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Type of the Paper (Article, Review, Communication, etc.)

Growth, Phycobiliproteins, Chlorophyll and Carotenoids content of the marine cyanobacteria *Phormidium* sp. and *Cyanothece* sp. as affected by white and colored artificial light in batch cultures

George N. Hotos [1]* and Theodoros I. Antoniadis[1]

[1] Plankton Culture Laboratory, Department of Animal Production, Fisheries & Aquaculture University of Patras, 30200 Messolonghi, Greece
* ghotos@upatras.gr

Abstract: Cyanobacteria are extensively studied and cultured because they can produce many value-added substances among which are pigments, mainly the phycobiliproteins phycocyanin (PC), phycoerythrin (PE), allophycocyanin (APC) and chlorophyll-a and carotenoids as well. As numerous cyanobacterial species await optimization for maximizing pigment production, we examined here two local marine species, *Phormidium* sp. and *Cyanothece* sp. batch cultured under 18-19.5 ^{o}C, at 40 ppt salinity with Walne's nutrient medium, using white LED light of low (2000 lux) and high (8000 lux) intensity and additionally blue, green and red LED light. Significant differences were found among the intensities and colors of light used. Maximum growth was induced by high white light in both species (2.15 g dw/L in *Phormidium* and 1.47 g/L in *Cyanothece*). Next to them was green light (1.25 g/L) in *Cyanothece* and low white and green (1.26 – 1.33 g/L) in *Phormidium*. Green light maximized phycocyanin content in *Phormidium* (0.45 mg/mL), while phycoerythrin was maximized (0.17 mg/mL) by blue light and allophycocyanin by all colors (~0.80 mg/mL). All colors maximized phycocyanin in *Cyanothece* (~0.32 mg/mL) while phycoerythrin and allophycocyanin were maximized under green light (~0.138 and 0.38 mg/mL respectively). In *Phormidium* maximization of chlorophyll-a (9.3 μg/mL) was induced by green light while total carotenoids and b-carotene (3.05 and 0.89 μg/mL respectively) by high white light. In *Cyanothece* both white light intensities along with green light maximized chlorophyll-a content (~9 μg/mL) while high white light and green maximized total carotenoids (2.6-3.0 μg/mL).

Keywords:: cyanobacteria; *Phormidium*; *Cyanothece*; culture growth; light;; chlorophyll; carotenoids; phycocyanin; phycoerythrin; allophycocyanin; phycobiliproteins

1. Introduction

Cyanobacteria comprise a vast assemblage of prokaryotic microalgae that are found in ecosystems highly diversified in terms of extreme conditions prevailing both seasonally and nutritionally [1]. They colonize every habitat due to their ability to adapt and cope with a great variation of light intensity and spectra, temperature and nutrient availability [2-4]. They possess oxygenic photosynthesis along with eukaryotic algae and share with them the same basic and accessory pigments (chlorophyll-a and carotenoids respectively) [5]. Additionally, cyanobacteria are equipped with unique photosynthesis aiding pigments of proteinaceous structure named phycobiliproteins (phycocyanin, allophycocyanin and phycoerythrin, blue, bluish-green and red colored respectively) that impart to them their characteristic bluish-greenish tint, hence their common name "blue-green" algae [6-8]. All the above mentioned pigments that function in the harness of light energy for algae to transform CO_2 to sugars in order to increase their biomass, have tremendous significance for certain sectors of bioindustry as healthy ingredients for humans and animals and for fine chemicals [9, 10]. In particular, the biliprotein phycocyanin, a water soluble pigment with wide and continuously expanded uses in biology, medicine and food

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industry [6, 11], presents front-line exploitable potential. Based on rough statistics [12, 13] its world market value is estimated to 50 million US$ per annum with commercial value from 0.13 to 25 US$ per mg depending on its purity level from food grade (the lowest) to analytical grade (the highest) [14].

Although various cyanobacterial species can widely be mass cultured in farms for various purposes [15], the lion's share is held by the Genus *Arthrospira* (*Spirulina*) [13, 16] overshadowing initiatives for other exploitable similar species. As cyanobacteria offer unique advantages for culture such as the independence from nitrogenous nutrient sources due to the atmospheric nitrogen fixation for some of them [15], their efficient light capture machinery [17], or the self-aggregation of their cell mass (e.g. *Phormidium*) a property much appreciated in culture procedure [18, 19], research on their mass production is worth the trials.

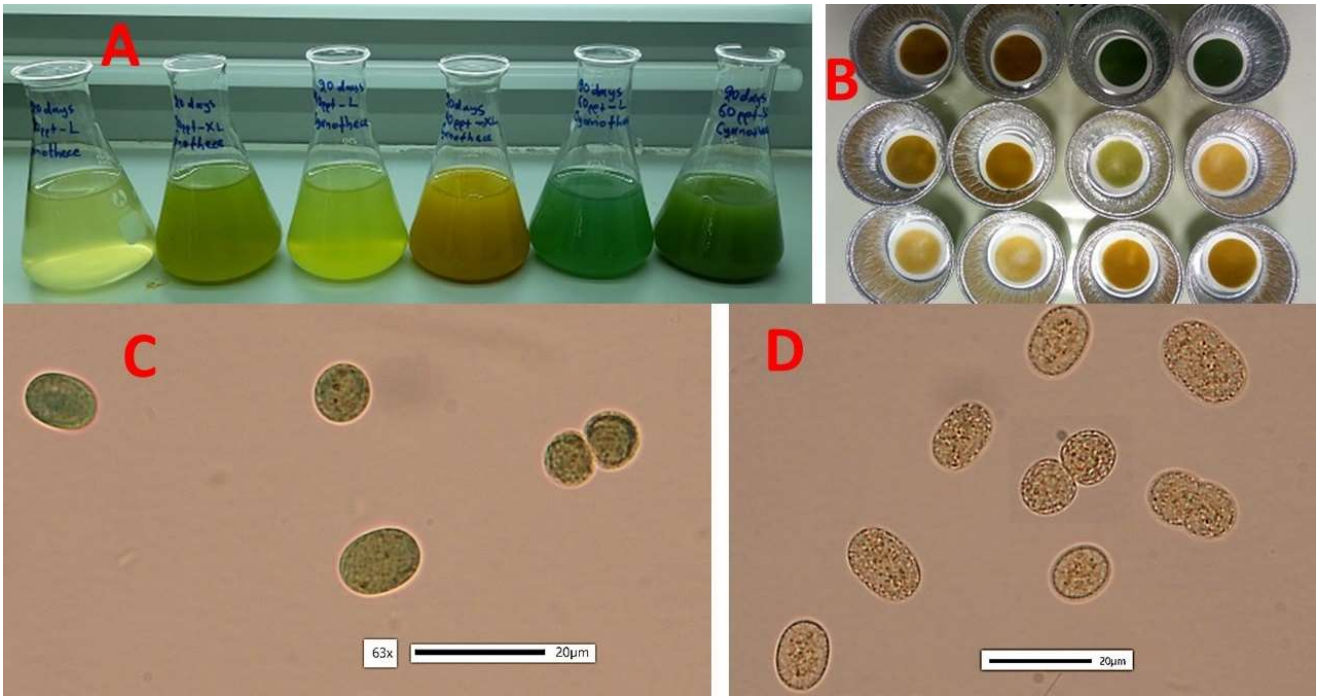

Figure 1. *Cyanothece* sp. **A**. Color of its cultures exhibiting intense differentiation depending on the age of culture. **B**. GF/C filters colored by sampling different stages of culture. **C**. Cells of *Cyanothece* sp. colored greenish on the 8th day of culture, 630x. **D**. Cells of *Cyanothece* sp. colored greenish on the 18th day of culture, 630x.

For all microalgae (prokaryotic and eukaryotic) the culture productivity on the one hand and their biochemical composition (photosynthetic pigments included) on the other, are greatly affected by physicochemical and nutritional factors prevailing in their cultivation [20]. Among them light is of paramount importance as both its quantity (intensity) and quality (color) of the visible spectrum utilized for photosynthesis, affect profoundly the productivity of the culture and the biochemical composition of the cells [21-23]. As the increased interest of the world market for carotenoids from natural sources [24] and biliproteins [6, 13, 25] substantiate the experimentation on optimizing their production, the parameter of light emerges as a key-role factor for accomplishing that [14, 19]. Although cyanobacteria are limitedly mentioned in the recent review work of [23] where the influence of light on microalgae is reviewed, there are several studies that focus on the effect of light intensity and its color on growth and cell constituents of several cyanobacterial species, mainly *Arthrospira* [14, 26-31], *Anabaena* [32], *Nostoc* [32], *Phormidium* [18, 19] and various other species [33-37]. On this ground we examined the effect of light on two locally

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isolated (saltworks of Messolonghi, W. Greece, [38]) cyanobacteria, the coccoid N-fixing *Cyanothece* sp. (Figure 1) and the filamentous non N-fixing *Phormidium* sp. (Figure 2). As both of them exhibited potential for fast growth in a wide range of salinity and light intensity in preliminary or elaborated cultures and additionally this *Phormidium* strain proved a profound phycocyanin producer [19], we decided to deepen into their response to different light regimes. Both of them exhibit intense chromatic adaptation as a result of ageing of culture in combination with light intensity, a phenomenon manifested in cyanobacteria [39, 40] that triggered our interest to monitor their pigment content in batch culture. Besides *Spirulina* (cyanobacterium) and *Porphyridium* (red algae) biliprotein commercial production that occupies the bulk of the business [13], there is always space for exploring other cyanobacterial species for the same purpose and additionally for chlorophyll and carotenoids. To the best of our knowledge this is the first study concerning the effect of colored light on species of *Cyanothece* and *Phormidium*.

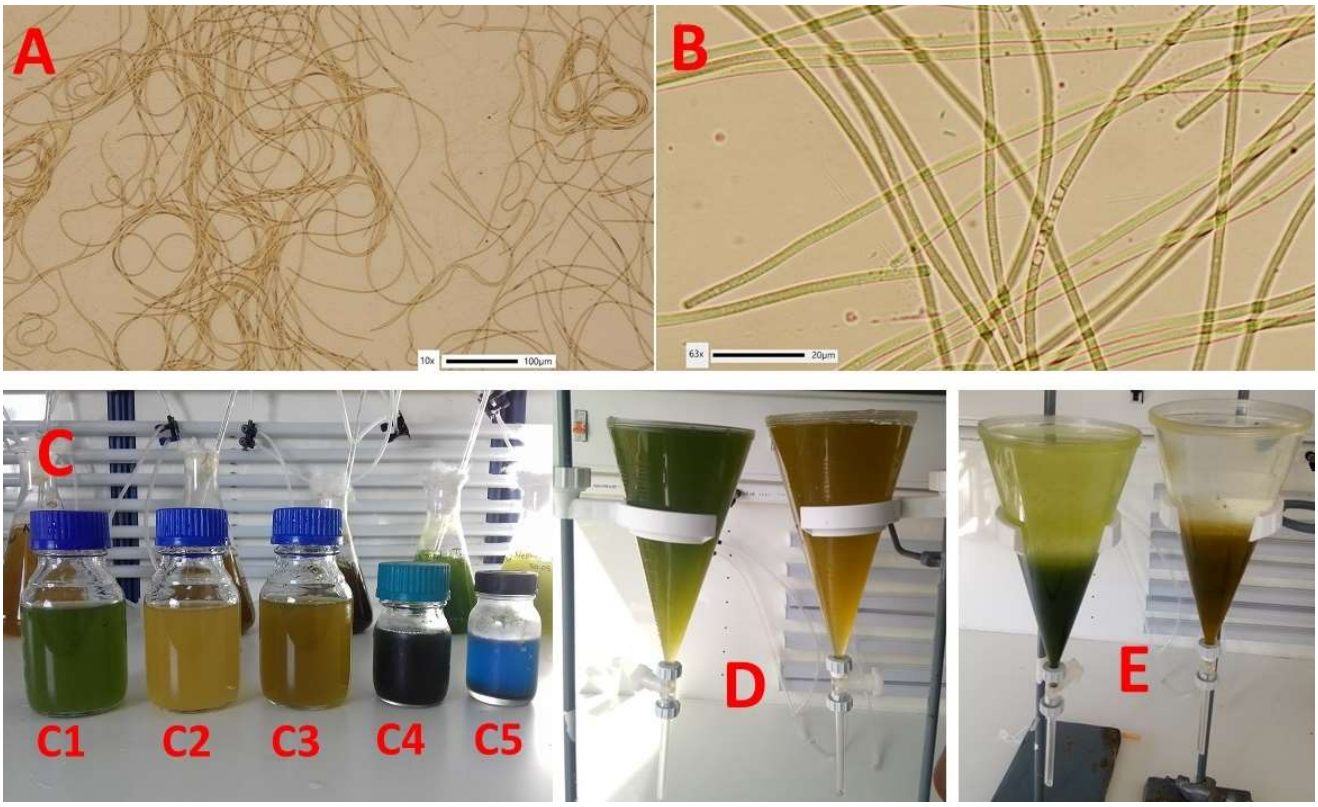

Figure 2. *Phormidium* sp. **A**. Dense mass of filaments at 100x magnification. **B**. Filaments at 630x magnification. **C**. Characteristic colors of samples from different stages of culture and treatment. **C1** from culture in exponential phase (8th day), **C2**. from stationary phase (13-15th day), **C3**, from old culture (>18th day), **C4**, a dense after centrifugation sample from the exponential phase to be frozen, **C5**, the same sample after thawing exhibited profound phycocyanin release. **D**. Samples from exponential (left) and late stationary phase (right) of culture just after collection. **E**. the same samples after about 1 hour exhibiting sedimentation of the cell mass.

2. Results

2.1. Biomass

In every light regime biomass increased during the culture period in both species (Figure 3) and the increase was much higher in white light in which the higher intensity (8000 lux)

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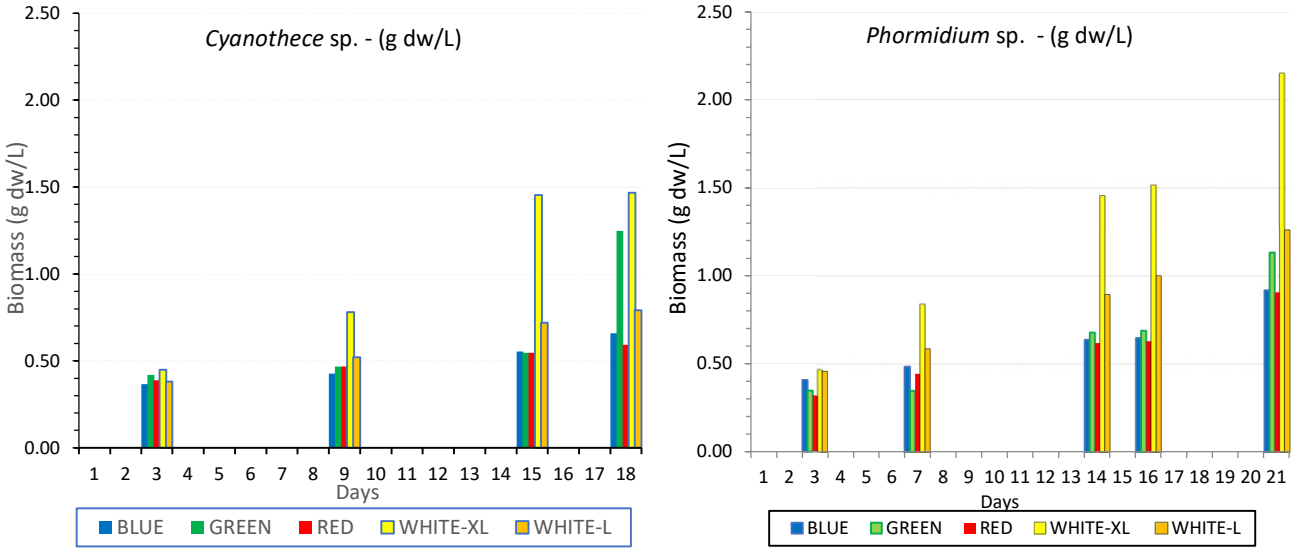
values were almost double of those of low intensity (2000 lux) after the 7th and 9th day in *Phormidium* and *Cyanothece* respectively. *Phormidium* grew faster than *Cyanothece* as evidenced also by the higher specific growth rate (Table 1) reaching maximum 2.15 g dw/L on 21st day, higher than 1.47 g dw/L of *Cyanothece* on 18th day. In both species growth was almost identical in all colors but from the 15-16th day, green color induced abruptly higher growth than blue and red reaching 1.25 and 1.14 g dw/L on 18th and 21st day in *Cyanothece* and *Phormidium* respectively, values statistically different ($p<0.005$) from the respective ones of all other colors. However specific growth rate fluctuated to low values for *Phormidium* (max. 0.131 at green light in *Phormidium*) and even lower in *Cyanothece* (max. 0.098 at high white light in *Cyanothece*). pH fluctuated throught the culture period between 8.3 to 9.5 whith remarcably higher values of greater than 9.0 in high white light in both species.



(**a**) (**b**)

Figure 3. Biomass production as g dry weight/L through the culture period in: (**a**) *Cyanothece* sp. and (**b**) *Phormidium* sp. WHITE-XL= white light of 8000 lux, WHITE-L= white light of 2000 lux, BLUE, GREEN, RED = specific color light used.

2.2. Chlorophyll and carotenoids

Significant differences were found in chlorophyll-a content between *Phormidium* and *Cyanothece* (Figures 4 and 5). In both species the maximum chlorophyll-a content was ~ 9 μg/mL but recorded at different days of the culture (earlier in *Cyanothece*) and with greater and statistically significant differences among the colors. In both species higher chlorophyll content was induced by white low light (2000 lux) compared to white high light (8000 lux, $p<0.005$). But the highest values in both species was recorded in green light and this phenomenon was much more pronounced in *Phormidium* after the 14th day whith values statistically greater ($p<0.005$) than any other light, while in *Cyanothece* the green value peaked later on the 18th day and was statistically equal to that of white low light ($p>0.005$). Worth mentioning is also the fact that in both species after about 7 days the ratio of values of chlorophyll-a between low and high white light remained almost the same.

In contrast to chlorophyll, much higher total carotenoids content was induced by high white light compared to low light in both species, 4.2 μg/mL in *Phormidium* on the 21st day and 3.0 μg/mL in *Cyanothece* on the 18th day (Figures 6 and 7). All other colors had a quite uniform influence on carotenoids content exhibiting a steady slow increase in the course of the culture period but with red and green in *Phormidium* and green in *Cyanothece* on 21st and 18th day respectively, peaking their influence at levels significantly higher ($p<0.005$)

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than their blue and blue and red counterparts respectively. The ratio of chlorophyll to total carotenoids was maximum in all lights during the early phase of the culture (Table 1) peaking on the 7th day in *Phormidium* with the higher value of 5.44 for the green light and the lowest 2.2 for the white high light. In *Cyanothece* the chl.:carot. ratio presented quite similar values among colors throughout the culture period with the lower values in blue light (2.0-3.0) and the highest in low white light (8.0-15.0). In *Phormidium* the total carotenoids : b-carotene ratio exhibited rather small differences among treatments ranging from 2.65 to 4.4 with higher values recorded at the final days of the culture in all light regimes. Unfortunately in *Cyanothece* due to a technical problem there were no data for b-carotene.

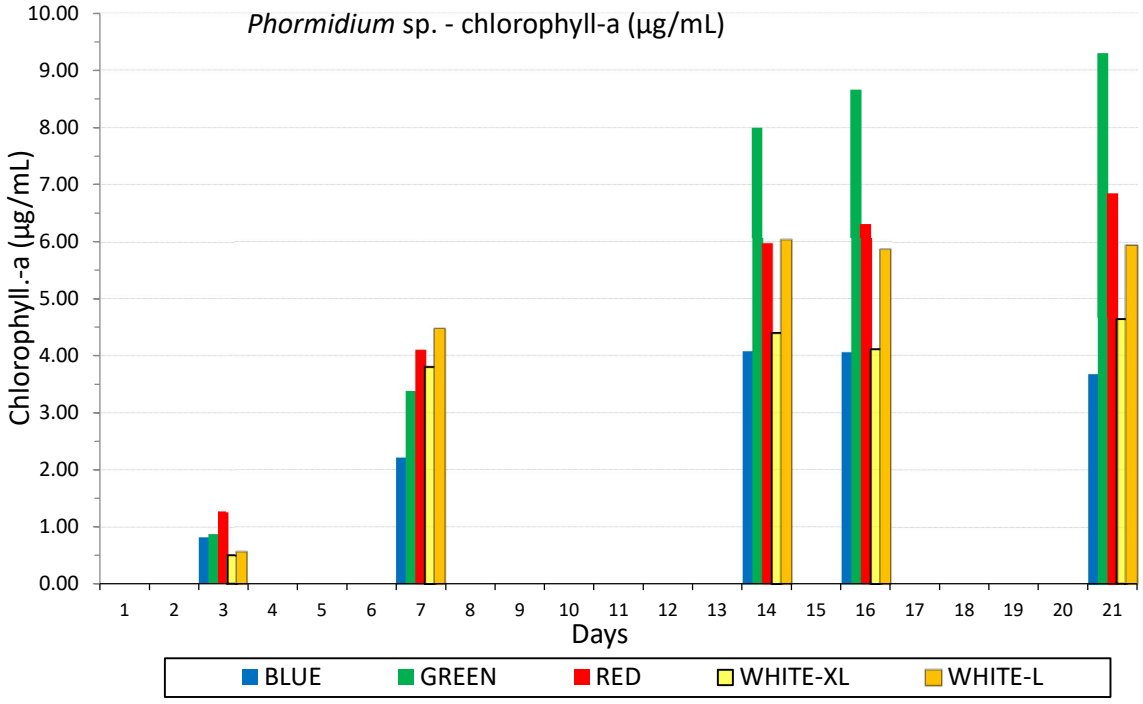

Figure 4. The development of chlorophyll-a content in µg/mL along the culture period of *Phormidium* sp.

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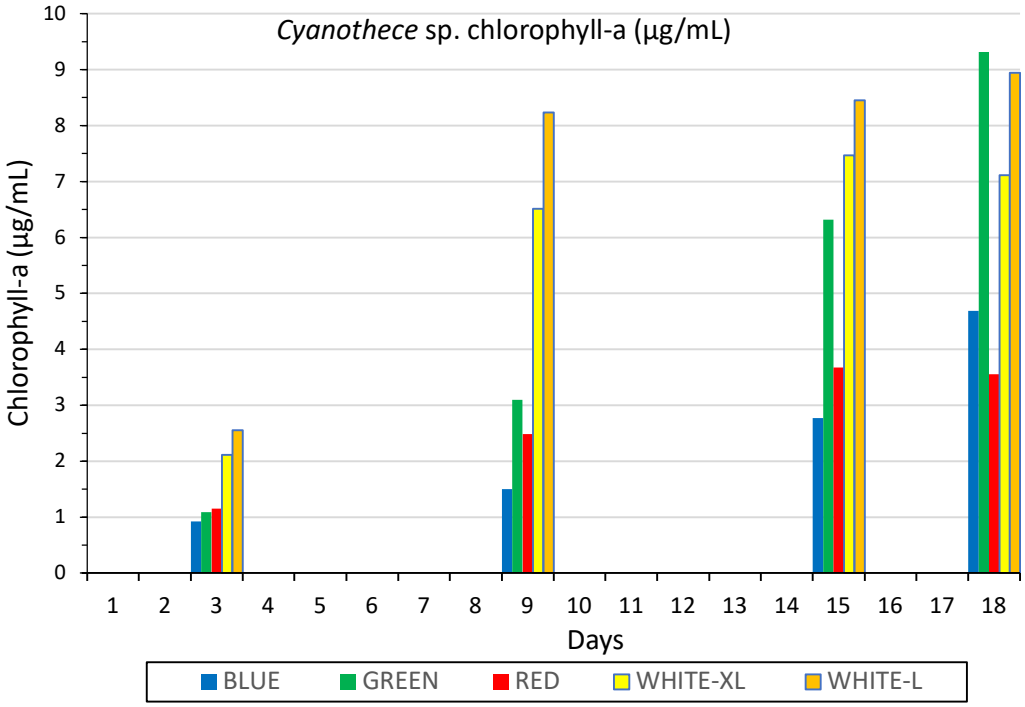

Figure 5. The development of chlorophyll-a content in µg/mL along the culture period of *Cyanothece* sp.

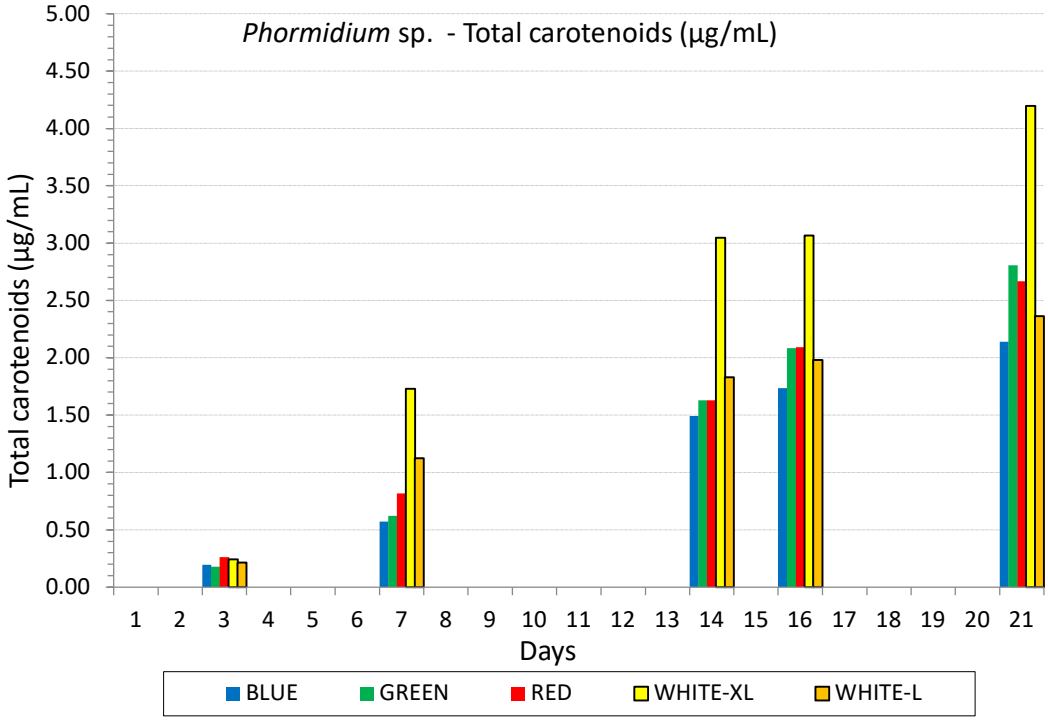

Figure 6. The development of total carotenoids content in µg/mL along the culture period of *Phormidium* sp.

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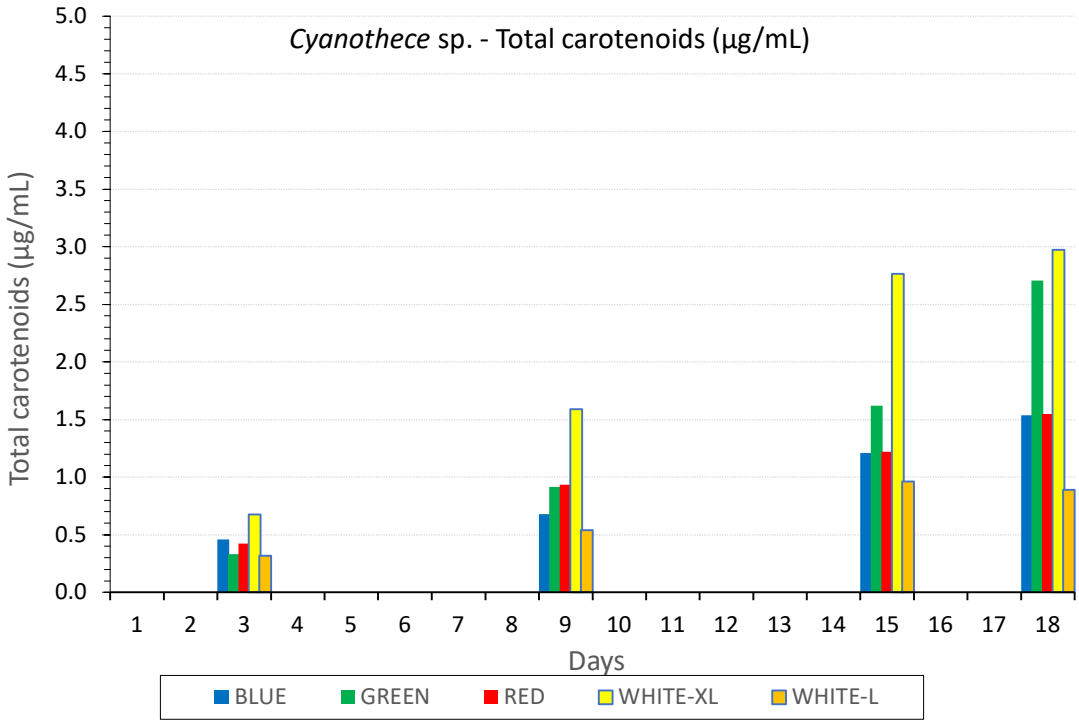

Figure 7. The development of total carotenoids content in µg/mL along the culture period of *Cyanothece* sp.

2.2. Phycobiliproteins

Great variation in every particular phycobiliprotein content was recorded among the lights used and among the days of the culture period. As illustrated in the exemplified absorption spectra diagramms of high white light for the start, middle and final stages of the culture of *Phormidium* (Figure 8), the relative content of chlorophyll-a (440 & 685 nm), carotenoids (500 nm), phycoerythrin (580 nm) and phycocyanin (625 nm) as evidenced by their characteristic peaks, were greatly modified along with culture age. While the peaks of chlorophyll-a remained stable, phycocyanin was maximized on the 7th day and phycoerythrin on the 21st day in accordance with the data presented in Figures 9 and 11 in respect to phycocyanin and phycoerythrin content of high white light in *Phormidium*.

Phycocyanin content was much influenced by all colors in *Phormidium* (Figure 9) and from the first days of the culture its concentration reached values close to 0.3 mg/mL that peaked on the 16th day (0.46 mg/mL) under green light, statistically different ($p<0.005$) from all other lights. Thereafter, on the 21st day the influence of the green light diminished. Red and blue light, except for the 16th day, induced also comparable to red and low white light production of phycocyanin. Apart from colored light the low white light after the 7th day induced much greater production of phycocyanin compared to high white light.

In *Cyanothece* (Figure 10) phycocyanin was increased much more delayed than in *Phormidium* from the very low values for all lights of ~0.03 mg/mL on the 3rd day to values around 0.3 mg/mL on the 7th day by low white and green lights. Thereafter the influence of white light diminished and red and blue colors reached the values of green in the region of 0.3 mg/mL. As a rough approximation we can claim that in both species phycocyanin was much favored by the colored light (especially in *Cyanothece*) reaching ~0.3 mg/mL.

Phycoerythrin exhibited a totally different image than phycocyanin between the two species although in a generalized consideration, maximum content was in the region of 0.15 mg/mL for both species but with a totally different pattern for each of them. In

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Phormidium (Figure 11) after the 7th day all colors induced much more content ($p<0.005$) than every kind of white light and finally on the 21st day blue light by far ($p<0.005$) induced 0.175 mg/mL, a value considerably higher than any other light. In *Cyanothece* (Figure 12) an astonishing and overwhelming influence on phycoerythrin content was recorded using green light which greatly surpassed all kind of lights ($p<0.005$) throughout the culture period reaching 0.13 mg/mL on the 18th day. In *Cyanothece* in contrast to the above mentioned case of phycocyanin for both species and phycoerythrin in *Phormidium*, high and low white lights had negligible influence on induction of phycoerythrin production.

A remarkably similar to phycoerythrin pattern of difference between the two species was also recorded in the case of allophycocyanin (Figures 13 and 14) as firstly, its content was much greater in *Phormidium* (max. 0.83 mg/ml) compared to *Cyanothece* (max. 0.37 mg/mL) and secondly, its maximization in *Cyanothece* was also induced (similarly to phycoerythrin) by green light far higher than any other kind of light ($p<0.005$). The two species differed in that allophycocyanin in *Phormidium* was favored by all colors over white light instead of only by the green as in *Cyanothece*.

Considering the total content of all biliproteins (Table 1) no clear pattern could be detected neither among days nor among light treatments. The higher value in *Phormidium* was recorded in green light on the 14th day (1.45 mg/mL with 0.374 mg/mL on the 3nd day) and the lowest in high white light on the 3nd day (0.026 mg/mL and maximum 0.136 mg/mL on 14th day). In contrast to *Phormidium*, *Cyanothece* exhibited a clear pattern of continuous increase of total biliproteins in all light treatments as the culture proceeded. Green light exhibited the over all other treatments maximal value of 0.768 mg/mL on the 18th day (with minimal 0.065 mg/mL on the 3nd day) and both white lights the lowest values in comparison to all colors (~0.02 min. to 0.250 mg/mL max.).

Totally different ratio paterns were recorded among the two species (Table 1) concerning the ratio of phycocyanin (PC) to phycoerythrin (PE). In *Phormidium* the PC:PE ratio exhibited the highest values on the 3nd day whith maxima in the green and blue light (39.3 and 40.3 respectively) and minima in the high white and red light (8.4) and the ratios continuously decreased to end up on the 21st day with values much smaller, 1.1 for high white light, 1.5 for blue, 2.4 for green, 6.2 for red and 10.3 for low white light which, with an initial value of 18.1 along with red light (from 8.4 to 6.2), exhibited the lower decrease. In *Cyanothece* contrary to *Phormidium*, the PC:PE ratio exhibited a reverse trend with low values in the beginning (3nd day), 6.3-6.6 for both white lights, 9.4 for blue and ~15.0 for red and green. With the advance of the culture in all lights, the ratios increased gradually to reach much higher values on the 18th day from 67.7 for red to 125-150 for white lights and 305 for blue light. Remarkable exception was the green light which contrary to all other lights exhibited a constant decrease in its values from 15.2 on the 3nd day to 1.9 on the 18th day.

The ratio of phycocyanin to allophycocyanin (PC:APC) fluctuated among very low rather similar values (Table 1) throughout the culture period in *Cyanothece* in all lights (0.7-3.5) and even lower in *Phormidium* (0.3-2.0).

The ratio of total phycobiliproteins (PBP) to chlorophyll and total carotenoids (PBP:Chl and PBP:Tcar respectively) exhibited quite similar pattern between the two species (Table 1). Both PBP:Chl and PBP:Tcar were steadily increased from the 3nd day till the 9th and then decreased. In *Phormidium* PBP:Chl values in general were much higher in all lights than *Cyanothece* (e.g. 447 vs 109 for blue light) and this was further exagerrated in PBP:Tcar (e.g. 1735 vs 240 also for blue light).

In terms of yield of phycocyanin and phycoerythrin the two species exhibited quite uniform values (Table 1) ranging from 18.52 to 31.76 mg/g dw phycocyanin in blue and green light respectively in *Phormidium* and from 18.34 to 34 mg/g dw for high white and red light respectively in *Cyanothece*. Considerably lower values were recorded for phycoerythrin in both species but overall *Phormidium* produced more. In both species the

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maximal amount was produced under green light (10.44 and 12.07 mg/g dw for *Phormidium* and *Cyanothece* respectively), but while in *Cyanothece* the value for green far exceeded any other from the rest of the colors, in *Phormidium* blue and red induced similar yields to that of green (10.62 and 10.87 mg/g dw respectively).

The purity of phycocyanin tested by recording the ratio of absorbance of crude extract between the wave lengths of 620 and 280 nm, considering the value of 0.7 as the border of satisfactory purity (above) and poor (below), was in most cases above 0.7 in both species. An example is given in Figure 15 where the absorbtion spectra of crude extract of phycocyanin from samples of the 14th day from all colors in *Phormidium* were put together. It is evident that the curves presented a greater hight between the peaks of phycocyanin at 625 nm and the region below 400 nm (characteristic of other proteins) in all colors as compared to low white light and the absence of purity of high white light. In general blue light proved the most efficient with values over 1.5, low white light just on the edge (~0.7) and high white light very low (~0.2).

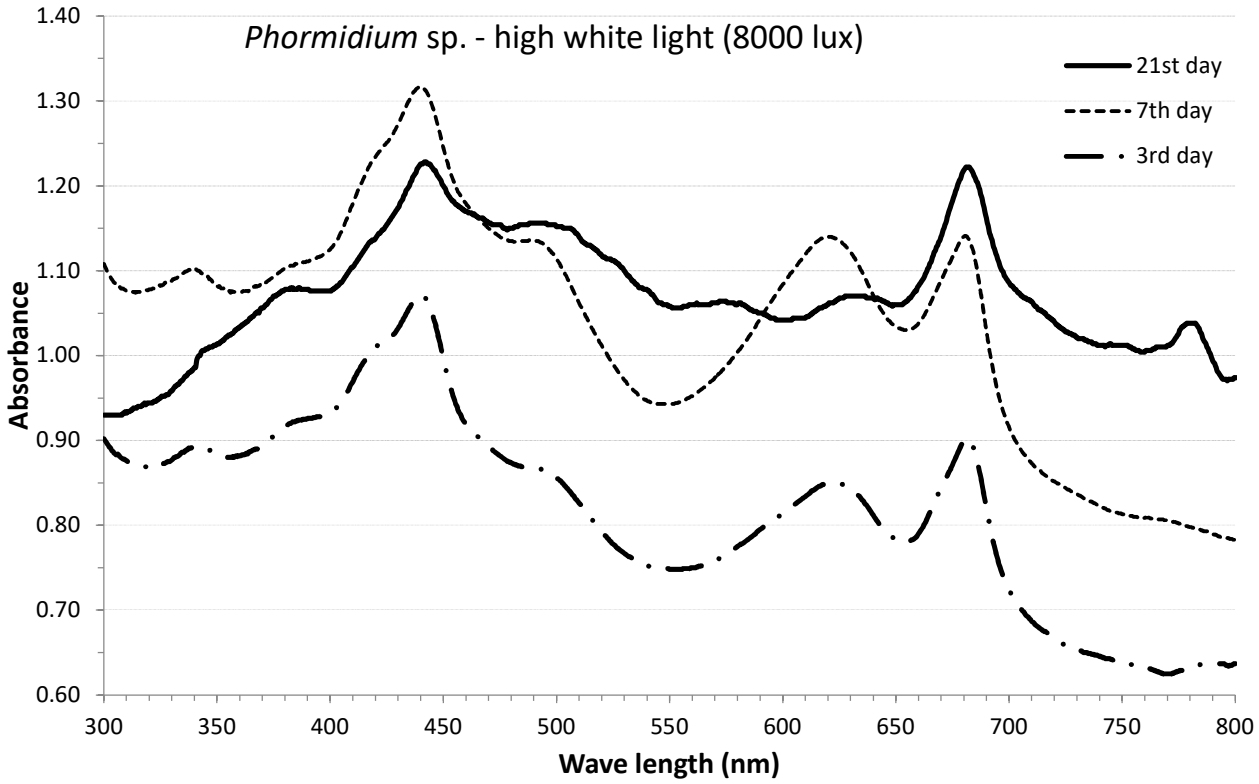

Figure 8. Absorption spectra of culture samples of *Phormidium* sp. under high intensity white light recorded at differed culture days.

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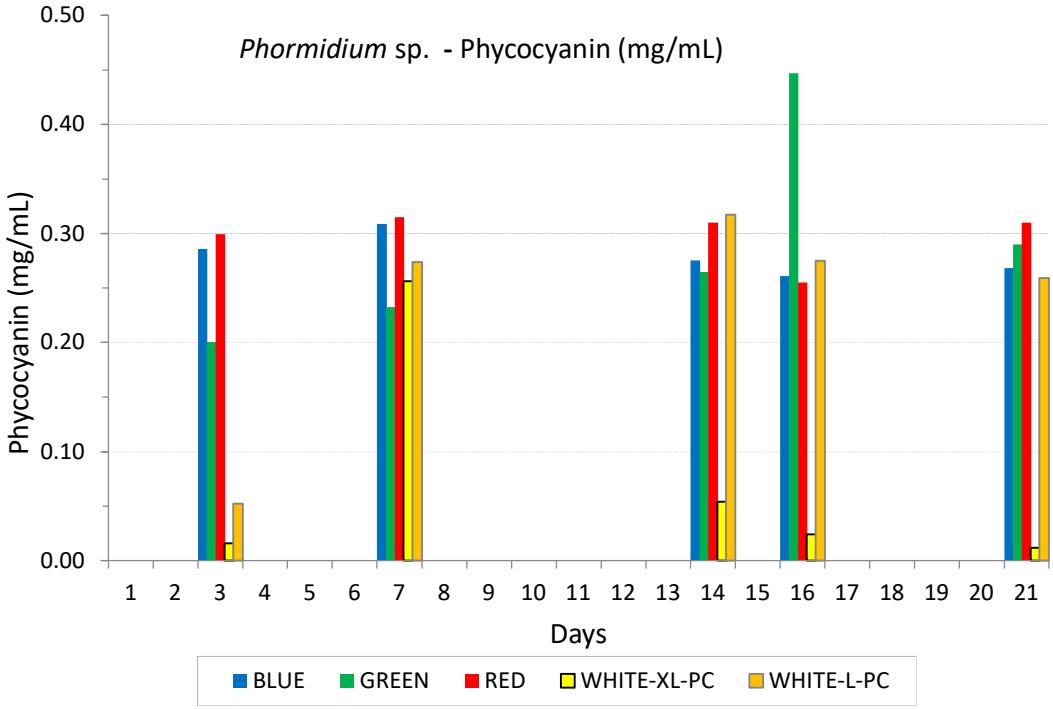

Figure 9. The development of phycocyanin content in mg/mL along the culture period of *Phormidium* sp.

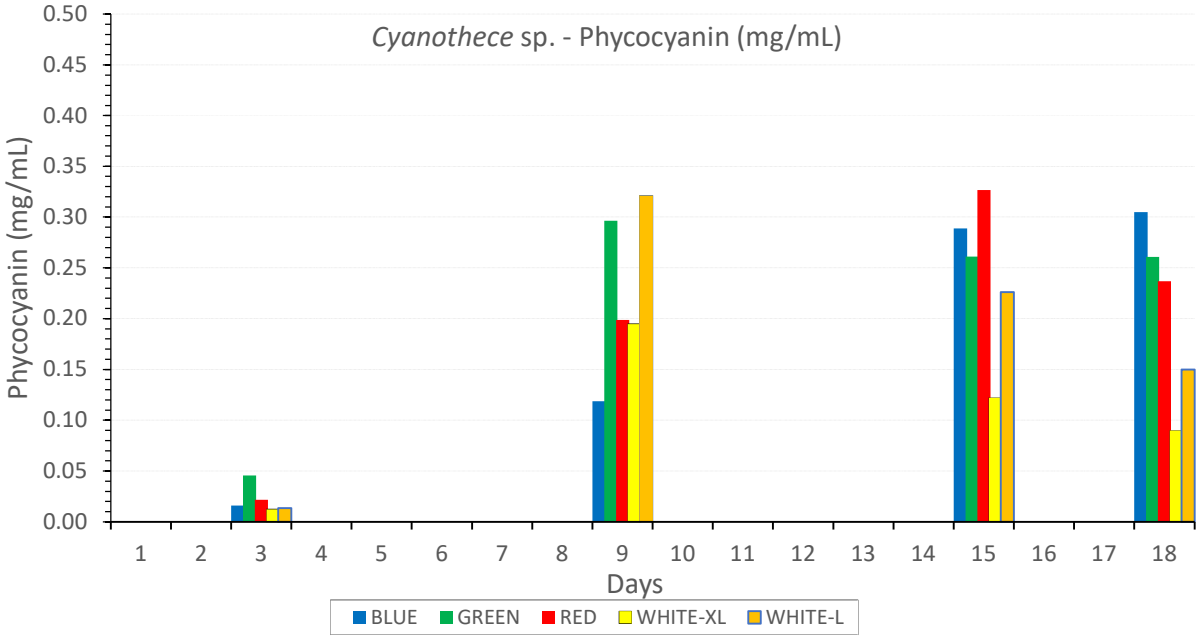

Figure 10. The development of phycocyanin content in mg/mL along the culture period of *Cyanothece* sp.

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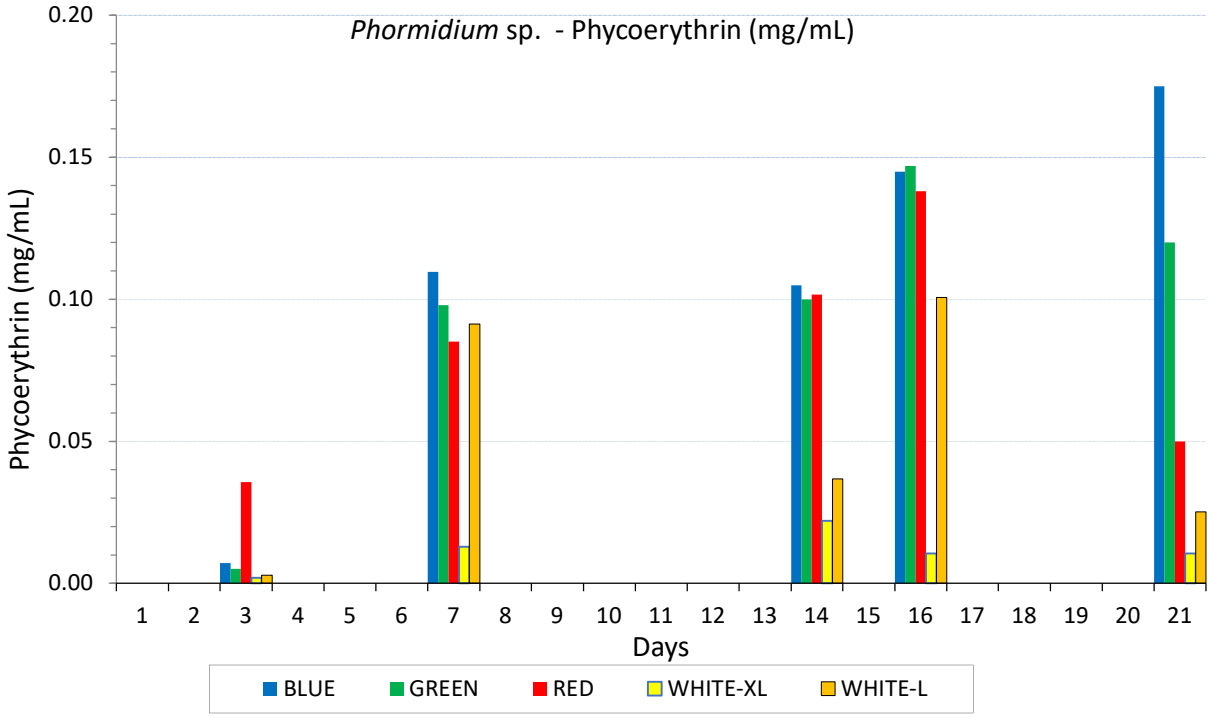

Figure 11. The development of phycoerythrin content in mg/mL along the culture period of *Phormidium* sp.

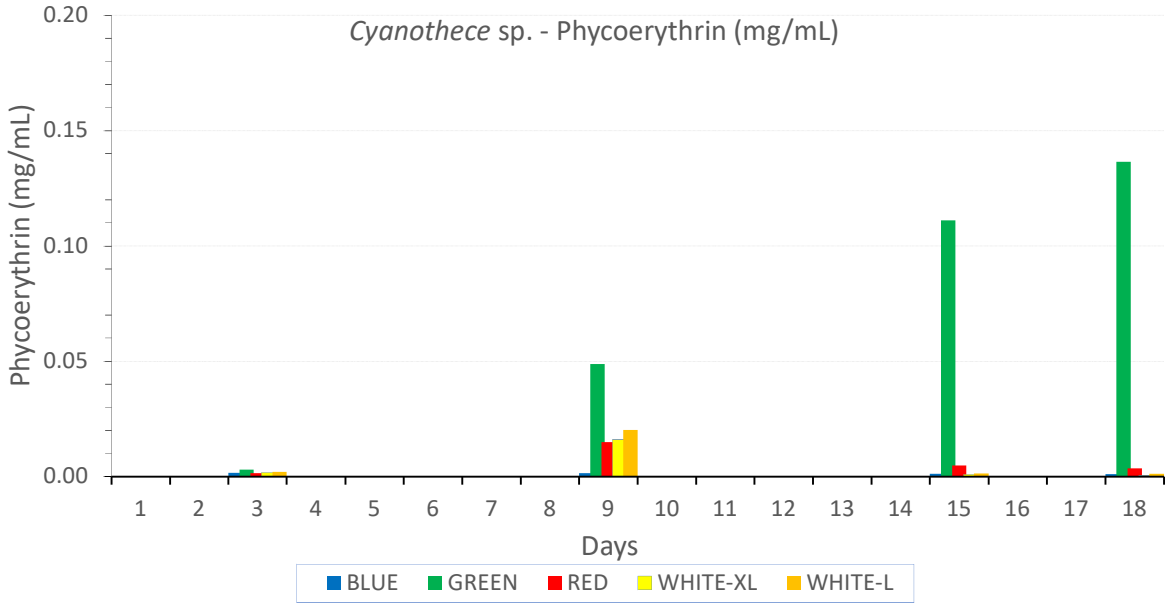

Figure 12. The development of phycoerythrin content in mg/mL along the culture period of *Cyanothece* sp.

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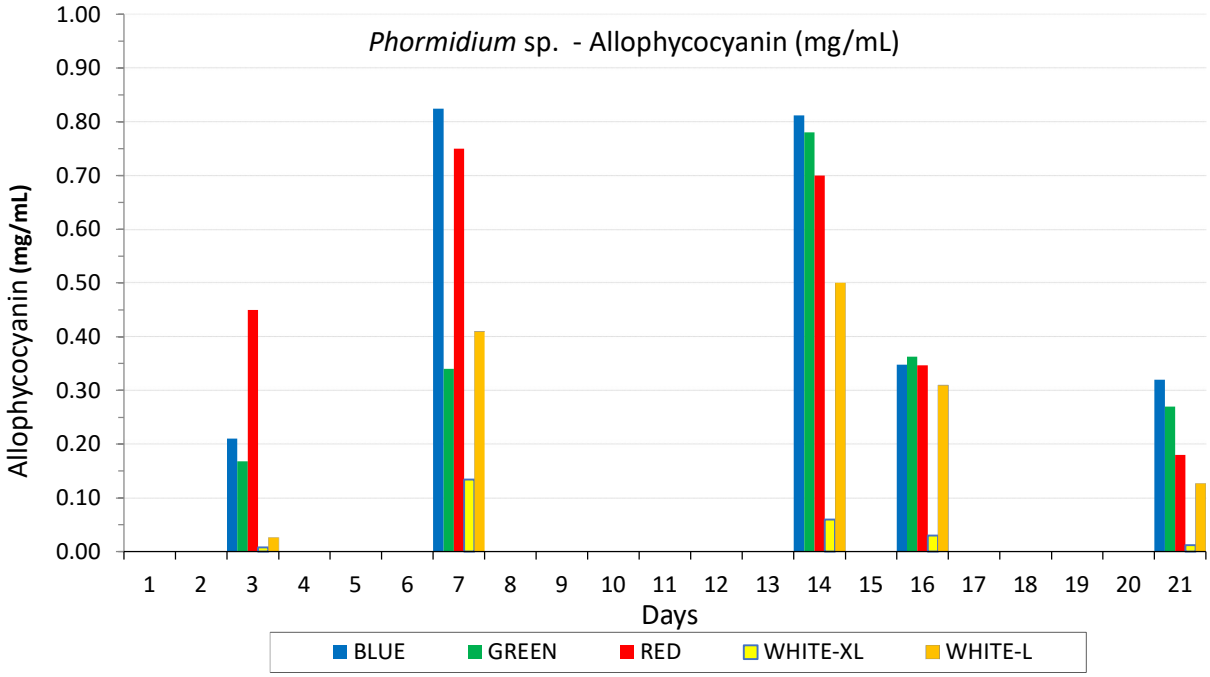

Figure 13. The development of allophycocyanin content in mg/mL along the culture period of *Phormidium* sp.

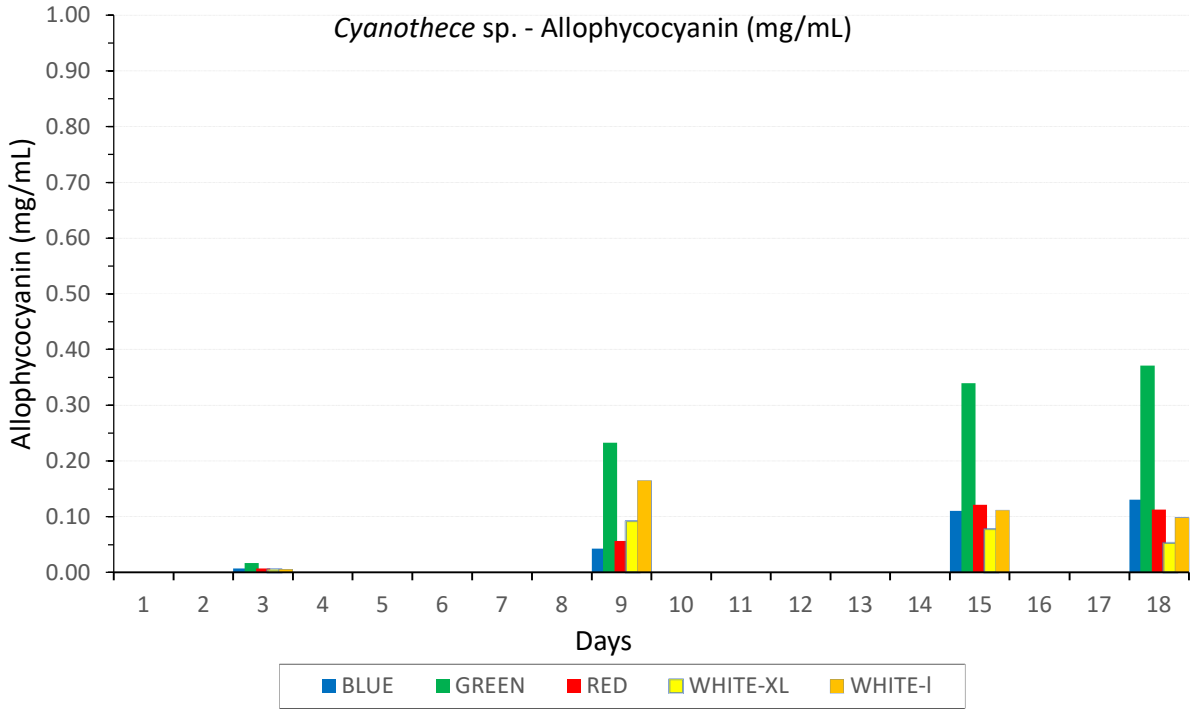

Figure 14. The development of allophycocyanin content in mg/mL along the culture period of *Cyanothece* sp.

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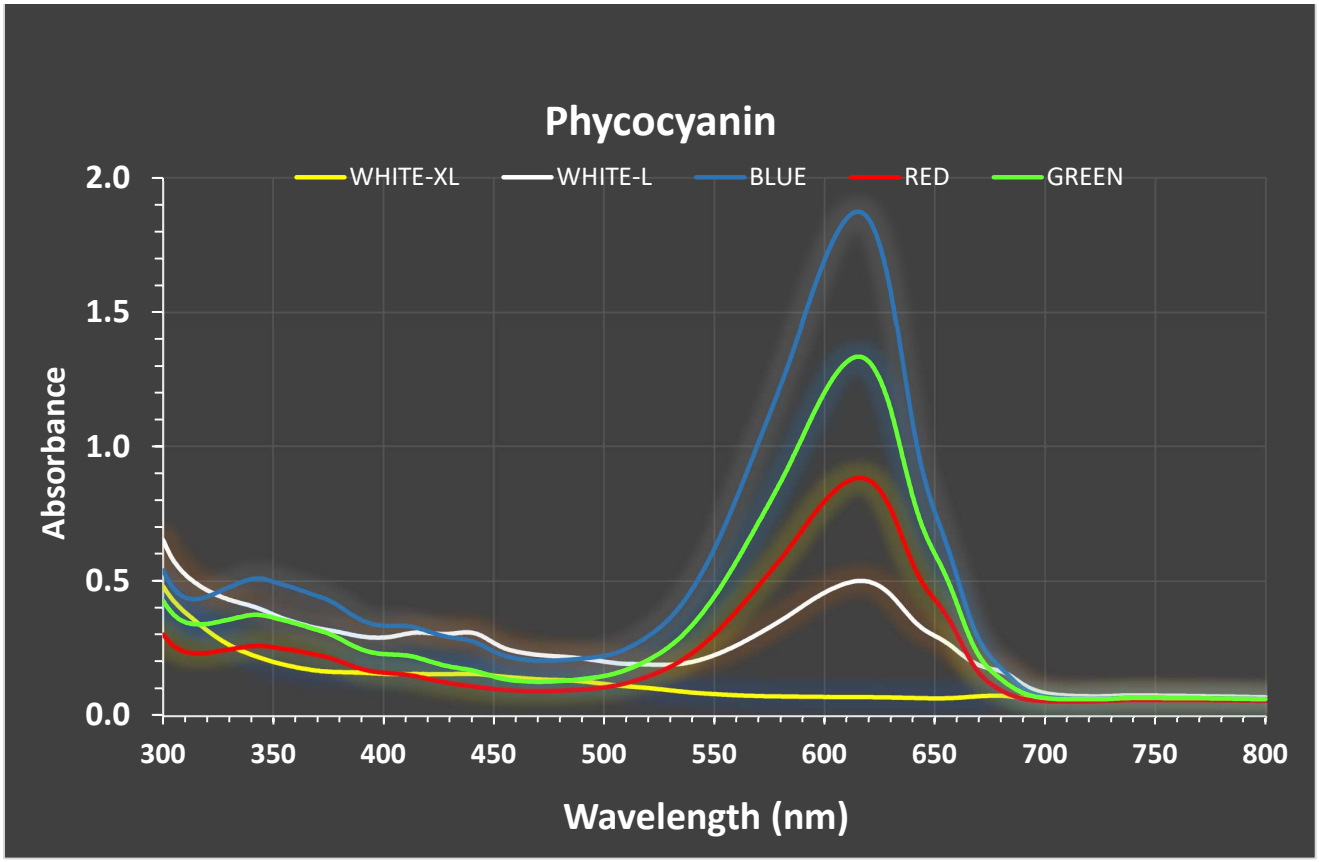

Figure 15. An array of absorption spectra of phycocyanin crude extracts on the 14th day of culture of *Phormidium* sp. under the different light treatments.

Table 1. Descriptive statistics of maximal values of different variables across light treatments in both *Phormidium* (suffix: Ph-) and *Cyanothece* (suffix: Cy-)*. W=white light, L=2000 lux, XL= 8000 lux, BLUE, GREEN, RED = colors of light used. Chl.-a= chlorophyll-a, Tot.carot.=total carotenoids, b-carot. = b-carotene, PC=phycocyanin, PE= phycoerythrin, APC= allophycocyanin, PBP= phycobiliproteins, SGR=specific growth rate, Tg = generation time.

Max. values ± SE	Ph-W-XL	Ph-W-L	Ph-BLUE	Ph-GREEN	Ph-RED	Cy-W-XL	Cy-W-L	Cy-BLUE	Cy-GREEN	Cy-RED
Chl.-a	**4.65**^{a,h}	**5.93**^{b}	**4.08**^{c}	**9.3**^{d}	**6.85**^{e}	**7.47**^{f}	**8.94**^{g,d,i}	**4.7**^{h,a}	**9.31**^{i,d,g}	**3.67**^{j}
(µg/mL)	±0.08	±0.045	±0.023	±0.07	±0.034	±0.11	±0.6	±0.078	±0.13	±0.05
Tot. carot.	**4.9**^{a}	**2.36**^{b}	**2.14**^{c}	**2.8**^{d}	**2.66**^{e}	**2.97**^{f}	**0.96**^{g}	**1.54**^{h,j}	**2.71**^{i}	**1.55**^{j,h}
(µg/mL)	±0.05	±0.006	±0.03	±0.021	±0.006	±0.025	±0.0115	±0.0261	±0.036	±0.0261
b-carot.	**0.946**^{a}	**0.64**^{b,e}	**0.494**^{c}	**0.783**^{d}	**0.637**^{e,b}					
(µg/mL)	±0.01	±0.003	±0.006	±0.005	±0.0034					
PC	**0.256**^{a}	**0.317**^{b,c}	**0.31**^{c,b}	**0.447**^{d}	**0.315**^{e,b,c,j}	**0.195**^{f}	**0.321**^{g,b,c}	**0.305**^{h,b,c,g}	**0.296**^{i}	**0.327**^{j,b,c,e,g}
(mg/mL)	±0.006	±0.0004	±0.009	±0.003	±0.0065	±0.0134	±0.025	±0.0025	±0.0147	±0.0148
PE	**0.022**^{a}	**0.1**^{b}	**0.175**^{c}	**0.147**^{d}	**0.138**^{e,i}	**0.016**^{f}	**0.02**^{g}	**0.0017**^{h}	**0.136**^{i,e}	**0.015**^{j}
(mg/mL)	±0.001	±0.0001	±0.002	±0.004	±0.003	±0.00031	±0.00038	±0.00007	±0.0014	±0.0002
APC	**0.134**^{a,h}	**0.51**^{b,i}	**0.824**^{c}	**0.78**^{d}	**0.751**^{e}	**0.092**^{f}	**0.165**^{g}	**0.131**^{h,a}	**0.371**^{i,b}	**0.121**^{j}
(mg/mL)	±0.003	±0.056	±0.008	±0.003	±0.005	±0.0015	±0.0035	±0.0005	±0.0056	±0.0008
Total PBP	**0.403**	**0.854**	**1.084**	**1.145**	**0.940**	**0.303**	**0.506**	**0.437**	**0.768**	**0.452**
(mg/mL)										
PCyield	**19.41**^{a,c,f,h}	**20.68**^{b}	**18.52**^{c,a,f,h}	**31.76**^{d}	**22.54**^{e}	**18.34**^{f,a,c}	**29.16**^{g}	**19.26**^{h,a,c,f}	**27.43**^{i}	**34.6**^{j}
(mg/g dw)	±0.44	±0.37	±0.71	±0.48	±0.47	±0.98	±1.95	±0.89	±0.98	±0.88

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PEyield (mg/g dw)	**1.09**[a] ±0.0006	**6.091**[b] ±0.005	**10.62**[c] ±0.11	**10.44**[d] ±0.005	**10.87**[e] ±0.057	**1.16**[f] ±0.075	**1.83**[g] ±0.078	**0.122**[h] ±0.0015	**12.07**[i] ±1.031	**0.151**[j] ±0.0137
Chl.: Tot. carot.	**2.2**	**3.99**	**4.18**	**5.44**	**5.04**	**4.1**	**15.24**	**3.04**	**3.9**	**3.01**
Tot. car.:b-car.	**4.44**	**3.64**	**4.32**	**3.58**	**4.18**					
PBP : Chl.	**106**	**173**	**616**	**428**	**620**	**47**	**62**	**109**	**187**	**109**
PBP :Tot. carot.	**233**	**690**	**1735**	**2096**	**3028**	**190**	**938**	**331**	**632**	**371**
PC : PE	**20**	**18.1**	**40.3**	**39.3**	**8.4**	**150**	**174**	**304**	**15**	**67**
PC : APC	**2.0**	**2.0**	**1.4**	**1.6**	**1.7**	**2.12**	**2.12**	**2.77**	**2.71**	**3.51**
SGR (doubl./d)	**0.122**[a] ±0.012	**0.055**[b,g] ±0.0021	**0.118**[c,e] ±0.0005	**0.131**[d] ±0.0004	**0.118**[e,c] ±0.0003	**0.098**[f] ±0.001	**0.05**[g,b] ±0.0057	**0.034**[h] ±0.003	**0.022**[i] ±0.0022	**0.028**[j] ±0.0028
Tg (days)	**6.14**	**12.51**	**6.32**	**5.7**	**6.34**	**7.1**	**13.87**	**20.3**	**24.6**	**31**

- *Values are means ± S.E. (standard error) of 3 measurements. The different superscripts indicate a statistically significant difference at the 0.05 level of confidence (statistical processing with ANOVA and then pair-wise comparison with Tukey's test). Where there is a second superscript it means statistically equal to the value of the condition of the corresponding letter.

3. Discussion

As cyanobacterial cultures are greatly affected in terms of output of biomass and pigments (let aside other constituents) by the conditions prevailing during the culture period, and light is of paramount importance [41-43], the proper adjustment of light quantity and quality is perhaps the most crucial task to be accomplished. As indicated in [44], a sound approach for optimizing production of value-added algal products is to establish first realistically attainable conditions of energy costly temperature range. Only if the species under investigation can grow well in the not so high temperature range of ~20-23 ^{0}C instead of the extreme 30-36 ^{o}C frequently encountered in culture optimization studies (e.g. [35, 45, 46]) there are real perspectives for the economics of a culture aiming to maximize pigments output by testing light manipulation. In this respect the present work follows that of [19] in which *Phormidium* sp. a filamentous non diazotrophic species was found as a very promising species for mass production in moderate temperature regime (~22 ^{o}C). Now, *Phormidium* and *Cyanothece* sp. a coccoid single-celled diazotrophic species [47] were tested under different light regimes as both in preliminary cultures exhibited high growth rates. Both of them exhibited at late stages of culture intense chromatic adaptation a feature much pronounced in cyanobacteria [48]. Their response to different colors of light in combination with the alterations of their biliproteins content during growth that finally is manifested in the turning of their green coloration in the exponential phase to yellow-green in the stationary phase with various hues of green in between, leaves no doubt that both belong to Group II cyanobacteria according to [49]. Their high content in phycoerythrin at late stages (especially in *Cyanothece*) which is very flexible biliprotein in adaptation to changing environmental conditions (especially light) [50], imparts to them their change of coloration and broadens the range of light-harvesting capability in the green area of the light spectrum [51, 52].

Reviewing the literature on the pigment content in cyanobacteria, the situation is perplexed and puzzling as there are highly variable and sometimes contradicted data. However, two things in general can be distilled from the overall bulk of findings. First, growth of cyanobacteria under different regimes of light quality is highly variable among species (e.g. [53-55]) and second, as there is not a fixed ratio of pigments in the cellular volume among the species, an open wide field of experimentation for optimizing yields exists. The first prerequisite of optimization is the satisfactory production of enough biomass. Without producing enough algal mass the quest for maximizing production of pigments is meaningless. In this respect the role of light is catalytic and we need to compromise between two opposite directions of adjusting light intensity. This is because cyanobacteria

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grow faster in continuous high light intensities [8, 19] and the findings of the present study support strongly this for both *Phormidium* with 1.52 g dw/L in high light of 8000 lux as compared to 1 g/L in low light of 2000 lux and *Cyanothece* with 1.62 and 1.02 g/L respectively. But on the other hand, cyanobacteria can cope effectively with low light intensities due to their low energy requirement for maintenance and their unique flexible pigment composition that by broadening the spectrum of available low light can balance the photon energy between PS I and PS II [46, 56]. As biliproteins constitute 20-40% of total proteins in cyanobacteria [52, 57] and outnumber greatly chlorophyll and carotenoids, it comes of no surprise their profound increase at low light intensities for extracting the maximum of photons available [45, 58, 59]. This was recorded in all treatments of low white light for both species in the present study and for all light colors used and proves that the underlying mechanism is not only the wavelength of the light that triggers a particular cellular response but the intensity per se as the intensities of the colored light used were less than 3000 lux. Furthermore, we recorded an increase in the amount of biliproteins as the culture growth period lengthened. Due to their high nitrogen amount biliprotein synthesis is directly linked and depends upon the cell growth status, the more advanced the culture the more the biliprotein content increases [46, 60, 61]. There are however some studies that report increase phycocyanin production induced by high illumination [35, 62] but we feel them as needing further verification. In the present study and especially in the case of *Phormidium*, beyond the middle of the exponential phase (7th day) the low intensity colored lights induced a profound increase of biliproteins in comparison with the respective amounts in low white light (2000 lux) and the respective ones were tremendously diminished in high white light (8000 lux) in corroboration to the similar findings of [63] for *Synechococcus* concerning phycoerythrin. Our results in phycocyanin and phycoerythrin production yield in *Phormidium* at low illumination of 37 and 5.7 mg/g dw and 28 and 1.9 mg/g dw respectively for *Cyanothece*, are quite close to those for *Nostoc* sp. [46] where at 2000 lux of white light and at high temperature of ~ 30 ^{o}C total biliproteins accounted for 43 mg/g dw. In another study for optimization of phycocyanin production in *Phormidium ceylanicum* [64] at ~27 ^{o}C and 6500 lux of white light, the amount obtained was 0.32 mg/mL and enhanced to 0.73 mg/mL after optimization with addition of certain chemicals. Also, at high temperature (28 ^{o}C) and at 3 light intensities of 4000, 6000 and 8000 lux white light in *Oscillatoria* sp. [57], the results indicated a rather uniform output between light intensities of phycoerythrin (~0.05 mg/mL), phycocyanin (~0.24 mg/mL) and allophycocyanin (~0.23 mg/mL) after 15 days of cultivation and with a biomass specific growth rate of 0.228 doubl./day. In another study [65], *Spirulina platensis* in mixotrophic culture produced 2.38 g/L biomass and 0.261 mg/mL phycocyanin while under continuous supplementation of CO_2 reached 7.27 g/L and 1.22 mg/mL biomass and phycocyanin respectively [66]. Quite similar results were recorded for *Anabaena*, *Nostoc* and *Spirulina* species [67]. Our results for maximum phycocyanin content of 0.32 mg/mL for both *Phormidium* and *Cyanothece* after 14 and 7 days respectively at ~19 ^{o}C and 2000 lux and without addition of CO_2 are quite in accordance with them and much encouraging for potential of further maximization using solely white light.

It is very important, mainly from the production perspective, to monitor the cellular content of pigments in order to decide when it is best to collect the biomass and extract them. The sooner the desired pigment content peaks, the better for the economics of culture. Usually, the peak occurs somewhere in the exponential phase of growth between the 10th and 14th day (e.g. [57]) but it can be protracted for more than 30 days (e.g. [68]). From data of the various studies in the literature no clear pattern can be drawn. The production of all kind of pigments being them chlorophyll, carotenoids or biliproteins exhibits great variation on the time that they are maximized among species, the particular pigment measured, conditions of culture and equipment used. In the present study the culture period lasted 18 days for *Cyanothece* and 21 days for *Phormidium* and we decided to end the cultures on these days as the concentration of most biliproteins had already peaked some days previously and in spite of the impressive fact that chlorophyll and carotenoids still were increasing. It is also noteworthy that among the biliproteins, allophycocyanin

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peaked earlier than phycocyanin and phycoerythrin in the culture of *Phormidium* and although several studies refer to allophycocyanin (and also phycoerythrin) as the least in cellular content compared to phycocyanin [14, 17, 69], while in [70] phycocyanin and allophycocyanin are the main biliproteins in *Phormidium*, *Spirulina* and *Lyngbya*, in the present study the allophycocyanin content exceeded that of phycocyanin in *Phormidium* and was close to half the concentration of phycocyanin in *Cyanothece* (all the cases refer to white light of 2000 lux). This finding implies that diversification of photosynthetic pigments content among cyanobacterial species is a fact, at least between filamentous and single-celled (coccoid form) of them. It is assumably one of the factors that contribute through the breakdown of nitrogen rich chlorophyll and biliproteins during the late phases of culture in high illumination where only carotenoids which are non-nitrogenous are retained and as a consequence the coloration of the culture becomes yellowish [71].

Considering the influence of monochromatic light on the composition of phycobilisomes of different species of cyanobacteria that is manifested as complementary chromatic adaptation purposed to maximize capture of available light [8, 39], the results of the present study revealed that this phenomenon is much intensified in both *Phormidium* and *Cyanothece* but with great differences among them not only on the biliprotein amount but on the biomass produced as well. In *Phormidium* by far the greatest amount of biomass was steadily induced by white light of 8000 lux since the 14th day (1.45 g dw/L) peaking on the 21st day (2.15 g dw/L), while the relevant values of colored cultures were half of the above. Completely opposite is the situation on the colored light induced fluctuation of biliprotein content along the culture period where after the 7th day all colors used gave far more greater amounts of biliproteins than those of low or high white light. In *Cyanothece* this phenomenon was less pronounced than in *Phormidium* in the case of biomass produced by white light because colored light and especially green reached similar to white light values of ~1.68 g dw/L on the 18th day. But considering the effect of colors on the biliprotein content, *Cyanothece* exhibited a profound supremacy of biliprotein production in every color used over white light from the 9th day. Contrary to some reports that consider blue light as less photosynthetically efficient for cyanobacterial growth compared to eukaryotic algae [31, 72], in our study blue light greatly enhanced phycoerythrin and allophycocyanin production (and by no means negligibly, phycocyanin) in *Phormidium*, while in *Cyanothece* induced the maximum phycocyanin production (similarly with red and green) while phycoerythrin and allophycocyanin were by far maximized under green light. Other studies however indicate blue as the most suitable light for phycocyanin concentration [29, 36, 37, 72] but not for promoting biomass and this is partly in accordance with our findings for *Cyanothece*. Similar to the profound accumulation of phycoerythrin under green light in *Cyanothece* was recorded in *Fremyella diplosiphon* [73] while red light induced phycocyanin maximization. Red light induced enhanced phycocyanin production in both *Phormidium* and *Cyanothece* but not to a lesser degree to blue light as in *Synechococcus* [28].

Reviewing the literature on the effect of light color on growth and biliprotein content among cyanobacterial cultures there are so many perplexed data that no solid conclusion can be reached, nor is it possible to manifest a guide-line for maximizing production of either biomass or (mainly) biliproteins. As an example of this stands the following. Green light was found most suitable for phycocyanin and allophycocyanin in *Nostoc* [46] and for phycoerythrin in *Fremyella diplosiphon, Calothrix, Gloeotrichia* and *Pseudoanabaena* [55, 74, 75] but not suitable for growth of *Synechococcus* [53]. Green light along with blue was found also to maximize total photosynthetic pigments (chlorophyll included) in marine *Synechococcus* sp. [76]. Blue light enhances phycocyanin to chlorophyll ratio in *Synechocystis* sp. [72], biomass and phycocyanin in *Arthrospira platensis* along with red light [31], phycocyanin in *Synechococcus* sp. [8], biomass in *Pseudoanabaena* [55], total biliproteins in *Anabaena ambigua* [41], *Westellopsis iyengarii* [77], *Spirulina fussiformis* [54] and *Nostoc sphaeroides* [78] and phycoerythrin in *Calothrix elenkinii* [75]. Minimum synthesis of phycocyanin and allophycocyanin was however reported in *Nostoc* sp. [46]. Red light enhances phycocyanin synthesis in *Calothrix elenkinii* [75], *Fremyella diplosiphon*, *Calothrix* sp.

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and *Spirulina platensis* [28, 50, 74] and *Pseudoanabaena* sp. [55], while inhibiting phycoerythrin synthesis in *Gloeotrichia* sp. and *Nostoc* sp. [74], maximum growth on *Synechococcus* sp. [53] and total biliproteins in *Anacystis nidulans* [79], *Synechococcus* sp. [58], *Calothrix* sp. [80] and *Nostoc* spp. [81, 82].

Concerning the universally present in all photosynthetic algae chlorophyll-a and carotenoids, our study found that green light greatly enhanced the chlorophyll content in *Phormidium* after 14 days of culture onward and the same occurred in *Cyanothece* but to a lesser degree. In *Cyanothece* white light rivaled green light in enhancing chlorophyll content and this occurred in a lesser degree in *Phormidium*. Chlorophyll content was more in white light of low intensity as compared to high intensity in the case of *Phormidium* and this is in accordance with the similar trend found in *Anabaena ambigua* [41]. White light of high intensity (8000 lux) by far exceeded all colors in enhancing total carotenoids and b-carotene in *Phormidium* and the same but to a lesser degree occurred in *Cyanothece*. Very limited information can be found in the literature about the effect of colored light on the cyanobacterial chlorophyll and carotenoids content as the majority of studies are focused on biliproteins. Even so, an indirect clue of the variation of chlorophyll content among cyanobacteria is the ratio of biliproteins to chlorophyll which depends upon the color of light in which they are growing [71]. In this respect the by far biggest values among all colors recorded in blue and red colors in *Phormidium* (~600:1) reflect the compensatory adjustment of cells in these wavelengths where chlorophylls absorb to the maximum but billiproteins cannot. Green light effected maximum chlorophyll content in *Phormidium* (9.3 μg/mL or 13.75 mg/g dw) and *Cyanothece* (11.55 mg/g dw) from the 14th day onwards quite similar to those reported for *Westelliopsis prolifica, Nostoc muscorum, Aulosira fertilissima, Westelliopsis fertilissima, Tolypothrix tenuis* and *Anabaena variabilis* [83] but differing from them in the respect that they did not mention neither kind of light nor conditions of culture used and in our cultures there was not a steep drop of values after the 14th day as they reported. Similar values of chlorophyll to ours are also reported for *Limnothrix redekei* and *Planktothrix agardhii* (9.95 and 6.07 mg/g dw respectively) using white light [84], while much reduced values of 1.6 and 2.0 mg/g dw of chlorophyll-a and chlorophyll-b respectively in green light were given for *Arthrospira platensis* by [85] but their value of chlorophyll-b should be rejected as cyanobacteria do not contain this pigment. Cultures of *Nostoc calcicola* [86] at 25 ^{o}C and under low intensity white light (1000-3000 lux), gave 0.8-1.1 g dw/L, 3-4 mg/g dw chlorophyll-a and 6-8 mg/g dw total carotenoids but the narrow range of light intensity they used and the absence of implementation of colored light does not allow for a sound conclusion about the influence on pigments content either of the intensity of light or of its color. Total carotenoids in the present study in *Phormidium* were much enhanced by high intensity white light (4.2 μg/mL on the 21st day) almost double of that for low intensity (2.36 μg/mL) and close to 3 μg/mL for green and red light. Lower values as compared to *Phormidium* were recorded in *Cyanothece* in high white light (2.97 μg/mL on the 18th day) and 2.7 μg/mL in green light. Transforming these values in mg/g dw, our results of total carotenoids with values of 2.3 – 3.1 mg/g dw for colored light and ~2.0 mg/g dw for white light in *Phormidium* and about 20% lower in *Cyanothece* are much higher than the respective ones for 5 cyanobacteria recorded by [83]. The ratio of total carotenoids to b-carotene in *Phormidium* kept quite uniform in all light treatments between 3:1 to 4.4:1 with maximum concentrations b-carotene in high white light (0.95 mg/L) followed by green light (0.78 mg/L). Although we did not find relative studies in the literature concerning cyanobacterial b-carotene content, we assume that our results are very promising in terms of production as compared to the notoriously known for accumulation b-carotene green algae *Dunaliella salina,* which under very intense light (~75,000 lux) can yield ~1-3 mg/L [87].

4. Materials and Methods

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The strains of *Phormidium* sp. and *Cyanothece* sp. used ensued from the hypersaline ponds of the nearby saltworks of Messolonghi. After serial dilutions and repeated renovations of cultures in 40 ppt salinity, eventually they were isolated as monospecific cultures to be used thereafter. The experiment took place at 18-19.5 ^{o}C in a 18.000 BTU air-conditioned laboratory. 2-L autoclaved glass conical Erlenmeyer flasks were used in triplicate for each light filled up to the 1.85-L mark with sterilized sea water of 40 ppt salinity enriched with nutrients according to Walne's formula. At start of the batch culture the inoculum of each cyanobacterium was 150 mL (to 1850 mL of 40 ppt water) taken from the mother cultures that were maintained in exponential phase at 40ppt salinity. In every case of salinity adjustments, this was done with sterilized distilled water. Light used was of two kinds. White light emitted by an array of 20 Watt 1600 lm LED lamps and colored light by blue, green and red LED (BarisLight neon silicon strip 9W/m, 400Lm/m) each housed in a properly constructed box the interior of which did not receive other light than the assigned color (Figure 16). In the case of white light the desired intensities of 2000 and 8000 lux were attained by properly placing the vessels at the proper distance from the lamps. Intensity in lux was measured at the middle of the outer surface of the vessels by means of a luxmeter (BIOBLOCK LX-101). In the case of colored lights the light intensities measured at the surface of the vessels positioned 1cm away from the LED stripes were 680, 2800, 3000 lux for red, green and blue LEDs respectively. Illumination in all cases was continuous (24hL:0hD). The cultures were kept in suspension and received CO_2 by means of coarse bubbling through a 2-mL glass pipettes (one in every vessel) supplying air at a rate of half culture volume/min. The pipettes were connected through sterilized plastic hoses to the 0.45 μm filtered central air supply system fed by a blower.

Culture progress was monitored spectrophotometrically by measuring the absorbance of a small sample at 750 nm using a Shimadzu UVmini-1240 UV-visible spectrophotometer. The measurements were transformed to dry biomass per volume (g/L) using a proper equation derived from previous calibration curves of dry weight vs absorbance using dense cultures of each species with serial dilutions and additionally more couples of values from culture samples taken every 3 days.

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 to 100 ^{o}C for 2 hours. After that they were weighted to the 4th decimal and the dry weight was calculated as g/L. In every daily sample taken from the culture, the pH was also measured using a digital pH-meter (HACH-HQ30d-flexi). The maximum specific growth rate (SGR) was estimated during the exponential phase of the culture's growth curve (from the 3nd till the 16th day in *Phormidium*, from the 3nd till the 15th day in *Cyanothece*) using the equation:

$$SGR= (\ln C_2 - \ln C_1)/(t_2-t_1)$$

where: C_1 and C_2 stand for g D.W. of cells at days t_1 and t_2 respectively (t2>t1).

From the above equation the generation time T_g of the culture was calculated as days for doublication using the formula:

$$T_g = 0.6931/SGR$$

Chlorophyll-a and total carotenoids were extracted from centrifuged culture samples with absolute methanol and their concentration (μg/mL) was calculated spectrophotometrically using the equations [88]:

$$\text{chl-a} = 12.9447\ (A665-A720)$$

$$\text{total carot.} = [1000\ (A470-A720)-2.86\ \text{chl-a}]\ /221$$

Beta-carotene (b-carotene) was extracted using a 2:4 mixture of hexane/methanol using a slightly modified recipe of [89] and its concentration (μg/mL) was calculated spectrophotometrically using the equation:

$$\text{b} - \text{carot} = \left(\frac{A453 - A665}{3{,}91}\right) \text{ x } 3{,}657 \text{ x } 2 \text{ x D}$$

With (A453−A665/3.91): absorbance of b-carotene corrected for chlorophyll

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contamination, 3.657: calibration factor derived from HPLC analysis of b-carotene concentration, 2: amount of mL hexane used, D: the dilution factor for hexane used for spectrophotometric measurement in cases when the initial absorbance exceeds the value of 2 [89].

Phycocyanin (PC), allophycocyanin (APC) and phycoerythrin (PE) content were extracted by freezing (-20 °C) for 24 hours a concentrated known amount of 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 darkness. The freezing/thawing procedure was repeated for two consecutive days. The sample's slurry was then centrifuged at 3000 rpm for 5 minutes and the supernatant was measured spectrophotometrically to calculate the amount of the pigments (in mg/mL) using the equations [90]:

$$PC = \frac{A_{615} - 0.474\,A_{652}}{5.34}$$

$$PE = \frac{A_{562} - [(2.41PC) - (0.849APC)}{9.62}$$

$$APC = \frac{A_{652} - 0.208\,A_{615}}{5.09}$$

From the above equations the yield in phycocyanin in mg PC/g dry weight was calculated using the equation [91]:

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

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

The purity of phycocyanin was recorded as the ratio of absorbance of 620 to 280 nm [92].

Statistical treatment of the different variables was done with ANOVA and Tukey's test for comparison of the means at the 0.05 level of significance using the free PAST3 software.

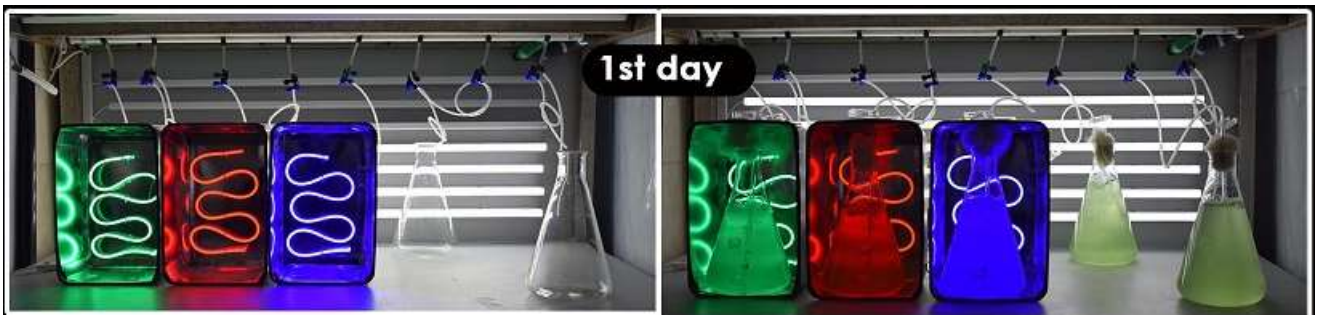

Figure 16. A set of the colors configuration setup used in the experimentation before (left) and after (right) the start of the culture.

5. Conclusions

In a quite low temperature of 18-19.5 °C which can be attained everywhere, the filamentous *Phormidium* sp. and the coccoid *Cyanothece* sp. were batch cultured in 2-L vessels under illumination by LED lights in 5 treatments. White light of 8000 lux was by far the best for biomass production in *Phormidium* yielding on the 21st day 2.1 g dw/L followed by white light of 2000 lux (1.3 g/L) and green light (1.13 g/L) while blue and red light produced 0.9 g/L. Biomass yields were also high in *Cyanothece* with 1.47 g/L for white high light (and half of that for low light), followed by green light (1.25 g/L) while blue and green produced 0.6-0.66 g/L. These biomass yields stand among the higher ones in the

literature for cyanobacteria and we assume that can be increased further as in most studies similar values were attained at significantly higher temperatures of ~28-35 ^{o}C. From the results concerning maximization of biomass production, the use of white light of at least 8000 lux intensity is recommended.

Under white light of low intensity (2000 lux) chlorophyll-a production was enhanced in both species with higher values in *Cyamothece* followed by green light while in *Phormidium* green light enhanced to the maximum degree compared to all other colors chlorophyll-a production. Total carotenoids (and b-carotene in *Phormidium*) were by far maximized under intense high light (8000 lux) followed by green light.

Phycobiliproteins production presented a quite perplexed situation among the two species. In *Cyanothece* all colors along with white low light enhanced phycocyanin production while in *Phormidium* green light produced the most. Phycoerythrin was by far much enhanced by blue light late in the culture of *Phormidium* while in *Cyanothece* it was the green light which overwhelmingly among all other colors produced the maximum of phycoerythrin. Allophycocyanin was maximized by green color in *Cyanothece* and by blue, red and green in *Phormidium*. Colored light enhanced also purity of phycocyanin over white light with values of greater than 1.0 (0.2-0.7 for white light) with blue light having the most beneficial effect followed by green and red. Overall production of >30 mg/g dw phycocyanin and >12 mg/g dw phycoerythrin can easily be produced by culturing both species using the appropriate colored light and these yields can probably be much increased using higher than 19 ^{o}C temperature, addition of pure CO_2, addition of special nutrients and photobioreactor technology.

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