


Article

# Culture Growth of the Cyanobacterium *Phormidium* sp. in Various Salinity and Light Regimes and Their Influence on Its Phycocyanin and Other Pigments Content

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**Abstract:** A strain of the filamentous non N-fixing cyanobacterium *Phormidium* sp. isolated from the Messolonghi (W. Greece) saltworks, was cultured in the laboratory at six different combinations of salinity (20–40–60 ppt) and illumination (low–2000 lux and high–8000 lux). At salinities of 60 and 40 ppt and in high illumination (XL–8000 lux), the growth rate ( $\mu_{max}$ ) presented the highest values (0.491 and 0.401, respectively) compared to the corresponding at 20 ppt (0.203). In general and at all salinities, the higher illumination (XL) gave the highest growth rates and shorter duplication time (tg) in comparison to the lower illumination (L). On the contrary, phycocyanin, phycoerythrin and allophycocyanin production was extremely increased in the lower illumination (L) in all salinities, from ~14 fold at 40 and 60 ppt to 269 fold at 20 ppt of those corresponding to higher illumination (XL). Similar analogies were also recorded for the other two billiproteins. Chlorophyll-a content was also higher in lower illumination at all salinities in contrast to total carotenoids that did not exhibit such a pattern. The high growth rate and high phycocyanin content along with the rapid sedimentation of its cultured biomass can set this marine *Phormidium* species as a promising candidate for mass culture.

**Keywords:** cyanobacteria; *Phormidium*; culture growth; light; salinity; phycocyanin; pigments



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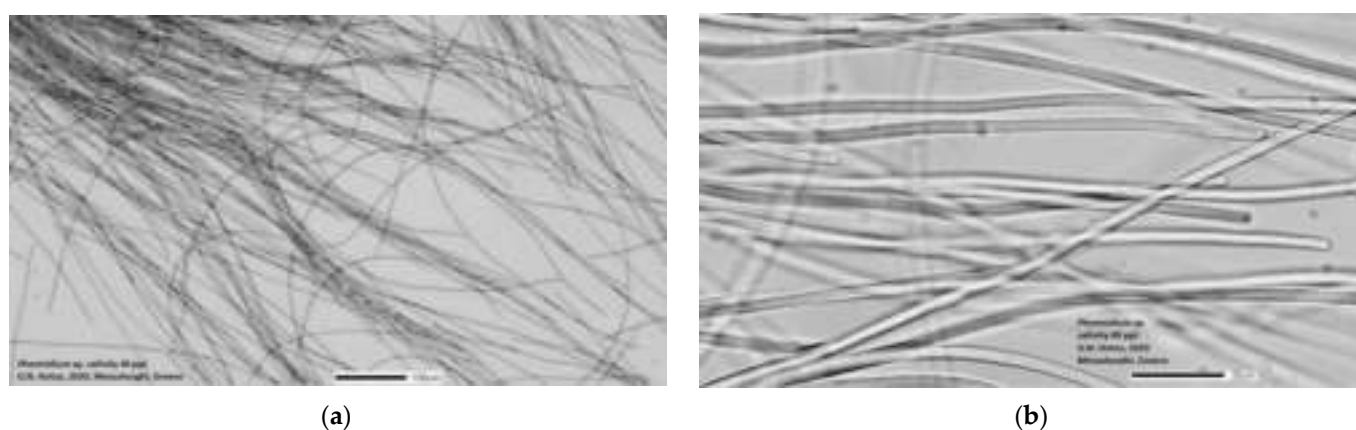
## 1. Introduction

Cultures of microalgae have long ago been established as a promising industry in terms of producing various value-added products, such as aquaculture feedstuff, bio-fuels, healthy food pills, antioxidants, pharmaceuticals, etc., to name but a few [1,2]. The catalogue of cultured species is continuously expanded as novel species reveal advantages over others in the production yield and the economic feasibility of the culture methods employed. A huge bibliography already exists supporting the information needed in order to maximize production of the cultured species, but a lot of issues still exist even for the well-established culture techniques, as the whole set of parameters of culture is receptive for improvement. This is particularly true for the least-studied species and novel species.

Among microalgae, cyanobacteria [3] occupy a large portion of the cultured algae mainly due to the tremendous expansion of the culture of the filamentous genus *Arthrospira* (*Spirulina*) [4] that has successfully been proven to be the “Holy Grail” for the worldwide production of a demanding healthy food market [5]. The production of *Spirulina* paved the way for expanding the spectrum of other potential mass cultured cyanobacteria of similar thallus morphology that can be to the same degree productive and can exploit other than freshwater (which is the usual case for *Spirulina*) waters, such as brackish, seawater or even hyper-saline.

For microalgae to be effective for production, some considerations must be satisfied in order to comply for the demand of a high yield: first, a satisfactory dry mass yield per unit volume or weight, second, high levels of valuable constituents (proteins, lipids, pigments, etc.) and third (but not last), ease of collection of the biomass produced. While the harvesting of the biomass remains a bottleneck in terms of lowering the cost of production

for the unicellular microalgae, this is not the case for filamentous species such as *Spirulina*, because its filaments tend to clump and although not a fast settler, and thus, they are easily mass gathered to be further processed. However, *Spirulina* culture has certain drawbacks as well, concerning the prerequisites for its mass production, which are: fresh or slightly brackish water of a very high pH and high temperatures. The algae culture industry cannot be confined in freshwater only, as an enormous area of the planet offers great advantages of using sites that can be supplied with water of various salinities for production of microalgae without depriving crops from irrigation water. There are other cyanobacterial species that also belong to the same order (Oscillatoriales) as *Spirulina* that are of filamentous nature and can thrive in salt water. One of them, *Phormidium* sp. (Figure 1), has been isolated [6] from the hypersaline waters (~75 ppt) of the Messolonghi saltworks (W. Greece) and proved to be very sturdy in culture conditions, producing a significant amount of phycocyanin (Figure 2).



**Figure 1.** Filaments of *Phormidium* sp. from culture: (a) Mass of filaments at 100× magnification; (b) Closer look at 630× magnification of the filaments of *Phormidium* sp.



**Figure 2.** Samples of extracted phycocyanin after freezing and thawing of *Phormidium* sp. mass.

Phycocyanin is a characteristic water-soluble phycobilliprotein that abounds in cyanobacteria, and in combination with phycoerythrin and allophycocyanin, comprises the highly efficient photon-gathering apparatus of the phycobillosomes [7]. It is blue colored and gives the characteristic bluish tint in cyanobacteria. Phycocyanin is highly valued in the market, as it is a potent antioxidant for human health and a natural dye in the food industry to name but a few of its uses. In that sense, exploring the culture variables of a sturdy cyanobacterial species that can be cultured in various salinities, produces a significant amount of mass, settles easily and fast enough to facilitate its collection and produces significant amount of phycocyanin deserves our attention and experimentation in order to standardize the optimum protocols of culture. This is the case of the aforementioned *Phormidium* sp.

## 2. Materials and Methods

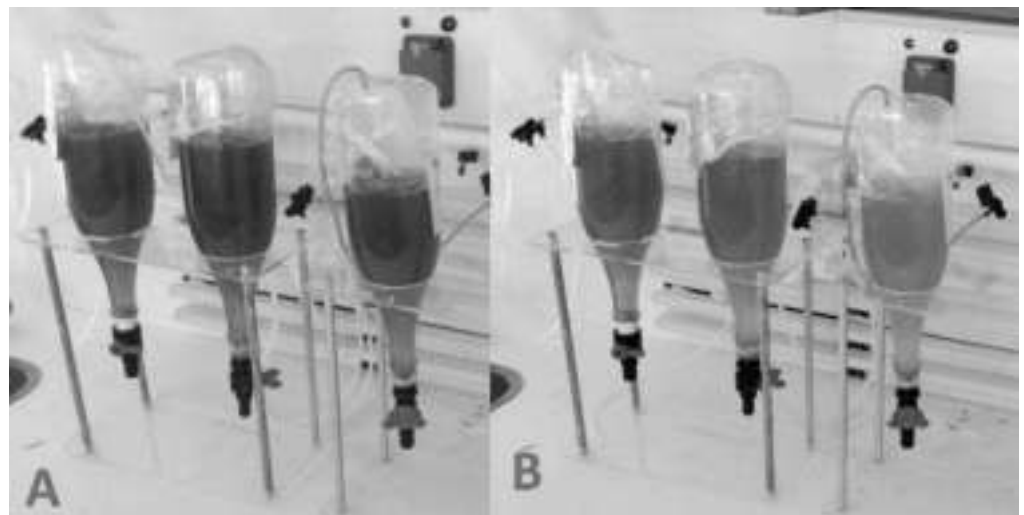
The strain of *Phormidium* sp. under experimentation was one of the species that ensued from a screening survey in local lagoons and saltworks of W. Greece. Through regular renovations of cultures, *Phormidium* sp. dominated the culture vessel, and after some more renovations, its monoculture became available in 40 ppt salinity-enriched water.

The experimentation consisted of batch cultures in 2-L conical glass Erlenmeyer flasks were filled with 2 L saline water of three selected salinities, 20, 40 and 60 ppt, each in triplicate, receiving light intensity of 8000 lux from 20 watt 1600 lm LED lamps, measured at the middle of the outer surface of the vessel (Lux meter BIOBLOCK LX-101). Another set of the same arrangement was set in such a distance from the lamps so as to receive 2000 lux of light. The timer-controlled photoperiod was kept for both sets at 16 hL:8 hD throughout the experiment.

Prior to start, the proper amount of clean seawater (40 ppt) was autoclaved, after which its salinity was adjusted properly either by dilution with sterilized distilled water (to the 20 ppt level) or by diluting sterilized artificial salt (Instant Ocean<sup>®</sup>, Blacksburg, VA, USA) in order to obtain a salinity of 60 ppt. The water was enriched with minerals contained in prepared Walne's media. The suspension of the filaments and the supply of CO<sub>2</sub> were accomplished using coarse bubbling through 2-mL glass pipettes (one in every vessel with a supply of one culture volume/min) connected through sterilized plastic hoses to the 0.45 µm filtered central air supply system fed by a blower. The temperature was maintained at 21–22.5 °C by a 18,000 BTU air condition.

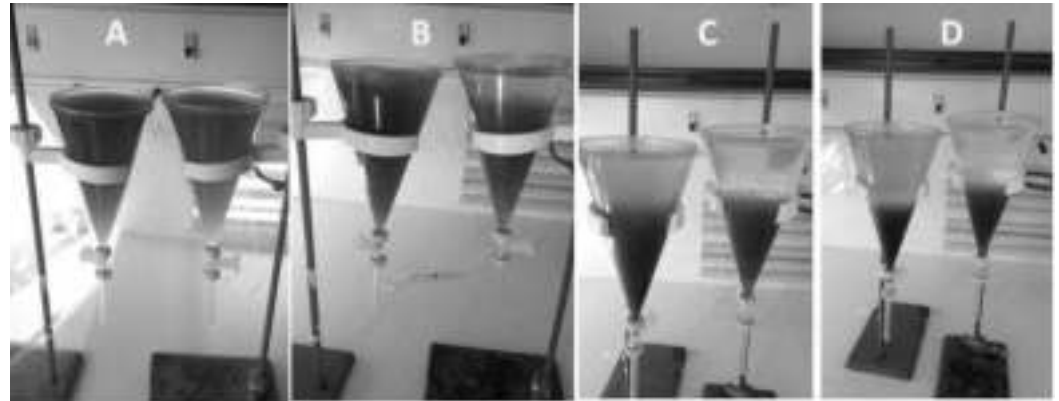
The progress of the cultures was monitored by daily measurements of optical density at 750 nm of the medium in each vessel using a visible-UV spectrophotometer (Shimadzu UVmini-1240 UV-visible, also used for pigment measurements) and transformation of the absorption values to values of g dry weight/L [8]. This was accomplished by using the proper equation from the calibration curve of weight vs. absorbance using a dense culture of *Phormidium* with serial dilutions and additionally more couples of values from culture samples taken every 3 days.

The cultures of *Phormidium* exhibited a remarkable phenomenon of changing color from bright green in the start, turning to dark yellow around the 12th–14th day, with a transient yellow-olive green tint 2–3 days before the complete change (Figure 3). This proved to be a stable phenomenon occurring in all vessels and was additionally verified in subsequent cultures.



**Figure 3.** Coloration of *Phormidium* sp. mass at an early stage (5th day) (A) and at a late stage (15th day) (B) of its culture.

When the aeration of the culture vessel stops, the mass of the filaments rapidly settles on the bottom, creating a thick green or yellow mass (depending on the culture age). This highly important (for harvesting the algae) phenomenon is characteristically shown in Figure 4 and from start until full sedimentation takes about 1 h.



**Figure 4.** The progress of sedimentation of the filaments of *Phormidium* sp. of an early (green colored) and a late (olive-yellow) culture lasting ~1 h between stage (A–D).

The maximum specific growth rate ( $\mu_{\max}$ , day<sup>-1</sup>) was estimated during the exponential phase of the culture's growth curve using the equation:

$$\mu_{\max} = (\ln C_2 - \ln C_1) / (t_2 - t_1)$$

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

From the above equation the generation time  $t_g$  of the culture was calculated as days until duplication using the formula:

$$t_g = 0.6931 / \mu_{\max}$$

The calculation of the dry weight was made by filtering a known amount of culture through 0.45  $\mu\text{m}$  GF/C filters in a vacuum pump (Heto-SUE-3Q), washing the filter with ammonium formate and drying the filters in an oven to 100 °C for 2 h. Then, the filters were weighted to the fourth decimal and the dry weight was calculated as g/L.

The pH was daily measured by a digital pH-meter (HACH-HQ30d-flexi). The pigment chlorophyll-a (chl-a) was extracted using the solvent DMSO, following a slightly modified method of Griffiths et al. (2011) [9], and its concentration ( $\mu\text{g}/\text{mL}$ ) was calculated spectrophotometrically using the following equation:

$$\text{chl-a} = 12.47(\text{OD}_{665}) - 3.62(\text{OD}_{649})$$

Total carotenoids (carot.-total) were extracted from another sample with absolute methanol [10] as solvent and their concentration ( $\mu\text{g}/\text{mL}$ ) was calculated by the following equations [11]:

$$\text{carot.-total} = [1000 (\text{OD}_{470} - \text{OD}_{720}) - 2.86 \text{ chl-a} (\mu\text{g}/\text{mL})] / 221$$

$$\text{chl-a} (\mu\text{g}/\text{mL}) = 12\,9447 (\text{OD}_{665} - \text{OD}_{720})$$

Phycocyanin (PC), allophycocyanin (APC) and phycoerythrin (PE) contents were extracted by freezing (−20 °C), for 24 h, a concentrated known amount of cells in 0.1 M sodium phosphate buffer (pH 7.1) as solvent at a ratio of 1:10 (algae mass:solvent) and then thawing at 4 °C in darkness. The sample's slurry was then centrifuged at 3000 rpm for

5 min and the supernatant was measured spectrophotometrically to calculate the amount of the pigments (in mg/mL) using the following equations [12]:

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

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

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

From the above equations, the yield in phycocyanin in mg PC/g dry weight was calculated using the following equation [13]:

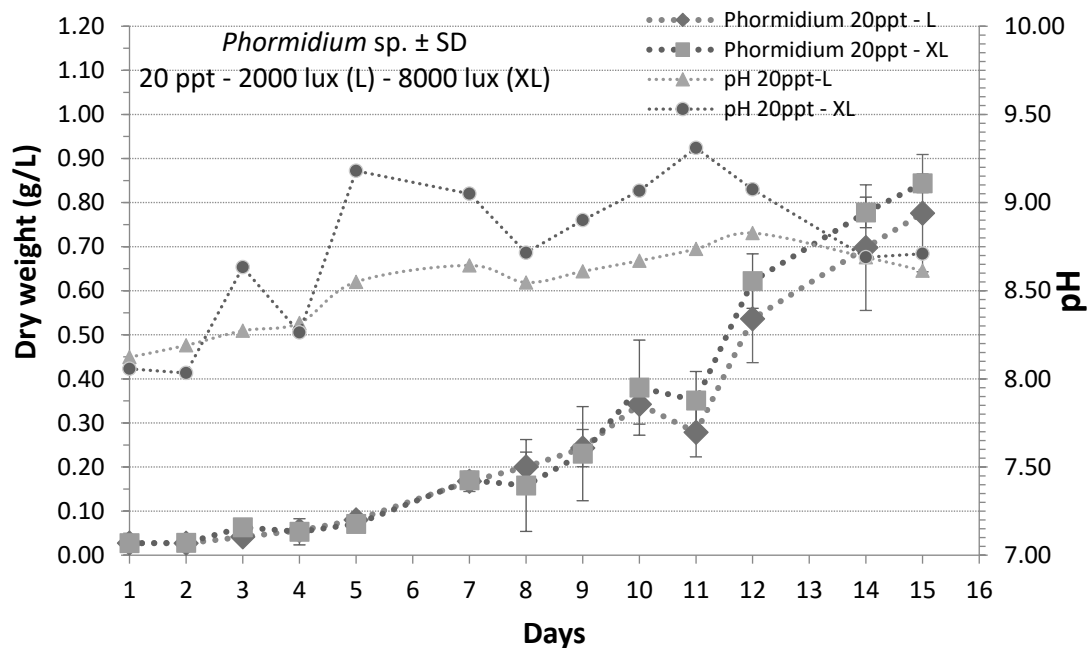
$$PC_{yield} = \frac{PC \left(\frac{mg}{mL}\right) \times V (mL)}{D.W. (g)}$$

where PC<sub>yield</sub> is the mg of phycocyanin per g algal dry weight, V is the volume of solvent used (mL) and D.W. is the grams of dry weight of the algal mass used.

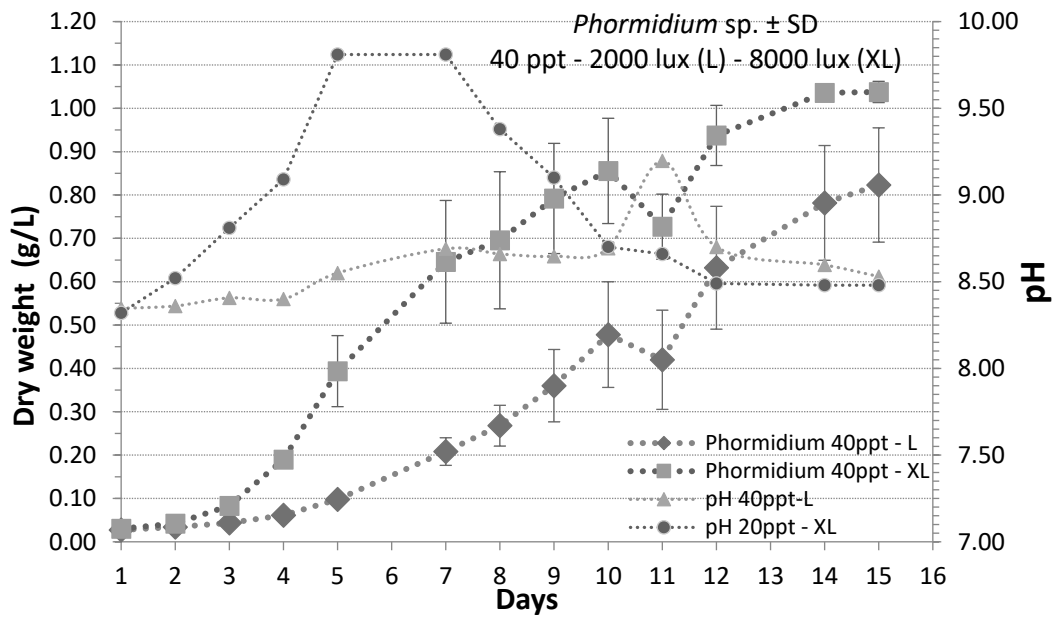
Statistical treatment of the different variables was done with ANOVA and pairwise Tukey’s test for comparison of the means at a 0.05 level of significance using the free PAST3 software.

### 3. Results

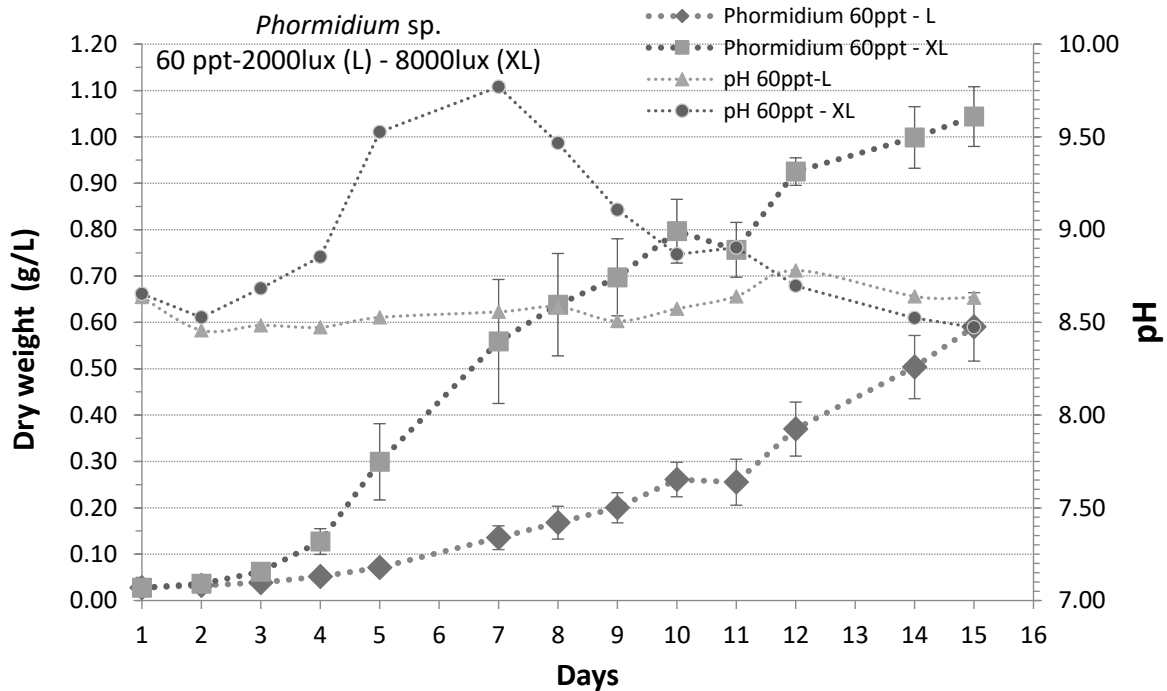
The cultures were monitored for 15 days, and Figures 5–7 depict the growth curves along with the daily pH values for each set of light-salinity treatment.



**Figure 5.** Growth curve and pH values for the period of a culture of *Phormidium sp.* at a salinity of 20 ppt in two light regimes: “high light”-“XL”-8000 lux and “low light”-“L”-2000 lux.



**Figure 6.** Growth curve and pH values for the period of a culture of *Phormidium* sp. at a salinity of 40 ppt in two light regimes: “high light”-“XL”-8000 lux and “low light”-“L”-2000 lux.



**Figure 7.** Growth curve and pH values for the period of a culture of *Phormidium* sp. at a salinity of 60 ppt in two light regimes: “high light”-“XL”-8000 lux and “low light”-“L”-2000 lux.

At a salinity of 20 ppt (Figure 5), the cultures of both light regimes presented almost an identical pattern of increase, with an initial lag period of 5 days and an exponential phase from day 5 until day 12. Then, the increase was lowered, reaching values of 0.83 g/L for XL and 0.77 g/L for L on the 15th day. The pH in the XL cultures from day 4 on was clearly kept in the high alkaline region (>9.0), higher than that of the L cultures (<9.0), and then, after the 12th day, this was reduced to almost identical values for both light regimes.

In the cultures with salinities of 40 and 60 ppt (Figures 6 and 7, respectively), the initial lag phase lasted only for 3 days (as compared to 5 days in the 20 ppt cultures) for both light regimes, and afterward, the growth curves entered the exponential phase, which was

more intense for the cultures receiving the higher level of light (XL-8000 lux), thus clearly demarcating two modes of growth curves, an elevated curve for high light (XL) and a substantially lower curve for the low light (L-2000 lux). This phenomenon was much more pronounced in the 60 ppt cultures compared to the ones of 40 ppt. Basically, growth was more retarded at low light and high salinity. On the 15th day, the XL cultures in both salinities attained values of >1.00 g/L, while those of low light (L) were at the same level as in the 20 ppt culture (~0.8 g/L) and in the relevant one of the 60 ppt culture, even lower (~0.5 g/L).

The pH values in the cultures of 40 and 60 ppt (Figures 6 and 7, respectively) exhibited a similar pattern to the cultures in 20 ppt, but here, the phenomenon was much more pronounced, with an abrupt increase towards the high alkaline area already after the 2nd day with the values of the XL cultures reaching the level of pH = 9.5 on the 7th day. This gradually subsided to values around 8.5, which characterized the cultures of low light (L) throughout the culture period.

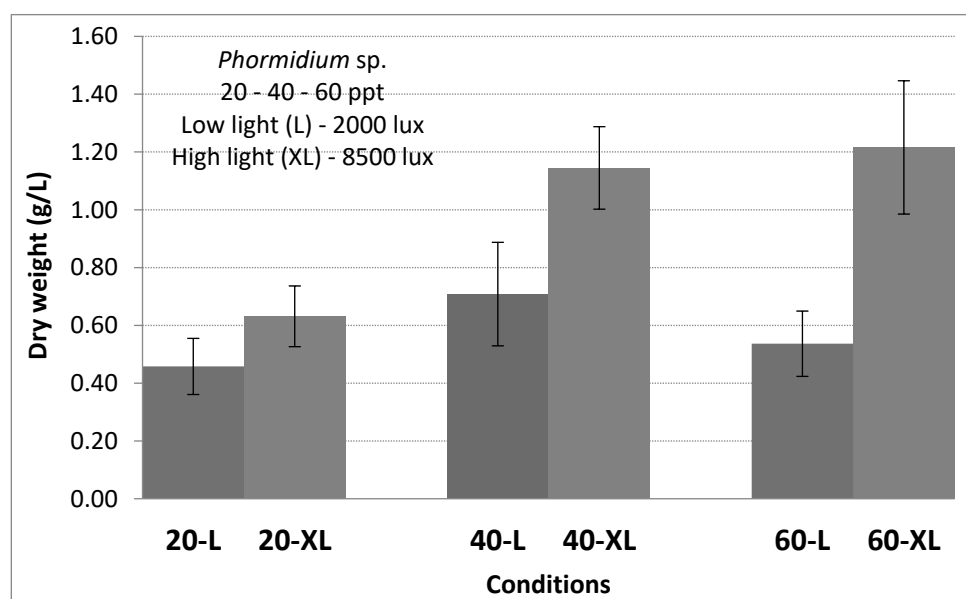
In Table 1, the growth characteristics of the cultures become more tangible with the arrangement of the values of the specific growth rate  $\mu_{max}$  and the generation time  $t_g$ . The highest  $\mu_{max}$  (0.491) and the faster doubling of the algal mass ( $t_g = 1.415$  days) were recorded in the culture of the highest salinity (60 ppt) and at a high level of light (XL-8000 lux), while the lowest  $\mu_{max}$  (0.203) and  $t_g$  (3.43 days) were recorded in the culture with a salinity of 20 ppt at high light (XL).

**Table 1.** Records of growth parameters of the cultures of *Phormidium* sp. in the conditions examined: L = light of 2000 lux, XL = light of 8000 lux,  $\mu_{max}$  = specific growth rate between days 4 and 7 of the culture period,  $t_g$  = days for doubling of the algal population. Values are means  $\pm$  S.D. of 27 measurements. Same or different superscripts (a, b, c, d, e) denote non-significant or significant differences, respectively, at a 0.05 level of confidence, examined by a pair-wise Tukey’s test.

Var.	20 ppt-L	20 ppt-XL	40 ppt-L	40 ppt-XL	60 ppt-L	60 ppt-XL
$\mu_{max}$	<sup>a</sup> 0.363 $\pm$ 0.03	<sup>b</sup> 0.203 $\pm$ 0.012	<sup>c</sup> 0.404 $\pm$ 0.045	<sup>a,c</sup> 0.401 $\pm$ 0.010	<sup>d</sup> 0.317 $\pm$ 0.038	<sup>e</sup> 0.491 $\pm$ 0.025
$t_g$	<sup>a</sup> 1.922 $\pm$ 0.169	<sup>b</sup> 3.426 $\pm$ 0.199	<sup>a,c</sup> 1.739 $\pm$ 0.207	<sup>a,c</sup> 1.791 $\pm$ 0.359	<sup>d</sup> 2.222 $\pm$ 0.290	<sup>e</sup> 1.415 $\pm$ 0.070
days	4th–7th	4th–7th	4th–7th	4th–7th	4th–7th	4th–7th
n	27	27	27	27	27	27

All cultures were left to mature further, and on the 20th day, a sample was collected from each vessel. Moreover, through filtration and drying, the yield in terms of dry weight per liter (g/L) was calculated, as depicted in Figure 8. Yields higher than 1 g/L were recorded in the high light cultures (XL) of 40 and 60 ppt salinities, while for the 20 ppt culture in both light regimes, the values were substantially lower.

The pigment content is summarized in Table 2. Phycobilliproteins were substantially higher both in terms of weight per culture volume (mg/mL) and weight per dry weight biomass (mg/g D.W.) in the low light regimes (L) as compared to their high light (XL) counterparts in all salinities, with the phycocyanin content many times higher than phycoerythrin. The salinity of the 20 ppt culture in low light (L) presented the highest yield of phycocyanin (>20 mg/g) and phycoerythrin (>5 mg/g), followed by the 60 ppt culture and the 40 ppt culture.



**Figure 8.** Yield in g dry weight per liter, of the cultures of *Phormidium* sp. at all treatments of combinations of salinity and illumination. Error bars represent the Standard Deviation. Symbols of the x-axis are explained in the caption inside the figure.

**Table 2.** Records of pigment content in the various culture conditions. PC = phycocyanin in mg per mL of culture volume (mg/mL), PC<sub>yield</sub> = weight of phycocyanin per biomass dry weight (mg/g D.W.), PE = phycoerythrin (mg/mL), PE<sub>yield</sub> = phycoerythrin (mg/mL), AllPC = allophycocyanin (mg/mL), Chl-a = chlorophyll-a (µg/mL), Carot. = total carotenoids (µg/mL). Values are means ± S.D. of four measurements. Same or different superscripts (a, b, c, d, e, f) denote non-significant or significant differences, respectively, at a 0.05 level of confidence examined by a pair-wise Tukey’s test.

Var.	20 ppt-L	20 ppt-XL	40 ppt-L	40 ppt-XL	60 ppt-L	60 ppt-XL
PC	<sup>a</sup> 0.277 ± 0.061	<sup>b</sup> 0.0011 ± 0.000	<sup>c</sup> 0.054 ± 0.0192	<sup>d</sup> 0.0037 ± 0.004	<sup>e</sup> 0.107 ± 0.039	<sup>d,f</sup> 0.0086 ± 0.09
min-max	0.216–0.350	0.001–0.0013	0.033–0.074	0.0002–0.0075	0.069–0.146	0.0005–0.0175
PC <sub>yield</sub>	<sup>a</sup> 22.658 ± 1.44	<sup>b</sup> 0.084 ± 0.012	<sup>c</sup> 5.750 ± 2.40	<sup>d</sup> 0.393 ± 0.413	<sup>e</sup> 9.631 ± 0.362	<sup>f</sup> 0.645 ± 0.664
min-max	21.30–24.06	0.075–0.098	3.428–8.001	0.014–0.837	9.284–10.006	0.030–1.276
PE	<sup>a</sup> 0.0462 ± 0.029	<sup>b</sup> 0.0004 ± 0.00	<sup>c</sup> 0.0056 ± 0.0015	<sup>d</sup> 0.0013 ± 0.0009	<sup>e</sup> 0.0107 ± 0.0056	<sup>d,f</sup> 0.0024 ± 0.002
min-max	0.0193–0.0744	0.0004	0.0032–0.0073	0.0002–0.0023	0.005–0.0162	0.0003–0.0053
PE <sub>yield</sub>	<sup>a</sup> 3.6030 ± 1.803	<sup>b</sup> 0.0307 ± 0.001	<sup>c</sup> 0.575 ± 0.1641	<sup>d</sup> 0.1392 ± 0.1221	<sup>e</sup> 0.9662 ± 0.1141	<sup>f</sup> 0.1787 ± 0.173
min-max	1.9325–5.2468	0.0291–0.0319	0.4101–0.7311	0.0148–0.2588	0.8188–1.0324	0.0202–0.3640
AllPC	0.1055 ± 0.053	0.0004 ± 0.00	0.0144 ± 0.0035	0.0028 ± 0.0025	0.0654 ± 0.0641	0.0058 ± 0.006
Chl-a	<sup>a</sup> 8.146 ± 0.298	<sup>b</sup> 3.457 ± 0.136	<sup>c</sup> 6.008 ± 0.387	<sup>d</sup> 4.808 ± 0.378	<sup>d,e</sup> 4.272 ± 0.855	<sup>f</sup> 1.438 ± 0.225
min-max	7.793–8.433	3.334–3.599	5.482–6.306	4.274–5.164	3.179–5.002	1.169–1.670
Carot	<sup>a</sup> 2.032 ± 0.183	<sup>b</sup> 1.210 ± 0.026	<sup>c</sup> 1.567 ± 0.090	<sup>d</sup> 1.976 ± 0.114	<sup>b,c,e</sup> 1.445 ± 0.304	<sup>b,f</sup> 1.153 ± 0.274
min-max	1.760–2.156	1.186–1.245	1.433–1.624	1.825–2.100	0.993–1.653	0.747–1.331

#### 4. Discussion

As the goal of an effective mass production of a certain microalgae (cyanobacterial or eukaryotic) depends on its growth rate and this, in turn, is affected by various factors, such as photoperiod, light intensity, nutrient composition, salinity, pH, etc. [14–21]. In the present study, the growth rate of *Phormidium* sp. was investigated in six combinations of salinity and light intensity on the ground that these two parameters can be set clearly at certain levels and remain constant throughout the culture period. The results (Figures 5–7, Table 1) clearly demarcated a first conclusion, namely, in elevated salinities of 40 and



60 ppt, growth was far higher than in the low salinity (20 ppt). The second conclusion was nested in the first, namely, with high light (8000 lux), growth was considerably higher than with low light (2000 lux) in the salinities of 40 and 60 ppt, while in the lower salinity (20 ppt), the effect of light was insignificant. Although the *Phormidium* group is far from being phylogenetically coherent [22] and considering the scarcity of data on its culture in the literature, we rely on the findings of certain species of this genus or other genera of filamentous cyanobacteria (e.g., *Phormidium foveolarum* and *Nostoc muscorum*) for a plausible explanation of the present findings. On this ground, it is possible that the particular strain of *Phormidium* cultured here apart from being halotolerant, prefers high salinities for fast propagation. In low salinity and low light, it probably suffers to a certain degree from oxidative stress, while in high light, the enhanced photosynthesis and the reinforced action of antioxidant enzymes compensate for or mitigate oxidative stress [23]. Similar tolerance to very high light intensity (25,000 lux) of the marine *Phormidium ceylanicum* was elsewhere recorded [24], which is probably an indication of a special physiological feature for the marine representatives of this genus that thrives even in hypersalinity [25]. Additionally, as this particular strain of *Phormidium* originated from the local salt-works, where extreme conditions of high salinity and periodically very high illumination prevail, it comes of no surprise that the findings of its higher productivity in high salinity and high light are contrary to what was encountered in other studies (e.g., [26]). Keeping and culturing this particular strain of marine *Phormidium* in the laboratory for over 2 years, I am highly impressed by its ability to dominate the cultures continuously, even in occasionally neglected and old vessels or after repeating renovations, and because of this, it may be one of the most promising species for maintaining monocultures. This advantage for a culture of *Phormidium* may probably be attributed to its ability to produce some kind of allelo-chemicals [27] that suppress the growth of other cyanobacteria or eukaryotic algae (and bacteria as well), a topic quite neglected in the literature.

The observed better growth boosted by higher levels of salinity and light was also confirmed in terms of dry weight production (Figure 8), attaining values well above 1 g/L in the 40 (1.18 g/L) and 60 ppt (1.22 g/L) salinities at high light illumination as compared to 0.5–0.6 g/L in all other conditions (20 ppt both low and high light, low light at 40 and 60 ppt). As data of either growth rate or productivity on *Phormidium* are hardly found in the literature, because most researchers focused on its pigment content, the present study can be considered as the first of its kind, creating a baseline for optimization of its mass culture conditions. The majority of relevant publications are referred to the dominant in production trials cyanobacterium *Arthrospira platensis* (*Spirulina platensis*) for which data abounds (e.g., [28–31]). Although the existing information on *Arthrospira* is not presented uniformly and the conditions of the cultures employed were quite different from the present study, an attempt was made here to compare the yield of *Phormidium* to that of *Arthrospira*. For *Arthrospira* cultures, production maxima varied from 0.2 g/L [31] to 1.7 g/L [30]. In this respect, the present data fall in the satisfactory range of ~1 g/L, which, under the preferred range of salinity and light mentioned above, can be easily achievable and set the basis for a routine management. Additionally, if the special dynamics in the culture of *Phormidium* are considered (i.e., its dominance when competes with other species in the culture vessels), then its advantages for mass culturing are further enhanced. The algal culture industry seeks sturdy species, tolerant to extreme conditions, stability of culture, fast growth rate and satisfactory production of dry mass. Last, but maybe most important, simple and easy collection of the algal mass is needed. In this respect, *Phormidium* has a tremendous advantage over other microalgae, as it settles completely (Figure 4) after about an hour from the stopping of aeration.

The second aim of the present study deals with the cellular content in phycocyanin and other pigments in order to find the optimum conditions for maximizing the output of these highly valued and highly commercialized substances. The results exhibited a profound production of phycocyanin, far bigger in the low light condition compared to the high light condition. This was recorded in all salinities tested, but its most outstanding expression

was in the lower salinity of 20 ppt, where in the low light, the phycocyanin amount both in terms of concentration or yield (0.277 mg/mL and 22.658 mg/g DW, respectively) was 250 times more than the relevant values recorded in the high light (0.0011 mg/mL and 0.084 mg/g DW, respectively). In the other two salinities (40 and 60 ppt), the same situation (higher production of phycocyanin in low light vs. high light) occurred, and although not in the same exaggerated manner as in 20 ppt, it was nevertheless impressively intense, with ratios of ~14:1 (L:XL).

The same pattern was recorded also for the other two billiproteins (phycoerythrin and allophycocyanin), with outstandingly higher amounts produced in low light of all salinities vs. high light. The phenomenon was also much more pronounced at the lower salinity of 20 ppt, where the ratios were 115:1 (L:XL) for phycoerythrin and 263:1 for allophycocyanin, as compared to ~4.5:1 for phycoerythrin and 5–11:1 for allophycocyanin at the higher salinities of 40 and 60 ppt.

Such data clearly indicate the catalytic effect of the lower illumination to the enhancement of cellular production of billiproteins. As a number of cyanobacterial species have been screened on their ability to produce phycocyanin [32] and significant amounts of other pigments (chlorophylls, carotenoids) [33], the spectrum of their cellular content in all species (dependent on the prevailing conditions) is very wide [34–36] and insinuates that the mechanization related to the proper manipulations of the cultures to achieve the desired production is open for improvement.

Attempting to compare the data on phycocyanin and on the other two billiproteins of the present study with those of the literature, rather few and fragmented data on *Phormidium* and other cyanobacteria can be found. However, the influence of a low level of irradiance on increased phycocyanin production of *Phormidium foveolarum* and *Nostoc muscorum* [23] was in accordance with the present data. Considering that billiproteins along with carotenoids (also present in cyanobacteria) constitute the accessory pigments of these algae [37], which help to maximize photon capture when feeble illumination prevails or shift roles in other sunlit environments to protect the photosystems from photolysis, an assumption (for cyanobacteria) based on evidence can be made. That is, billiproteins have the sole mission to gather the maximum amount of light possible. For this, in conditions of inadequate levels of light for maximum photosynthesis, the cell initiates excessive production of phycocyanin (mainly) and phycoerythrin-allophycocyanin accordingly. On the other hand, carotenoids have a double mission, i.e., light gathering and photo-protection, both of which affect their cellular concentration, but in a less pronounced way than that of billiproteins. Data from the literature on that topic are rather puzzling, as they note an increase of carotenoids in high light [23,38] in species of *Phormidium*, *Nostoc* and *Anabaena*. This is contrary to the results of the present study, which did not record great differences in carotenoids between low and high light in all three salinities tested (Table 2), with the biggest difference recorded at the lower salinity of 20 ppt, 2.032 µg/mL vs. 1.210 µg/mL (L vs. XL). On the other hand, chlorophyll-a content was much increased at all salinities in low light as compared to high light (Table 2), with the biggest values recorded at the lower salinity (20 ppt).

Summarizing all of the above, concerning the pigment content of this particular strain of *Phormidium* as affected by different light and salinity regimes, a profound influence of the low level of illumination on the increase of billiproteins and chlorophyll cell content was recorded beyond any doubt. This was much exaggerated in the lower salinity, which causes us to consider the influence of salinity on the cellular pigment content. The increased level of carotenoids recorded at 60 ppt for the coccoid cyanobacterium *Synechocystis* sp. PCC 6803 [39] was present in the present study, where carotenoids more or less remained at similar levels in all salinities. This can be probably attributed to differences among species regarding their tolerance and adaptation ability to various salinities. However, when phycocyanin is concerned, contrary to some studies in which a considerable drop in the phycocyanin content and overall photosynthesis was recorded when cultured in high salinity (60 ppt), attributable to the induced damage on phycobilisomes [40] or on proteins

of the PS II reaction center [41,42] of *Spirulina platensis* and *Synechococcus* sp., respectively, the present study suggests the opposite for *Phormidium*. This can probably be attributed to the special physiology of this species, which seems to grow better in high salinities (note that it originated from salt-works). The evidence for an elevated rate of photosynthesis in higher salinities (and thus, faster growth), is also validated by the more alkaline pH in the conditions of high light at 40 and 60 ppt salinities, probably due to an elevated rate of subtraction of protons from water [43] (and not to a reduction in CO<sub>2</sub>, which was constantly supplied by aeration) in order to enhance NADPH levels for more sugar production.

Summing up the data on culture growth and phycocyanin output in order to construct a competitive production scheme for this novel high salt-preferring species of *Phormidium*, the following process is advised:

1. Culture of the cyanobacterium with Walne's medium-enriched water of over 40 ppt salinity (suggested range 40–60 ppt), with a light intensity of 8000 lux or more for about 15 days in order to enhance best biomass production.

2. Stop aeration and initiate filament's sedimentation, which takes about an hour, after which the dense settled biomass can be collected. To facilitate the sedimentation and collection of algal biomass, it is advised to use transparent tubular culture vessels, preferably with a conical bottom. After the completion of sedimentation, the supernatant is discarded.

3. The collected wet biomass is transferred to new culture vessels filled with fresh nutrient-enriched water of 20 ppt salinity, lit by LED lamps, producing no more than 2000 lux, metered on the surface of the vessels, in order to maximize phycocyanin production. Aeration supplied to the vessels should be continuous at a rate of about one culture volume of air per minute.

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