 and 10.24 ± 0.258 in blue light, respectively) were higher in blue light (P<0.05) and β-carotene while higher in blue light on 15th day (4.82 ± 0.201 vs 4.20 ± 0.081 in green light, P<0.05), on 18th day both colors had equal values (5.01 ± 0.124 in green and 5.05 ± 0.137 in blue, P>0.05).

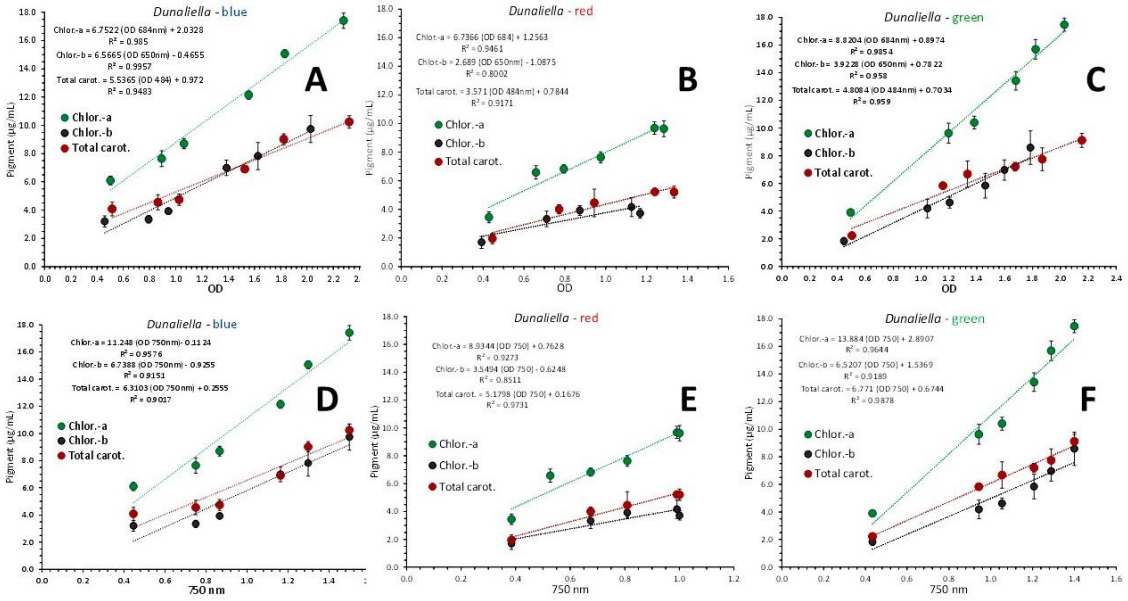

Figure 7. Regression lines of chlorophyll-a, chlorophyll-b and total carotenoids concentration as µg/mL ± SD vs OD at each wavelength exhibiting the maximum absorbance for each pigment and versus the OD at the wave length of 750 nm (C), in Dunaliella cultures, using data from blue (A and D), red (B and E) and green light (C and F).

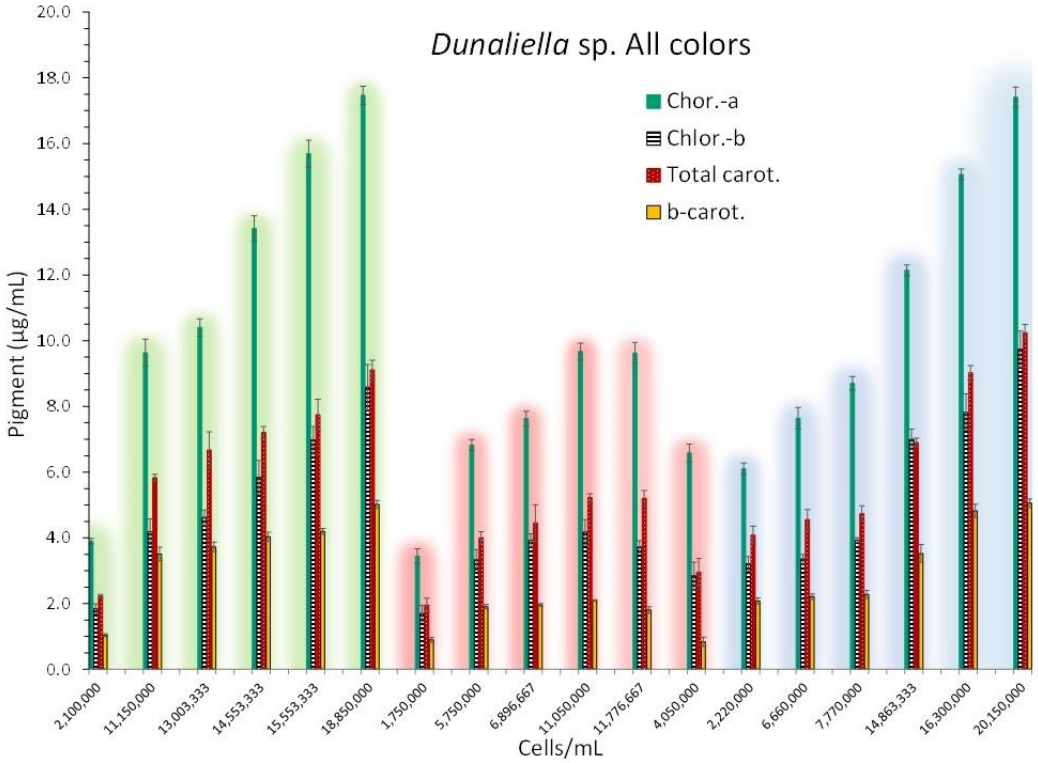

Figure 8. Pigment content in µg/mL ± SE for chlorophyll-a, chlorophyll-b, total carotenoids and β-carotene at 6 different culture densities of Dunaliella sp. in each light color used at different cell densities along the culture period starting from the 3rd day and every 3 days till the 18th day. Each set of measurements for each light color is depicted by the appropriate coloration.

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3.2. Anabaena sp.

3.2.1. Effect of low (2000 lux) and high (8000 lux) white light illumination

The two sets of absorption spectra during the culture period of *Anabaena* sp. in both L-light and XL-light regimes were quite similar (Figure 9) in terms of recorded biomass (g d.w./L) of the spectra taken every 3 days starting from the 3rd and ending on the 15th day. The most remarkable difference between the two light intensities was the more prominent peak at 630 nm that characterizes phycocyanin in all spectra of L-light (Figure 9A) compared to the relevant ones of XL-light (Figure 9B) from the 9th till the 15th day

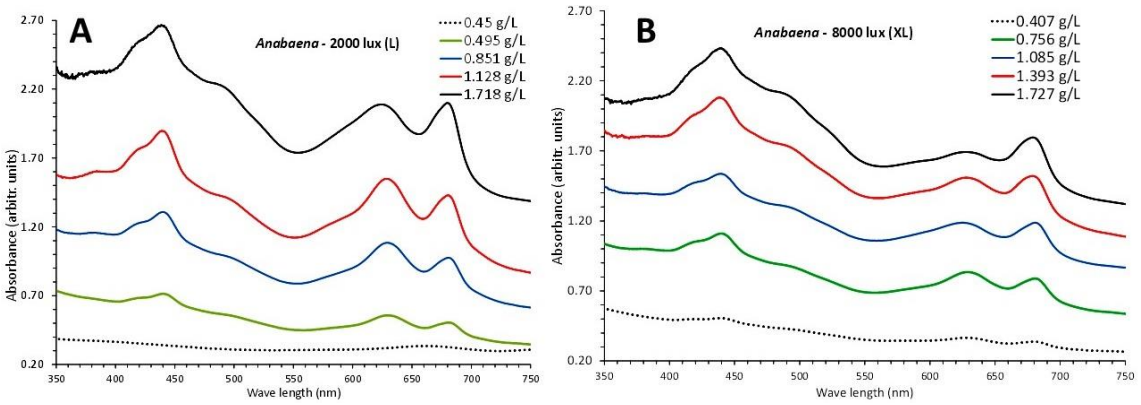

Figure 9. The absorption spectra at 5 consecutive days (3rd to 15th) taken every 3 days with their respective culture density (in g d.w./L) of the cultures of Anabaena sp. exposed to low 2000 lux (A) and high illumination of 8000 lux (B).

Biomass expessed as g d.w./L ± SE while presented significantly higher ($P<0.05$) values from the 3rd day till the 12th in XL-light compared to L, finally, on the 15th day, both recorded densities were equal ($P>0.05$) with 1.748 ± 0.101 in L and 1.727 ± 0.078 in XL.

Using the values of absorbance (OD) at 750 nm from the spectra of Figure 9 the regression line of cell density vs absorbance from both light regimes (L and XL) resulted in perfect fitness (Figure 10A) with equation of: g d.w./L = (1.2134 x OD 750 nm) + 0.0812 with

$R^2 = 0.9974$. Before using as above the corresponding values of OD at 750 nm for examining the correlation between the content of chlorophyll-a and total carotenoids with their spectrum's value of OD at 750 nm, their OD values corresponding to the peak at the spectrum for chlor.-a (682 nm) and total carot. (490 nm) were examined (Figure 10B). A strong relation was recorded for both pigments: Chlor.-a (μg/mL) = (5.3105 x OD 682nm) - 1.0738 ($R^2 = 0.9721$) and Total carot. (μg/mL) = (2.6585 x OD 490nm) - 1.0684($R^2 = 0.9816$). After the ascertainment of the above strong relation between the pigment content and their relevant OD of maximum absorbance, the use of OD at 750 nm was applied (Figure 10C), resulting in: Chlor.-a (μg/mL) = (7.9347 x OD 750 nm) - 1.3155 ($R^2 = 0.9769$) and Total carot. (μg/mL) = (4.3358 x OD 750nm) - 1.1666 ($R^2 = 0.9907$).

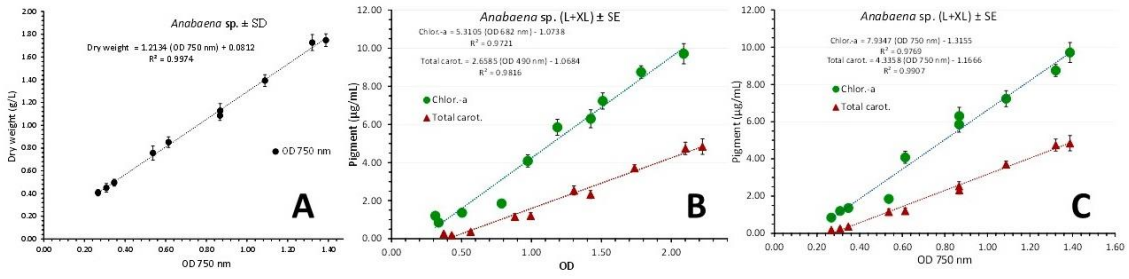

Figure 10. A: Regression line of cell density (in g d.w./L ± SD) vs optical density (OD) at 750 nm using values from both light regimes. B and C: Regression lines of chlorophyll-a, and total carotenoids concentration as μg/mL ± SE vs OD at each wavelength exhibiting the maximum absorbance for each

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pigment (B) and versus the OD at the wave length of 750 nm (C) in Anabaena sp. culture using values from both light regimes.

Applying the above described correlations of chlorophyll-a and total carotenoids concentrations to their OD value of maximum absorbance and to OD at 750 nm separately for each light regime (Figure 11), equally strong relations were recorded. In L-light (Figure 11A and C): Chlor.-a (μg/mL) = (4.9671 x OD 682nm) - 0.7367 (R^2 = 0.9938), Total carot. (μg/mL) = (2.5244 x OD 490nm) - 1.0189 (R^2 = 0.9772) and Chlor.-a (μg/mL) = (8.0337 x OD 750 nm) - 1.1314 (R^2 = 0.9905), Total carot. (μg/mL) = (4.2402 x OD 750nm) - 1.191 (R^2 = 0.9914). In XL-light (Figure 11B and D): Chlor.-a (μg/mL) = (5.8413 x OD 682nm) - 1.6415 (R^2 = 0.9614), Total carot. (μg/mL) = (2.7752 x OD 490nm) - 1.1191 (R^2 = 0.9966) and Chlor.-a (μg/mL) = (8.0234 x OD 750 nm) - 1.6417 (R^2 = 0.9758) and Total carot. (μg/mL) = (4.37 x OD 750nm) - 1.1026 (R^2 = 0.9959).

A similar to chlorophyll-a and total carotenoids procedure as above was applied to the correlations of phycocyanin concentrations to its OD value of maximum absorbance and to OD at 750 nm separately for each light regime (Figure 12). Using cumulatively values from both L and XL-light the relation between phycocyanin (in mg/mL ± SE) and its OD of its peak on the spectra (630 nm) resulted in (Figure 12A): Phycoc. L+XL (mg/mL) = (0.9178 x OD 630nm) – 0.1906, (R^2 = 0.8094) and using OD values of 750 nm (Figure 12B), in: Phycoc. L+XL (mg/mL) = (1.1724 x OD 750nm) – 0.056, (R^2 = 0.6389). Next, using separately the values from each light regime resulted in (Figure 12C): Phycoc. L (mg/mL) = (1.1176 x OD 630nm) – 0.2267, (R^2 = 0.985) and Phycoc. XL (mg/mL) = (0.5562 x OD 630nm) – 0.0253, (R^2 = 0.98) and using 750 nm (Figure 12D), in: Phycoc. L (mg/mL) = (1.7797 x OD 750nm) – 0.2299, (R^2 = 0.9687) and Phycoc. XL (mg/mL) = (0.6984 x OD 750nm) – 0.0765, (R^2 = 0.9717). So a much stronger relation is produced using OD values for each light regime separately than using values pooled from both light regimes.

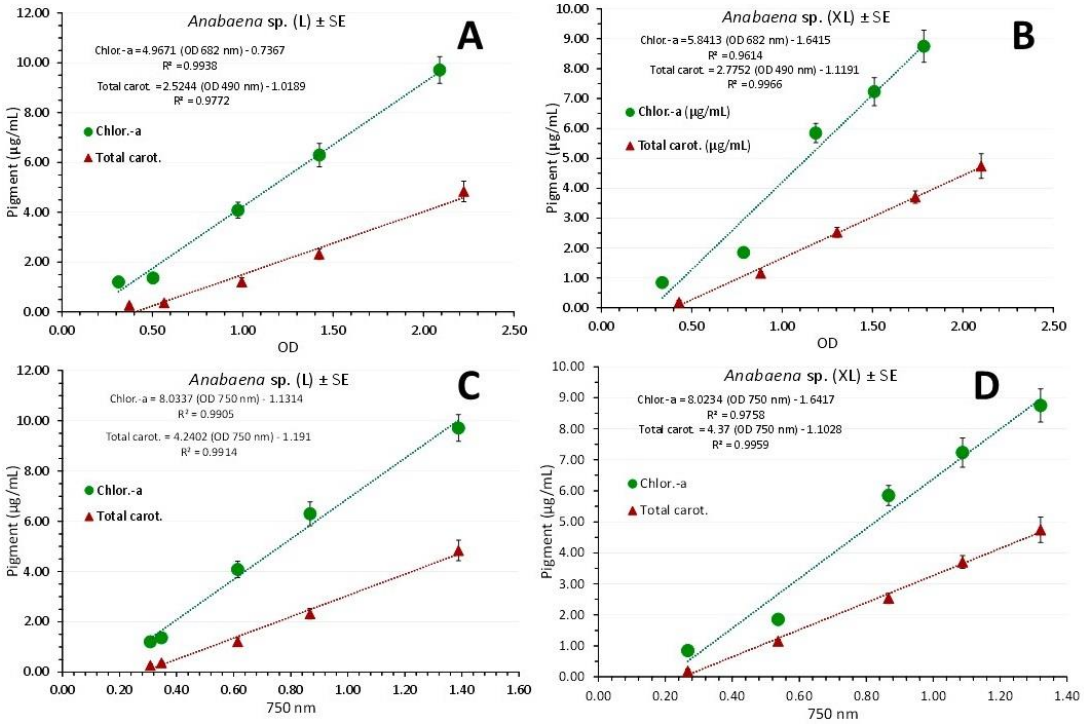

Figure 11. Regression lines of chlorophyll-a and total carotenoids concentration as μg/mL ± SE vs OD at each wavelength exhibiting the maximum absorbance for each pigment in L=light (A) and XL-light (B) and versus the OD at the wave length of 750 nm in L and XL-light (C and D) respectively in Anabaena sp. culture.

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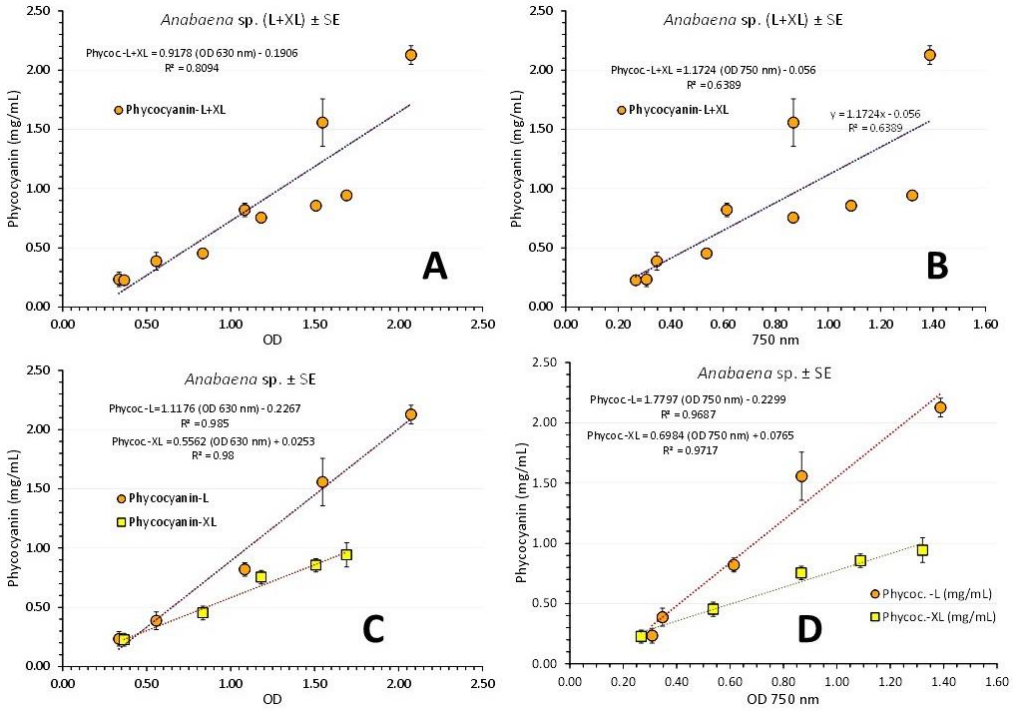

Figure 12. Regression lines of phycocyanin concentration as mg/mL ± SE vs OD at its wavelength (630 nm) exhibiting the maximum absorbance in pooled data from L+XL-light (A) and versus the OD at the wave length of 750 nm (B). The same using data from each light regime for 630 nm (C) and 750 nm (D) in Anabaena sp. culture.

In Figure 13 are depicted the measured concentrations of chlorophyll-a and total carotenoids in μg/mL ± SE, phycocyanin in mg/mL ± SE and phycocyanin yield in mg/g d.w. ± SE, for every light regime. Chlorophyll-a presented its highest concentration of 9.713 ± 0.533 on 15th day in L-light significantly higher ($P<0.05$) than the respective value in XL-light (8.750 ± 0.323). On the contrary, the previous days (6th, 9th and 12th) chlorophyll exhibited significantly higher values in XL-light. Almost the same pattern was found for total carotenoids with higher values in XL-light but finally on the 15th day the concentrations were statistically ($P>0.05$) equal (4.939 ± 0.411 in L-light, 4.792 ± 0.325 in XL-light).

Concerning phycocyanin a clear trend was found with equal concentrations ($P>0.05$) on 3rd, 6th and 9th days of culture, but significantly higher values in L-light (maximum 2.125 ± 0.078 on the 15th day) vs 0.943 ± 0.108 in XL-light for the same day. Similarly, the phycocyanin yield was higher ($P<0.05$) in L-light (Figure 13C) on the 15th day (29.785 ± 1.687) compared to 24.697 ± 0.366 in XL-light (Figure 13D) on the same day.

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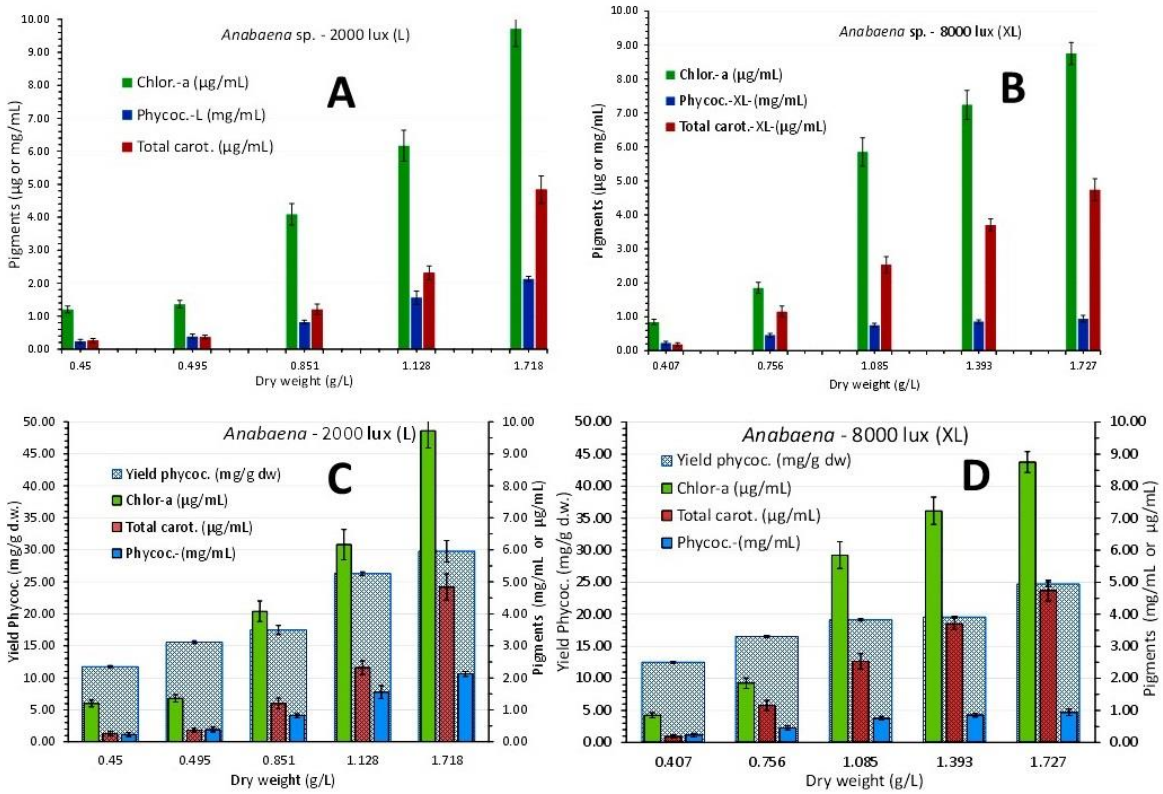

Figure 13. Pigment content in µg/mL ± SE for chlorophyll-a and total carotenoids and in mg/mL ± SE for phycocyanin at 5 different culture densities of Anabaena sp. in L-light (A) and XL-light (B) and additionally for phycocyanin yield in mg/g d.w. ± SE for L-light (C) and XL-light (D). The five biomass densities on "X" axis correspond to 3rd, 6th, 9th, 12th and 15th days of culture.

3.2.2. Effect of colored (green, blue and red) light illumination

In all three colors used the spectra (Figure 14) presented a remarkable similarity for the first 3 days of measurement (2nd, 3rd and 6th) with almost identical peaks for chlorophyll-a (682 and 440 nm), total carotenoids (490 nm) and phycocyanin (630 nm) between green and red. From the 10th day till the 16th day the spectra in green and red were more elevated compared to the respective ones in blue, but on 19th day the spectrum in red collapsed (Figure 14C), while in blue (Figure 14B) just barely raised from that of the previous measurement (16th day) and in green (Figure 14A) kept rising. Phycocyanin peaks (630 nm) became evident from the 6th day onwards in all colors with almost identical height till the 10th day. From the 12th day the phycocyanin peaks subsided in green till the last measurement of 19th day while the respective ones in blue and red (with the exception of the 19th day) kept rising even above the peak of chlorophyll-a (682 nm), especially in blue color.

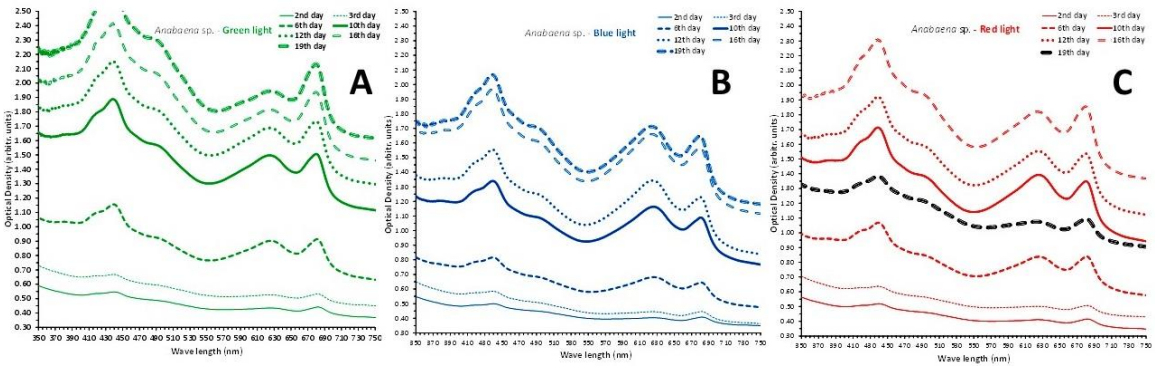

Figure 14. The absorption spectra at 7 different days (2nd – 3rd – 6th – 10th - 12th -16th -19th) of the cultures of Anabaena sp. exposed to green (A), blue (B) and red (C) light.

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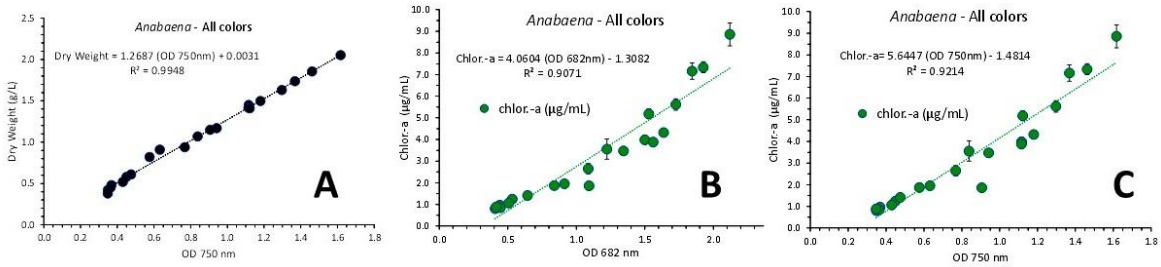

Figure 15. A: Regression line of cell density (in g d.w./L) vs optical density (OD) at 750 nm using values from all colored light regimes. B and C: Regression lines of chlorophyll-a, as µg/mL ± SE vs OD at 682 nm (B) and versus the OD at the wave length of 750 nm (C) in Anabaena sp. culture using values from all colored light regimes.

At all days of the culture (2nd-3rd-6th-10th-12th-16th-19th) the recorded biomass densities were significantly higher ($P<0.05$) in green light compared to blue light, reaching on the 19th day 2.053 ± 0.056 (SE) g d.w./L in green vs 1.498 ± 0.137 (SE) g d.w./L in blue. The relevant values in red light were significantly higher ($P<0.05$) than their relevant ones of the same days in blue light and equal ($P>0.05$) to those of the green light but on the 19th day the culture obviously collapsed to 1.15 ± 0.07 (SE) g d.w./L from 1.737 ± 0.071 (SE) g d.w./L on its 16th day's recording.

A perfect fit ($R^2 = 0.9948$) of the regression line of the relation between the OD at 750 nm of the pooled data from all light colors and the culture density (in g d.w./L) was found described by the equation: Dry Weight = 1.2687 x OD 750nm + 0.0031 (Figure 15A). A very good fit was also found relating chlorophyll-a content from all colored lights and either OD at 682 or 750 nm (Figures 15B and C with $R^2 = 0.9071$ and 0.9214, respectively) and even better fit when considering the relevant relations of chlorophyll-a vs OD of 682 or 750 nm for each color separately. In that case (Figure 16), the first order equations for green, blue and red illumination gave $R^2 = 0.9411$, $R^2 = 0.9816$ and $R^2 = 0.9477$, respectively using the OD of 682 nm (Figure 16A, B and C, respectively) and $R^2 = 0.9598$, $R^2 = 0.9664$ and $R^2 = 0.9757$, respectively using the OD of 750 nm (Figure 16D, E and F, respectively).

Excellent fits were found considering total carotenoids vs OD of 490 nm (their peak wave length) or 750 nm (Figure 17) using either pooled data from all colors or using each color separately. Using pooled data correlation coefficients were: $R^2 = 0.9056$ (Figure 17A) using 490 nm and $R^2 = 0.918$ (Figure 17B) using 750 nm.

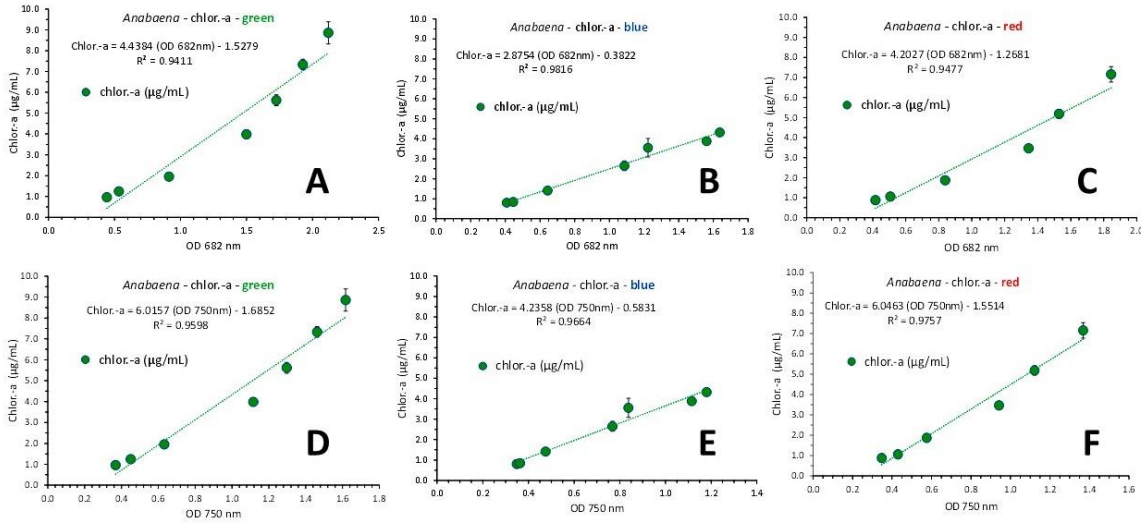

Figure 16. Regression lines of chlorophyll-a, as µg/mL ± SE vs OD at 682 nm in green (A), blue (B) and red (C) light and versus the OD at the wave length of 750 nm (D, E and F for green, blue and red color, respectively) in Anabaena sp. culture.

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Same or even better fits were found when considering the relevant relations of total carotenoids vs OD of 490 or 750 nm for each color separately. In that case (Figure 18), the first order equations for green, blue and red illumination gave R^2 = 0.944, R^2 = 0.9863 and R^2 = 0.9462, respectively using the OD of 490 nm (Figure 18A, B and C, respectively) and R^2 = 0.9433, R^2 = 0.9824 and R^2 = 0.9577, respectively using the OD of 750 nm (Figure 18D, E and F, respectively).

Considering phycocyanin and in contrast to the case of using pooled data from both light intensities (Figure 12), the use of pooled data from all color lights using either the wave length of 630 nm (Figure 17C) or that of 750 nm (Figure 17D), gave very high correlation coefficients (R^2 = 0.9332 and R^2 = 0.9358, respectively). Alternatively, using data for each colored light separately (Figure 19), the first order equations for green, blue and red illumination gave R^2 = 0.944, R^2 = 0.9863 and R^2 = 0.9462, respectively using the OD of 630 nm (Figure 19A, B and C, respectively) and R^2 = 0.9433, R^2 = 0.9824 and R^2 = 0.9577, respectively using the OD of 750 nm (Figure 19D, E and F, respectively).

The overall picture of pigments content among the colors of illumination along the culture growth (Figure 20), revealed significant differences between colors and between stages of culture density. Green light induced significant higher ($P<0.05$) content of chlorophyll-a and total carotenoids after the 3rd day compared to either blue or red light. Its highest values of 8.859 μg/mL ± 0.532 for chlorophyll-a and 5.766 μg/mL ± 0.472 for total carotenoids on 19th day (Figure 20A), were significantly higher ($P<0.05$) than the relevant ones of blue light (4.301 μg/mL ± 0.122 and 2.322 μg/mL ± 0.176, respectively) and red light (7.16 μg/mL ± 0.383 and 4.04 μg/mL ± 0.122 on the 16th day, respectively and 1.862 μg/mL ± 0.112 and 0.765 μg/mL ± 0.123 on the 19th day, respectively when the culture obviously collapsed).

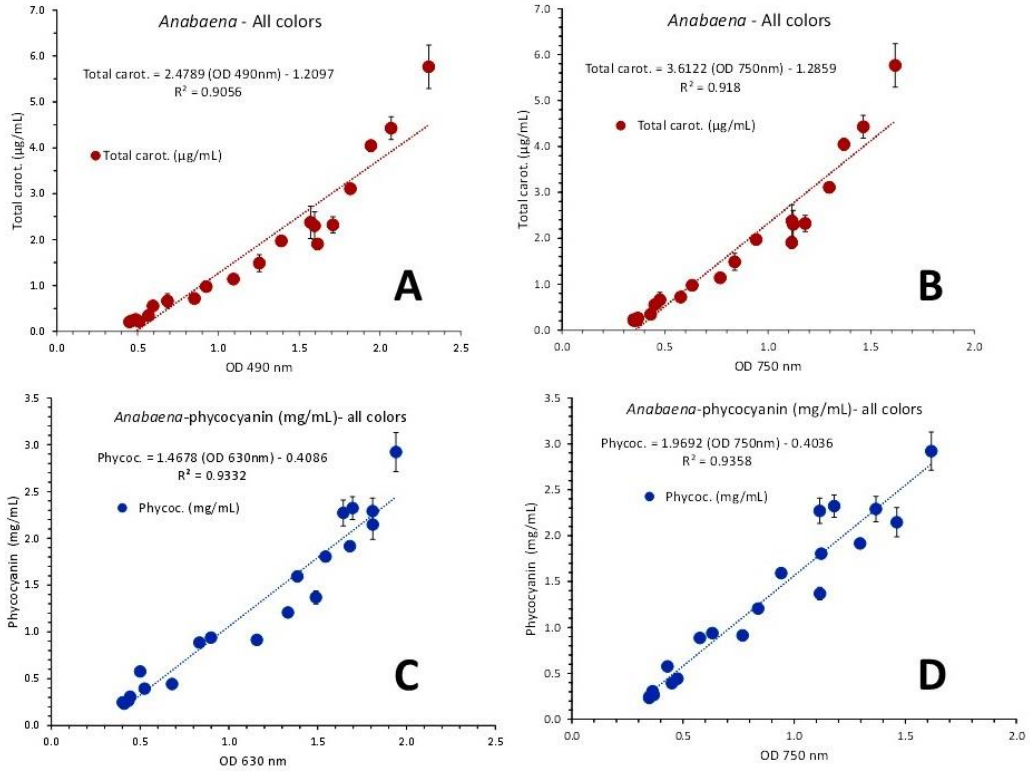

Figure 17. Regression lines of total carotenoids, as μg/mL ± SE vs OD at 490 nm (A) and versus the OD at the wave length of 750 nm (B) and phycocyanin as mg/mL ± SE vs OD at 630 nm (C) and versus the OD at the wave length of 750 nm (D), in Anabaena sp. culture using values from all colored light regimes.

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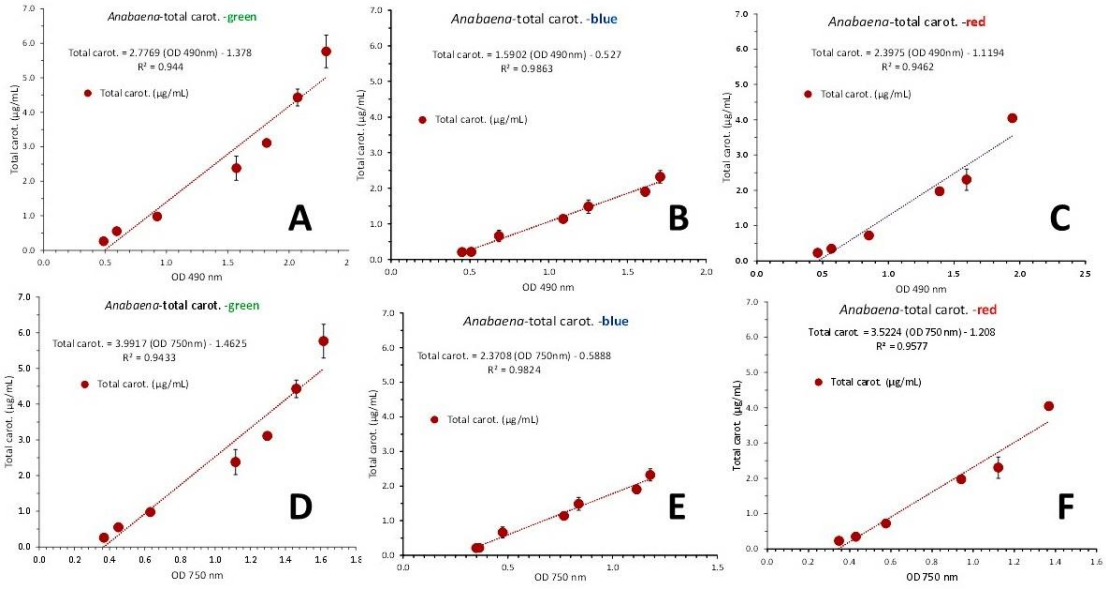

Figure 18. Regression lines of total carotenoids as µg/mL ± SE vs OD at 490 nm in green (A), blue (B) and red (C) light and versus the OD at the wave length of 750 nm (D, E and F for green, blue and red light, respectively), in Anabaena sp. culture.

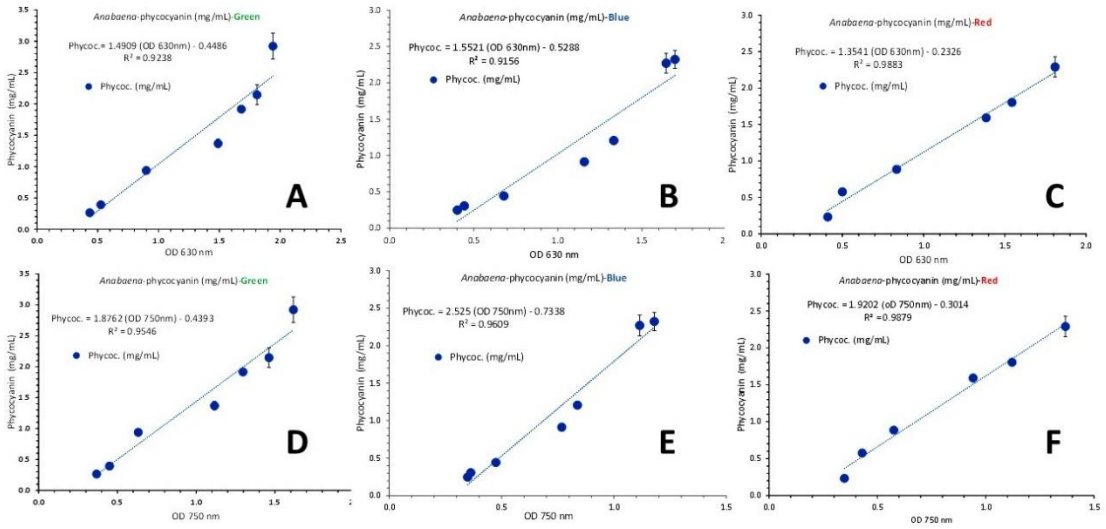

Figure 19. Regression lines of phycocyanin as mg/mL ± SE vs OD at 630 nm in green (A), blue (B) and red (C) light and versus the OD at the wave length of 750 nm (D, E and F for green, blue and red light, respectively), in Anabaena sp. culture.

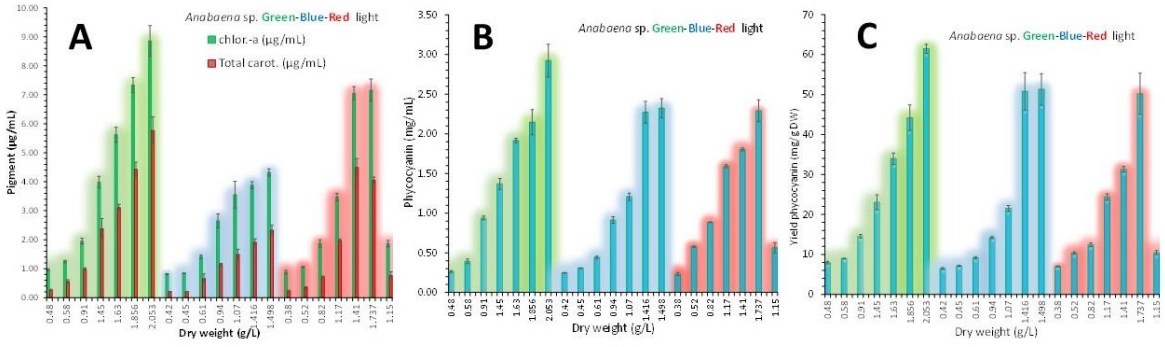

Figure 20. A: Chlorophyll-a and total carotenoids as µg/mL ± SE at each culture density in each colored light regime (indicated by the proper coloration of each bar cluster). B: phycocyanin as mg/mL

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± SE and C: phycocyanin yield as mg/g d.w. ± SE in each colored light regime (indicated by the proper coloration of each bar cluster), in Anabaena sp. culture.

In green light phycocyanin also presented its highest values either as concentration (Figure 20B) or yield (Figure 20C) on the 19th day (2.922 mg/mL ± 0.207 and 61.467 mg/g d.w. ± 1.126, respectively) significantly higher than the relevant values of the same day in blue (2.323 mg/mL ± 0.122 and 51.294 mg/g d.w. ± 3.954, respectively) and red light (2.291 mg/mL ± 0.139 and 50.23 mg/g d.w. ± 5.19 on the 16th day, respectively and 0.565 mg/mL ± 0.065 and 10.458 mg/g d.w. ± 0.489 on the 19th day, respectively when the culture obviously collapsed).

4. Discussion

In the present study the influence of light intensity on one hand and the color of the light on the other, were studied in a well known eukaryotic microalga (Dunaliella sp.) and in an also well known cyanobacterium (Anabaena sp.). The first objective of the study was to investigate if the quantity and quality of light has a remarkable influence on the biomass growth and the content of the algal pigments and the second, if there is a simple and reliable method using absorption spectra to infer about the status of these variables. In a continuously growing industry of extracting valuable bioactive compounds from microalgae [27–29] every method facilitating the rapid estimation of their pigment content is welcomed. In this perspective and before anything else, it should be clarified that the present work aims to help growers estimate culture density and photosynthetic pigments, if not with the precision of an analytical method, but at least by establishing a clear trend in the development of a culture of a certain species of microalgae. As a tool for this purpose, it is shown that the absorption spectrum can give a reliable picture of the state of pigments from a qualitative and quantitative point of view on the one hand and, on the other hand, supply the observer with the values of the optical density of the peaks of the wavelengths that characterize each pigment type as well as the value at 750 nm where no algae pigment absorbs. Key to the applicability of the findings of the present study is the use of the 750 nm wavelength as a predictor of both algal density and photosynthetic pigments. It is well known that the optical density (OD), also known as absorbance or turbidity, of several unicellular algae species and other unicellular microbes, is widely employed as a quick and non-destructive assessment of biomass [30,31], as the relationship between the amount of light absorbed by a suspension of cells and their mass or number is dependent on the size, shape, and refractive index of the particles [32]. Thus, the mass or number of cells in a suspension can directly affect how much light is absorbed by the suspension but the relationship between particle number and OD in order to construct a standard curve is intricate. That is because microalgae have a disproportionately high pigment content, primarily made up of carotenoids and chlorophylls and additionally phycobiliproteins in cyanobacteria, that are subjected to variation not only among species but also depending on culture conditions [33–35]. Thus, constructing a calibration curve correlating algal biomass to a wave length at which a certain pigment peaks, can produce great error if the pigment content changes. Such wave lengths are those of 400-460 nm or 650-680 nm where absorbance of chlorophylls is maximized and are frequently encountered in the literature. Although these wave lengths can be used by means of standard curves as predictors of chlorophyll content and the same is true for other wave lengths assigned to other pigments peaks [36–38] they are not the best predictors of biomass as found in the present study. Instead the wavelength of 750 nm can reliably predict with sufficient accuracy algal density [39–45] because this wavelength avoids the light absorption by photosynthetic pigments which might affect its absorbance value created by turbidity alone [31,42,46]. In both species of microalgae studied, its correlation with the algal density gave very high correlation coefficients [42,47–50] so as to leave us with no doubt for its usefulness in the daily monitoring routine of an algal culture. Further on, when we correlated 750 nm with each pigment content, again, strong correlations ensued and strange as it may be, we found no mention of such a relation in the literature. But in this case (correlation of 750 nm with pigment content) some precautions must be taken. First, such a correlation should be assigned uniquely only for a certain species and for the set of culture conditions prevailing (light intensity, light color etc). Second, a lot

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of pigment measurements should have been made in advance and then a correlation analysis of them with their respective absorbance at the relevant wavelength peak for the examined pigment should lead to a calibration curve. Only if the correlation is strong, the next step, namely the correlation of the pigment with 750 nm, can be useful. In the present study having done all the above and using the 750 nm values from the series of absorption spectra during the course of each culture, it was found a very strong predictive ability for chlorophylls and carotenoids content using pooled data for both low (2000 lux) and high (8000 lux) illumination of white light in both microalgae, but not for phycocyanin in which case correlations are much stronger when examined separately for each light intensity.

Since light provides the energy source for autotrophic growth, it is essential for the majority of microalgae. Irradiance and light quality both influence the growth of cultured microalgae through photosynthesis [51–53]. From an ecological and physiological perspective, the various pigments found in microalgae show differences in the capacity of algae to use light with various spectral compositions for photosynthesis and to adapt to various lighting regimes [53–58]. As a result, the spectrum makeup of light is a crucial growth parameter for producing microalgae [59–61]. In the present study substantial differences were recorded in both maximum biomass, content of pigments and pigment ratios among the intensities of white light and among the three light colors used in both microalgae examined. In both microalgae the maximum biomass was induced by the higher intensity (8000 lux) of white light when compared to all other light regimes (white 2000 lux, or the 8000 lux of green, blue and red light). Considering that in the present study the range of illumination used of 2000-8000 lux (40 - 160 μmol photons/m²/s) is included within the range of 26 - 400 μmol photons/m²/s which according to [53] are the ideal light intensities at which the maximum growth rate is seen for various taxonomic groups and species of algae, we feel confident about our results.

The response of the two examined algae to the quantity and quality of light presented generally similarities and differences. This was of course to be expected as the composition of the photosynthetic pigments in their photosynthetic centers are different between eukaryotic and cyanobacterial microalgae and in addition, there are differences in their physiological mechanisms in response to various environmental conditions. In the present study as the temperature was similar (19-20 °C) for both species, we hypothesize that at other higher temperatures where according to other studies microalgae in general show faster growth and biomass yield [56,58,62], their response will be greater in terms of growth rate and density as well and as possibly in terms of their pigment content.

A common feature between both species is the much faster growth and higher density at the light intensity of 8000 lux compared to 2000 lux. This was more prominent in Dunaliella while in Anabaena the differences were minimal. No relevant references were found specifically on this, and presumably these differences arise from the amount of energy that the photosynthetic apparatus of each species can absorb to carry out photosynthesis. When the maximum rate of photosynthesis is attained, the extra light flux is still absorbed by the cell, which causes photosynthesis to reach a condition of light saturation. As a result, the rate of photosynthesis and algal growth both decline concurrently [63–66].

5. Conclusions

As a result of the calibration dependences we have discovered that it is possible to quickly, easily, and fairly accurate estimate biomass and pigment content in the species of microalgae with industrial importance Dunaliella sp. and Anabaena sp. using their absorption spectra. High correlation coefficients attest to this method's accuracy. Additionally, biomass production in both species is enhanced by white light of 8000 lux compared to 2000 lux, chlorophyll and carotenoids in Dunaliella sp. while 2000 lux enhances phycocyanin production over 8000 lux in Anabaena sp. Using colored light it was found that green light enhances phycocyanin in the cyanobacterium Anabaena sp. and carotenoids and chlorophyll in Dunaliella along with blue over red light.

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Conflicts of Interest: The author declares no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of the data; in the writing of the manuscript; nor in the decision to publish the results.

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