

## Article

# Influence of Medium Frequency Light/Dark Cycles on the Cultivation of *Auxenochlorella pyrenoidosa*

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**Abstract:** Light (wavelength, intensity, and light/dark cycle) have been considered as one of the most important parameters for microalgae cultivation. In this paper, the effect of medium frequency intermittent light on *Auxenochlorella pyrenoidosa* (formerly *Chlorella pyrenoidosa*) cultivation was investigated. Three parameters of intermittent light, light intensity, light/dark ratio, and light/dark cycle were employed and the influence of these parameters on the productivity of *Auxenochlorella pyrenoidosa* was studied. The biomass yield and growth rates were mainly affected by the light fraction and cycle time. Light with  $220 \mu\text{E m}^{-2} \text{s}^{-1}$  light intensity was determined as the optimal light intensity for biomass production. At the light intensity of  $420 \mu\text{E m}^{-2} \text{s}^{-1}$ , the results indicated that the intermittent light improved the biomass production with larger light/dark ratio compared with the continuous light. At a lower mean light intensity over time, the intermittent light should be more suitable for biomass growth and the decrease in the light/dark ratio (L/D) will lead to a higher biomass productivity. The light/dark cycle time has little influence on the biomass yield.

**Keywords:** light-emitting diode (LED); *Auxenochlorella pyrenoidosa*; medium frequency; light/dark cycle

## 1. Introduction

Photosynthetic microorganisms have received growing attention, especially for mass cultivation of microalgae, due to their possible production of health foods, biofuels, and also for carbon dioxide reduction. Light attenuation in microalgae cultivation systems has been reported as a major bottleneck in microalgal production. In general, light attenuation leads to the light zone, a well-illuminated volume of the culture near the light source, and the dark zone, a self-shaded zone of culture [1,2]. Light condition is one of the main factors that affects the microalgae photosynthesis kinetics.

Numerous studies have indicated that light intensity and light wavelength change the results in increasing or decreasing the biomass and metabolite yields [3,4]. Khalili et al. [5] reported that the energy associated with photons with a wavelength of 680 nm is the exact energy level required by chlorophyll to initiate photosynthesis. Carvalho et al. [6] summarized previous studies that have suggested that a light source with narrower spectral outputs close to the photosynthesis absorption spectrum (680 nm) are photosynthetically more efficient. Hence, a red light source results in higher biomass accumulation compared with the other light wavelength. When the light intensity is insufficient, it has been reported that the blue light can also be absorbed by chlorophyll because of the blue light contains 40% more energy than the other light wavelength [5,7,8]. For light intensity, the optimal light intensity for microalgae cultivation ranges from 2000 to 10000 lux [9]. The excessive and insufficient light intensity result in decreases to the biomass productivity. Due to the light attenuation in microalgae cultivation systems, an efficient light energy supply has been reported as the greatest scientific and technological challenge in the research and the development of the cultivation of photosynthetic microorganisms [10].

Regarding the effect of the light/dark cycle, Grobbelaar [11] reported that microalgae growth can be influenced by three ranges of light/dark cycle: (1) high frequency, namely, the flashing light effect; (2) medium frequency, fluctuation cycles of seconds to minutes; and (3) low frequency, fluctuation cycles of hours to days and years. It was reported that proper flashing light under high cell concentrations will enhance microalgae growth rate and metabolite productivity [6,12], while a reduction in biomass production with a decrease in the duration of light periods was evident in low frequency [13,14].

For medium frequency light/dark cycles, conflicting results have been reported in the literature. Merchuk et al. [15] reported that the red microalga *Porphyridium* sp. has a maximal growth rate at cycle times of 27 s, 60 s, and 110 s, when the maximal dark period reaches about 5.6 s and cool white fluorescent light reaches an intensity of  $300 \mu\text{E m}^{-2} \text{s}^{-1}$ . It clearly indicates that the longer light period could not lead to a higher growth rate. In contrast, Lee and Pirt [16] reported that the duration of the dark was 9.2 s (with a total cycle time of 40 s) when the specific growth rate of *Chlorella vulgaris* (*Chlorophyta*) was at its maximum value, but the increase in the dark period leads to a decrease in the growth rate. The same results were reported by Grobbelaar [17] at a light/dark cycle from 4.1 s to 50 s and light intensity of  $800 \mu\text{E m}^{-2} \text{s}^{-1}$ , where the growth rate of *C. vulgaris* depends on the light/dark ratio; the larger the light/dark ratio, the more productivity and photosynthetic efficiency. In addition, Wu et al. [18] investigated the effect of light/dark ratio on the growth rate of *Porphyridium* sp. at a low concentration of  $1.0 \times 10^7$ – $1.20 \times 10^8$  cell  $\text{mL}^{-1}$  with three intensities of 110, 220, and  $550 \mu\text{E m}^{-2} \text{s}^{-1}$ . The results indicate that for the longer light period, the larger microalgae yield at light/dark cycle ranges from 28.3 s to 45 s. This discrepancy may be caused by the experimental setups. In these experimental setups, the different light/dark cycles were obtained by changing the flow rate of the suspension through light and dark zones. For these methods, the frequency of the light/dark cycle is difficult to precisely control because microalgae movement caused by turbulent fluctuation is random. The other one is the light attenuation, where the inner diameter of the tube in experimental setups of the aforementioned literatures were 9 cm [15], 0.48 cm [17], and 0.7 cm [16,18]. In addition, according to the description of light/dark cycle by Nedbal et al. [19], the continuous and intermittent light aforementioned experiments were in the same light intensity, and the continuous and intermittent light of equal mean irradiance over time in the medium frequency have attracted little attention. Therefore, the effects of medium frequency light/dark cycles on microalgae are still not thoroughly understood.

Based on the above discussion, the previous studies aimed to reveal the relationship between the intermittent light and the productivity of the biomass. However, the limitation in the previous experiments was neglecting the additional light and dark areas brought by the light attenuation in the cultivation systems. Therefore, the hypothesis in this study is that the additional light/dark cycle will decrease the productivity of biomass. Compared to the light/dark deduced by flow through light and dark zones in previous studies, the method of controlling the light on and off allows the frequency of the light/dark cycle to be precisely adjusted. To decrease the light attenuation, an experimental system with a low cell concentration and a short light-path was conducted to keep the microalgae cells in the same light condition. By doing this in this way, the medium frequency light/dark cycle can be easily applied to the microalgae. Due to narrow band wavelength, high brightness, and fast start without delay, five light-emitting diode light (LED) sources (blue, green, red, white, and yellow) and four light intensities were conducted in this study. The effect of two kinds of intermittent light with medium frequency light/dark cycles on the growth of the microalgae *A. pyrenoidosa* was investigated. The study will provide guidance in designing more efficient microalgae cultivation systems.

## 2. Materials and Methods

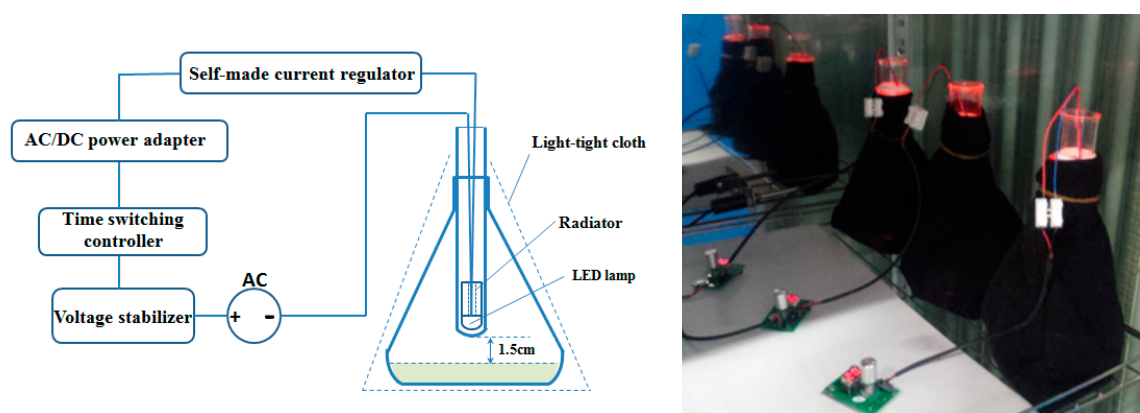
### 2.1. Organism and Culture Medium

The microalgae *Auxenochlorella pyrenoidosa* was used to conduct this study and provided from the Fisheries College of Jimei University. Microalgae were cultivated using the f/2 medium [20] and

was prepared using sea water containing ( $\text{mg L}^{-1}$ ):  $\text{NaNO}_3$  74.8,  $\text{NaH}_2\text{PO}_4$  4.4,  $\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$  10, ferric citrate 0.0039,  $\text{ZnSO}_4 \cdot 4\text{H}_2\text{O}$  0.023,  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  0.178,  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  0.012,  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$  0.0073,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  0.001, and  $\text{NaEDTA} \cdot 2\text{H}_2\text{O}$  0.00435. The f/2 medium was sterilized for 20 min at  $121^\circ\text{C}$ .

## 2.2. Cultivation Conditions

The cultures were carried out in a 500 mL Erlenmeyer flask containing 100 mL of inoculation and placed in a thermo-stabilized cabinet at  $25 \pm 1^\circ\text{C}$  for seven days. The interior of the flask was equipped with a glass test tube and a LED was fixed in the bottom of the glass test tube [21]. There was a round hole in the tube wall for ventilation, and the hole was covered with filter paper to prevent contamination. The distance between the bottom of the test tube and the upper surface of the cultivation medium was 1.5 cm. In addition, the Erlenmeyer flask was covered with a light-tight cloth to isolate the light, as seen in Figure 1. The flask was shaken twice over 24 h to avoid the sedimentation of cells. All experiments were replicated three times.



**Figure 1.** Schematic diagram of the experimental system.

In each scenario, the initial density of the culture ( $\text{OD}_{680}$ ) was adjusted to 0.09 by controlling the dilution rate, which means that the initial biomass of the microalgae was  $0.02 \text{ g L}^{-1}$ . At this density, and with the low work volume inside the Erlenmeyer flask, light path and mutual shading was low. This is a prerequisite to ensure that the flash rhythm of the LED would be sensed by the cells.

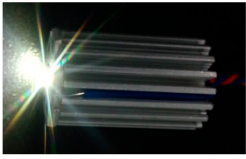
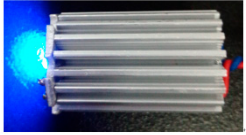
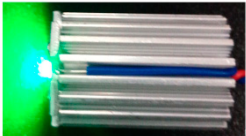
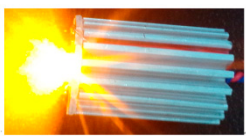
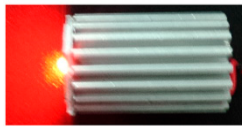
## 2.3. Light Regime

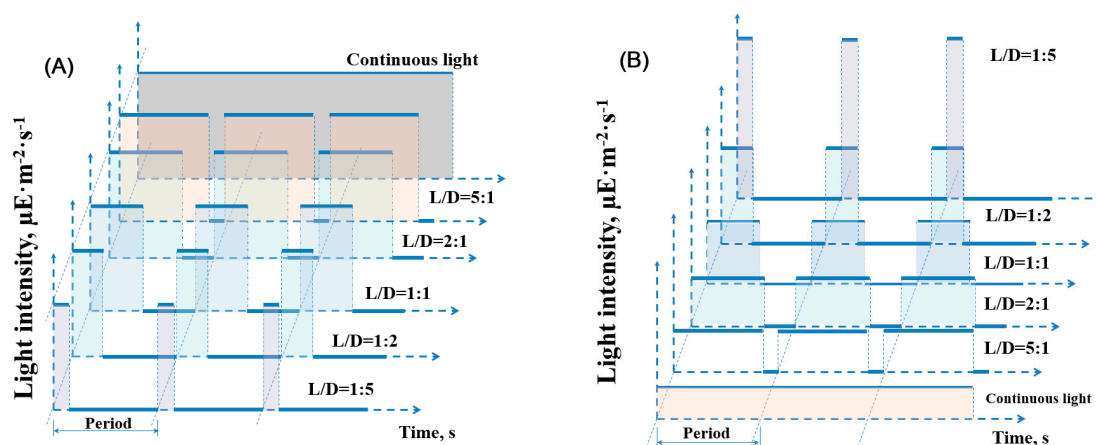
Five LEDs (CREE, USA), 3 W, emitting blue, green, red, white, and yellow light are shown in Table 1 and were built as the light source for microalgae cultivation. All of the LEDs were driven by a 12 V 1A AC–DC power adapter (SDK-0605, China) and illumination intensity was tuned via a self-made pulse-width modulation (PWM) constant current regulator.

It has been reported that the optimal light intensity for microalgae cultivation ranges from 2000 to 10,000 lux, and  $1 \mu\text{E m}^{-2} \text{ s}^{-1} = 52 \text{ lux}$  [9]. Therefore, experimental values of light intensity of 50, 110, 220, and  $420 \mu\text{E m}^{-2} \text{ s}^{-1}$  were conducted. In addition, all the LED lights had aluminum radiators.

In this study, three light/dark cycles (30 s, 60 s, and 120 s) and five light/dark ratios ( $L/D = 5:1$ ,  $2:1$ ,  $1:1$ ,  $1:2$ , and  $1:5$ ) were used to investigate the effect of intermittent light on microalgae cultivation. Therefore, the experimental light regime of the LEDs was controlled by a time switching controller with voltage stabilizer. Two kinds of intermittent light were conducted, as shown in Figure 2. Figure 2A shows the intermittent and continuous light with the same light intensity, and the intermittent and continuous light with the same time-average light intensity is shown in Figure 2B.

**Table 1.** The parameters of light-emitting diodes.

Model Number	Color	Wave Length/nm	Voltage/V	Current/mA
XPE-WHT		380–760	3.5–3.7	700–1000
XPE-BLU		465–485	3.5–3.7	700–1000
XPE-GRN		520–535	3.5–3.7	700v1000
XPE-AMB		586–595	2.2–2.5	700–1000
XPE-RED		620–630	2.3–2.5	700–1000



**Figure 2.** Schematic diagram of light regime of intermittent and continuous light. (A) Light regime with the same light intensity, and the total energy incident on the culture surface depends on the L/D. (B) Light regime with the same time-average light intensity, and the total energy incident on the culture surface remains constant per light/dark period.

The photon flux density (PFD) was measured via a quantum sensor, (QSL 2100, Biospherical Instrument Inc., San Diego, CA, USA). The PFD of 1.5 cm away from the bottom of the tube within the LEDs was measured and modulated in a darkroom before the start and after seven days of the experiment.

#### 2.4. Biomass Analysis

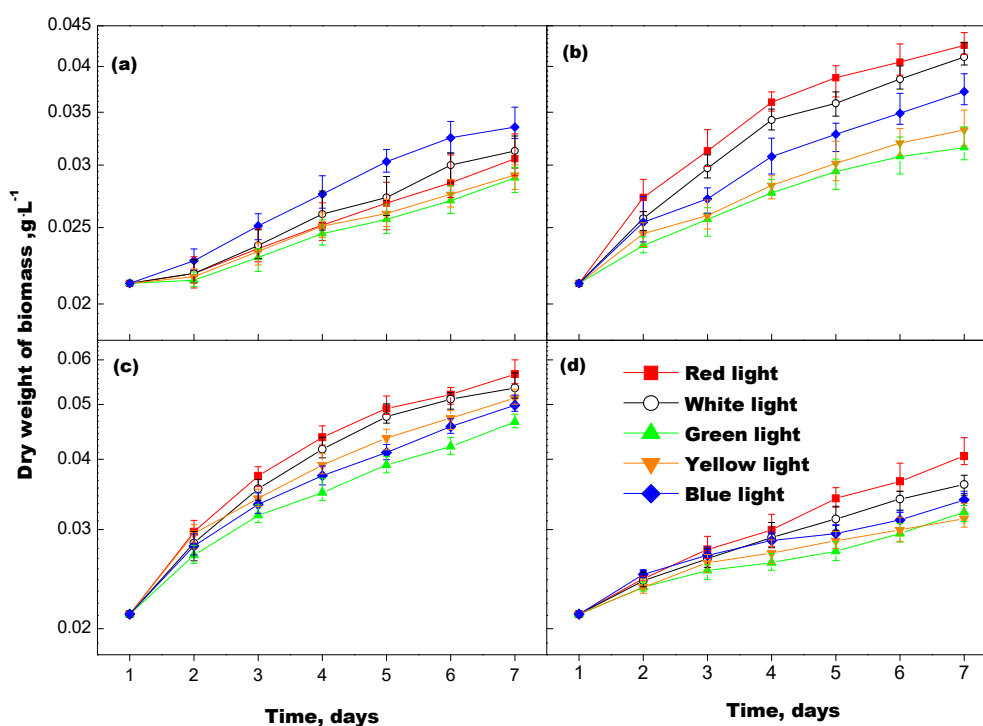
During seven days, the 3 mL/day of inoculum of *A. pyrenoidosa* was extracted for experimental measurement. The optical density (OD) of inoculum was obtained by measuring the absorbance of the

broth at 680 nm in a spectrophotometer (721G, INESA, Shanghai, China). The dry cell weight of the microalgae was calculated according to the linear equation between  $OD_{680}$  and the dry cell biomass of the microalgae, which was [22]:  $\text{dry weight (mg/mL)} = 0.207OD_{680} + 0.0022$  ( $R^2 = 0.9984$ ).

### 3. Results

#### 3.1. Continuous Light

*Auxenochlorella pyrenoidosa* growth under different light sources (red, white, green, yellow, and blue) with various light intensities ( $50, 110, 220$ , and  $440 \mu\text{E m}^{-2} \text{s}^{-1}$ ) for an incubation period of seven days is shown in Figure 3. The results obviously indicate that the microalgae biomass increased at first, and then decreased with the increase in light intensity. This means that there is an optimal light intensity to achieve the maximum biomass productivity. The growth of *A. pyrenoidosa* in the light intensity of  $220 \mu\text{E m}^{-2} \text{s}^{-1}$  was higher when compared with the light intensity of  $50, 110$ , and  $420 \mu\text{E m}^{-2} \text{s}^{-1}$ . The light intensity of  $50$  and  $110 \mu\text{E m}^{-2} \text{s}^{-1}$  was low to support the growth of *A. pyrenoidosa*, but the light intensity of  $420 \mu\text{E m}^{-2} \text{s}^{-1}$  was high enough to induce photo-inhibition. Hence, the light intensity of  $220 \mu\text{E m}^{-2} \text{s}^{-1}$  is suitable for *A. pyrenoidosa* cultivation, which agreed well with the previous study [16]. Excessive ( $420 \mu\text{E m}^{-2} \text{s}^{-1}$ ) or insufficient ( $50$  and  $110 \mu\text{E m}^{-2} \text{s}^{-1}$ ) incident light constrains optimal performance in terms of biomass yield.



**Figure 3.** Cell growth of *Auxenochlorella pyrenoidosa* at various light wavelengths and intensities: (a)  $50$ , (b)  $110$ , (c)  $220$ , and (d)  $420 \mu\text{E m}^{-2} \text{s}^{-1}$ .

At the light intensity of  $50 \mu\text{E m}^{-2} \text{s}^{-1}$ , the blue light achieved the highest biomass productivity. The biomass productivity of blue light increased by  $7.3\%$ ,  $9.7\%$ ,  $15\%$ , and  $16\%$  when compared to white, red, yellow, and green, respectively. The red light achieved higher biomass growth than the other lights when the light intensity was greater than  $110 \mu\text{E m}^{-2} \text{s}^{-1}$ . The maximum biomass productivity was  $0.057 \text{ g/L}$ , which was conducted at the red light with a light intensity of  $220 \mu\text{E m}^{-2} \text{s}^{-1}$ . When compared with the white light, the red light improved the biomass productivity of  $3.6\%$ ,  $5.9\%$ , and  $12.4\%$  at the light intensity of  $110, 220$ , and  $420 \mu\text{E m}^{-2} \text{s}^{-1}$ , respectively.



### 3.2. Intermittent Light

Based on the experimental results of continuous light, the red LED was the most responsive light in the cultivation of *A. pyrenoidosa*. Therefore, the red light was selected as the light source in the following studies. In addition, the results below were based on the statistical average of the dry weight of biomass of three experimental results.

#### 3.2.1. Intermittent and Continuous Light with the Same Irradiance

In the light regime as shown in Figure 2A, the total energy incident on the culture surface of intermittent light is less than the continuous light because of the ratio of light/dark (L/D). For example, at the light intensity of  $220 \mu\text{E m}^{-2} \text{s}^{-1}$  and the L/D = 1:1, the total energy incident on the culture surface of intermittent light is only half of the continuous light. It obviously indicates that the total energy incident on the culture surface increased with the increase in L/D.

Based on Figure 3, the light intensity less than or equal to  $220 \mu\text{E m}^{-2} \text{s}^{-1}$ , where the light intensity corresponds to the light-limited region. Therefore, the intermittent light leads to a decrease in the biomass productivity because the light energy support to photosynthesis by intermittent light is less than that of continuous light. The results obviously indicate that the biomass productivity increased with the increase in L/D, which agreed well with previous studies [16,17]. As shown in Figure 4A, one-sixth reduction of the total energy support led to a 10.9% reduction of biomass productivity. Similarly, the reduction in biomass productivity was 7.8% at the light intensity of  $220 \mu\text{E m}^{-2} \text{s}^{-1}$  (Figure 4B), which means that the greater the light intensity, the smaller the gap of biomass productivity between continuous light and intermittent light when the light intensity is conducted in the light-limited region.

At the light intensity of  $420 \mu\text{E m}^{-2} \text{s}^{-1}$  (Figure 4C), the intermittent light with L/D of 1:5, 1:2, 1:1, 2:1, and 5:1 corresponded to the light intensity of continuous light of about 70, 140, 210, 280, and  $350 \mu\text{E m}^{-2} \text{s}^{-1}$ , respectively. Due to the intermittent light reducing the effect of photo-inhibition, photo-oxidation, and mutual shading, more cells receive enough light to perform photosynthesis. Therefore, the intermittent light achieved a larger biomass productivity than continuous light when the light intensity is in excess of that necessary for photosynthesis. In addition, with the increase in L/D, the biomass productivity increased first, and then decreased for the excessive light intensity. This means that there is an optimal value of the light/dark ratio, which led to an improvement in the biomass yield of intermittent light when compared with the continuous light.

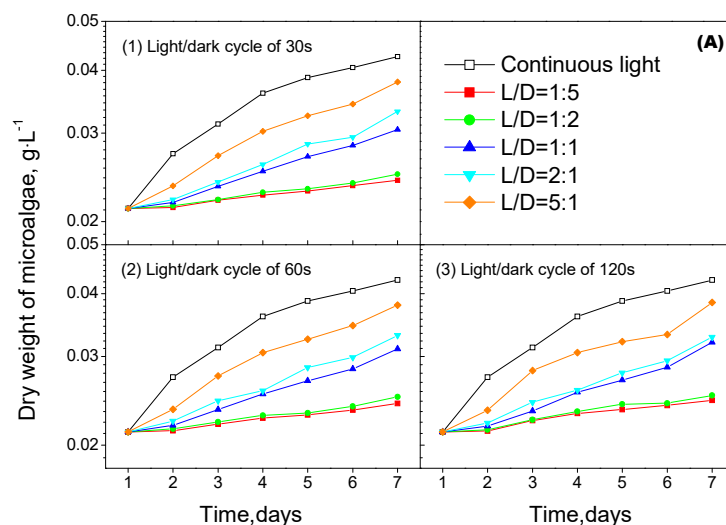
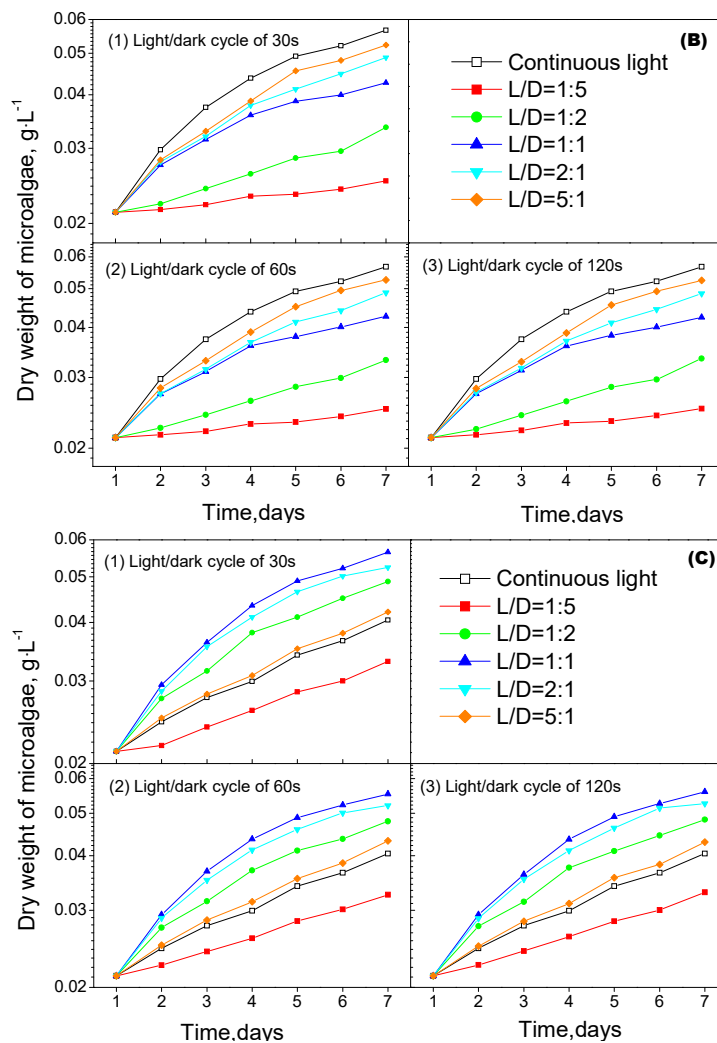


Figure 4. Cont.



**Figure 4.** Time courses of *A. pyrenoidosa* dry weight under the conditions of continuous light and intermittent light with the same irradiance and light intensity of  $110 \mu\text{E m}^{-2} \text{s}^{-1}$  (A),  $220 \mu\text{E m}^{-2} \text{s}^{-1}$  (B), and  $420 \mu\text{E m}^{-2} \text{s}^{-1}$  (C).

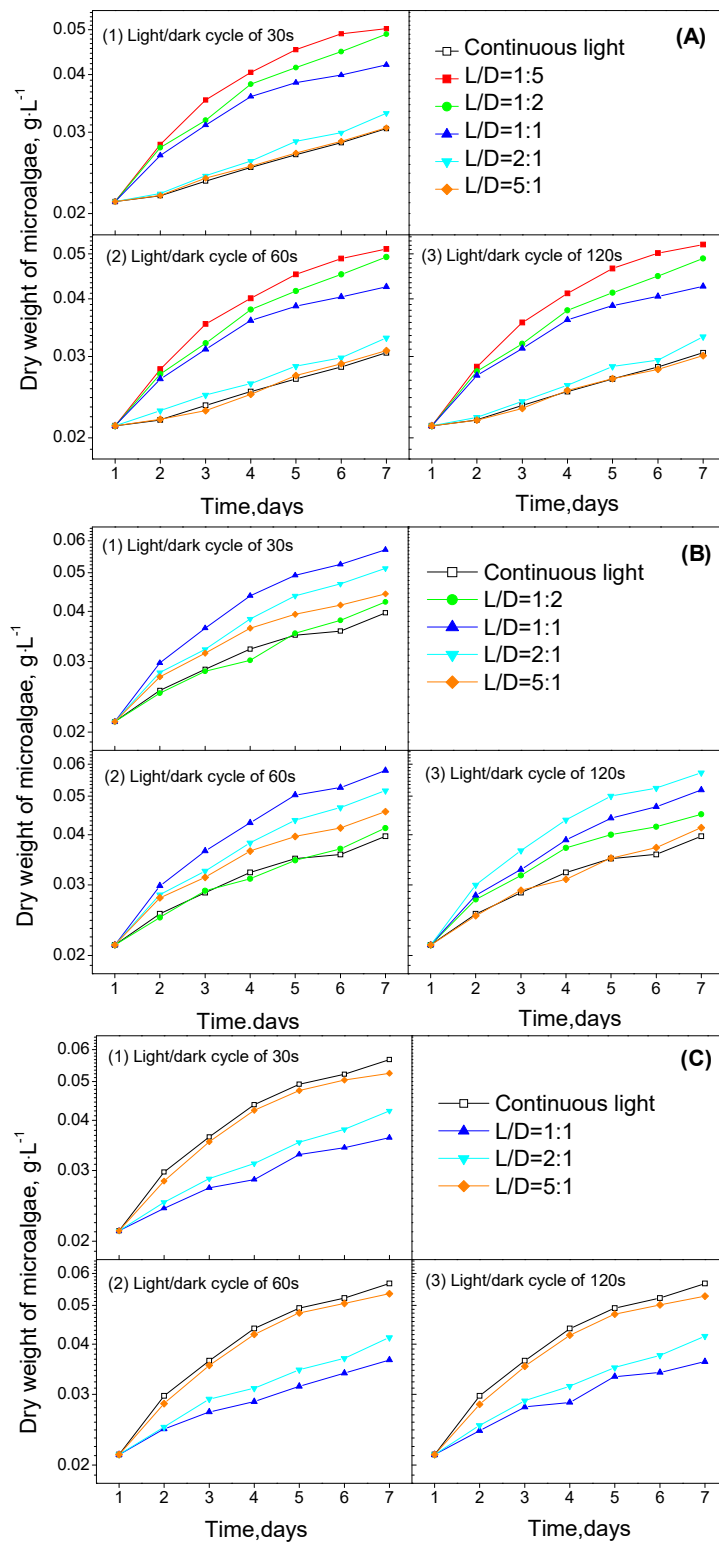
In summary, Figure 4 obviously indicates that the effect of the L/D cycle on the biomass production of intermittent light is only slight. The biomass productivity of medium frequency intermittent light is mainly related to the light intensity and the ratio of light to dark.

### 3.2.2. Intermittent Light and Continuous Light with the Same Time-Average Light Intensity

In the light regime shown in Figure 2B, the total energy incident on the culture surface of intermittent light is equal to the continuous light in the same time period. The intermittent light with three light/dark cycles (i.e., 30 s, 60 s, and 120 s) and five L/Ds (i.e., 5:1, 2:1, 1:1, 1:2, and 1:5) were studied and the results were compared with the continuous light with the same time-average light intensity.

At the time-average light intensity of  $50 \mu\text{E m}^{-2} \text{s}^{-1}$  (Figure 5A), the intermittent light of L/D (i.e., 5:1, 2:1, 1:1, 1:2, and 1:5) corresponded to the continuous light with light intensity of 60, 75, 100, 150, and  $300 \mu\text{E m}^{-2} \text{s}^{-1}$ , respectively. As the increased light intensity may help photons penetrate deeper into the culture, and thus reduce mutual shading by intermittent light, the intermittent light achieved a larger biomass production than continuous light. The same results were shown at the time-average light intensity of  $110 \mu\text{E m}^{-2} \text{s}^{-1}$  (Figure 5B), and the intermittent light of L/D (i.e., 5:1, 2:1, 1:1, and 1:2) corresponded to the continuous light with a light intensity of 132, 165, 220 and  $330 \mu\text{E m}^{-2} \text{s}^{-1}$ ,

respectively. At the L/D of 5:1, 2:1, and 1:1, because the light intensity is in the area of light-limited, the biomass production of intermittent light is larger than continuous light.



**Figure 5.** Time courses of *A. pyrenoidosa* dry weight under the conditions of continuous light and intermittent light with equal mean irradiance over time of 50  $\mu\text{E m}^{-2} \text{s}^{-1}$  (A), 110  $\mu\text{E m}^{-2} \text{s}^{-1}$  (B), and 220  $\mu\text{E m}^{-2} \text{s}^{-1}$  (C).



At the time-average light intensity of  $220 \mu\text{E m}^{-2} \text{s}^{-1}$  (Figure 5C), the intermittent light of L/D (i.e., 5:1, 2:1 and 1:2) correspond to the continuous light with the light intensity of 264, 330, and  $440 \mu\text{E m}^{-2} \text{s}^{-1}$ , respectively. The higher light intensity resulted in a decrease in growth rate. Therefore, the biomass productivity of intermittent light is lower than that of continuous light.

In conclusion, the increase in light cycle in light/dark cycle leads to the decrease in biomass production at a low time-average light intensity of intermittent light and the effect of medium light/dark cycle on microalgae biomass production is only slight.

## 4. Discussion

### 4.1. Light Source

In general, the blue light could support more than 40% more photon energy than other light wavelengths at the low light intensity. Therefore, the blue light may play an essential role in the regulation of *A. pyrenoidosa*, which leads to a rise in the biomass yield, as shown in Figure 3a. In addition, Figure 3b,c obviously showed that the red light resulted in the higher biomass productivity compared with the white light. These may be because the red light wavelength (620–630 nm) was close to the photosynthetic absorption spectrum of 680 nm when compared with the white light (380–760 nm). Light sources with narrower spectral outputs that are close to the photosynthetic absorption spectrum means that more energy can be absorbed by chlorophyll. Thus, the results indicate that red light should be the optimal light source for the cultivation of *A. pyrenoidosa* when the light is supported at sufficient condition ranges from 110 to  $220 \mu\text{E m}^{-2} \text{s}^{-1}$ .

At excessive light ( $420 \mu\text{E m}^{-2} \text{s}^{-1}$ ), the biomass productivity was higher than the insufficient light of  $50 \mu\text{E m}^{-2} \text{s}^{-1}$ . This may be due to the non-photochemical quenching, which dissipates the excess thermal energy that occurs when microalgae cells are exposed to light levels in excess of those necessary for photosynthesis.

### 4.2. Light Regime

As light attenuation induces light zones (well-illuminated area) and dark zones (insufficient illuminated area), the microalgae cells do not experience continuous illumination, but a pattern of light and darkness as they move through the light and dark volumes of the broth. This illumination of cells is called intermittent light, which has the same irradiance as the continuous light as per the light regime in Figure 2A. In general, this intermittent light ranges from seconds to minutes, and is also known as medium frequency light/dark cycle in practical microalgae cultivation systems. The results obviously indicate that the light/dark cycle caused by light attenuation in microalgae culture systems leads to a decrease in biomass productivity, as shown in Figure 4A,B. These also prove that the previous hypothesis in this study was correct.

In addition, the results of Figure 4C or Figure 5 also indicate that improving the light intensity with an optimal value of the light/dark ratio (L/D) would be helpful in enhancing the biomass productivity in medium frequency light/dark cycle. Furthermore, the product of the light intensity and the light/dark ratio is ultimately reflected in the total energy absorbed by the microalgae cells. Therefore, the results of Figures 4 and 5 obviously indicate that biomass productivity strongly depends on the light intensity and the light/dark ratio in intermittent light. The same results have been reported for continuous light, and some parameters such as average light intensity and volume average light intensity have been used to describe the relationship between the growth rate.

In conclusion, these results show a new method to improve the biomass productivity in practical microalgae cultivation systems with artificial light. Based on Lambert–Beer's law, the light intensity decreases rapidly with the increase in light path and cell concentration. Therefore, as the concentration of microalgae increases during the cultivation process, the light intensity could be appropriately increased. Moreover, the increases in light cycle time in the light/dark cycle could enhance the biomass productivity in practical microalgae cultivation systems.

## 5. Conclusions

The effect of light intensity, light/dark cycle, and light/dark ratio of intermittent light on *A. pyrenoidosa* was studied. Based on the experimental results, the blue light may be more suitable for microalgae cell growth when the light intensity is lower. The biomass productivity of *A. pyrenoidosa* is mainly related to the light intensity and the ratio of light/dark (L/D). The effect of medium frequency light/dark cycle on biomass production is low. When the intermittent light had the same light intensity with the continuous light, the intermittent light decreased the biomass productivity compared with the continuous light. Furthermore, the larger the L/D, the higher the biomass productivity reaches. In addition, to improve the biomass productivity in practical microalgae cultivation systems with artificial light, the light should be conducted at a higher light intensity and lower L/D.

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**Conflicts of Interest:** The authors declare no conflicts of interest.

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