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Phase-Dependent Effects of Photoperiod on Growth and Microcystin-LR Production in Two *Microcystis* Strains: Insights from Batch Culture for Bloom Management

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Abstract

The escalating threat of cyanobacterial blooms necessitates a deeper understanding of the environmental factors regulating their toxicity. While light intensity effects are well-documented, it remains unclear whether photoperiod regulates microcystin (MC) production. This study investigates the effects of five light–dark (LD) cycles on the growth and MC-LR production of two *Microcystis* strains in batch culture under a light intensity of $25 \mu\text{mol m}^{-2} \text{s}^{-1}$. Longer photoperiods enhanced early growth, although long-term biomass accumulation proved strain-dependent. Regarding toxin production, cellular MC-LR (total toxin per cell) during the initial 9-day phase was analyzed using a mixed-effects model. The analysis revealed significant main effects of photoperiod and cell density, supporting both direct and growth-mediated indirect effects of photoperiod. Moreover, a significant strain $\times$ photoperiod $\times$ day interaction ($p < 0.001$) was observed, indicating additional strain-specific and time-dependent regulation. Conversely, a general linear model of the strictly intracellular MC-LR at the 27-day endpoint showed significant independent effects of photoperiod and cell density, with no interaction. The photoperiod effect strengthened after controlling for cell density. These findings reveal a phase-dependent regulation of toxicity, suggesting that risk assessment based solely on biomass is inadequate. Sustainable bloom management should therefore incorporate photoperiod dynamics and adopt phase-specific strategies.

Keywords: *Microcystis*; microcystin-LR (MC-LR); light–dark cycle; photoperiod; water management



Academic Editor: Andrea G. Capodaglio

Received: 8 October 2025

Revised: 6 November 2025

Accepted: 11 November 2025

Published: 13 November 2025

Citation: Xiao, W.; Wang, X.; Wang, L.

Phase-Dependent Effects of Photoperiod on Growth and Microcystin-LR Production in Two *Microcystis* Strains: Insights from Batch Culture for Bloom Management. *Sustainability* **2025**, *17*, 10156. <https://doi.org/10.3390/su172210156>

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1. Introduction

Algal blooms represent a pervasive global threat to water security, primarily occurring in freshwater systems such as lakes, rivers, and reservoirs, in addition to coastal marine environments. Cyanobacterial blooms not only disrupt aquatic biodiversity and sustainable development but also endanger public health through the potential release of cyanotoxins. Microcystins (MCs) are a group of cyanotoxins produced by several genera of cyanobacteria, such as *Microcystis*, *Planktothrix*, and *Aphanizomenon* [1–3]. These toxins are frequently and persistently detected in diverse water bodies during warm seasons [4–6]. The primary health risk posed by MCs arises from their hepatotoxicity, which can cause fatal liver damage at high doses and act as a tumor promoter at low doses [7–9]. Furthermore, water blooms are difficult to eliminate, resulting in sustained pollution and making them a persistent challenge to environmental management and public health.

Cell biomass and toxin levels stand as the key parameters for the management of cyanobacterial pollution. Cell biomass is the defining parameter for assessing algal bloom pollution, and the concentration of toxins represents the health risk. These measures vary greatly depending on the dominant species, their physiological status, and the physico-chemical conditions of the environment [10–12]. Understanding how environmental factors influence cyanobacterial growth and toxin production is therefore essential for developing control strategies that exploit these conditions to suppress biomass and toxin accumulation. Several factors, such as nitrogen [13–15], phosphorus [16–18], and iron [19–21], and some physical conditions, including temperature [22–24], pH [25] and wind [26–28], can affect cyanobacterial growth and influence cyanotoxin yield. Light, as a critical factor for cyanobacterial growth, is necessary for the formation of water blooms. The authors of existing studies have extensively examined the effects of light intensity and spectral quality on *Microcystis*, a common bloom-forming genus [29–31]. In some of these studies, it has been reported that light intensity can influence cell growth, MC production and the expression of microcystin synthetase (*mcy*) genes [32–34]. In their study, Kaebernick et al. found that the light quality can affect the transcript levels of *mcyB* and *mcyD* in *Microcystis aeruginosa* PCC7806, with red light promoting and blue light reducing their mRNA levels [35].

In addition to the two light parameters, the duration of light exposure in a light–dark (LD) cycle may play an important role in cyanobacterial growth and their toxin yields. Both increasing light intensity and extending photoperiod can raise the total light integral; it remains unclear whether their effects on growth and toxin production follow similar trends. Although a few research groups have explored MC fluctuations under fixed LD conditions, such as the 12 h:12 h cycle, revealing diurnal variations in intracellular MC levels and *mcy* gene expression [36,37], these investigations were limited to a single photoperiod and short-term (24 h) observations. However, the duration of daylight changes with seasons in the natural conditions, and the influence of prolonged or shortened light exposure time in the LD cycle on the cyanobacterial toxin production remains poorly understood. In addition, the long-term physiological and metabolic adaptations of *Microcystis* to different photoperiods, particularly in terms of growth dynamics and toxin production, have yet to be systematically investigated.

To address these knowledge gaps, this study seeks to establish a conceptual framework linking photoperiod to cellular growth and MC synthesis in *Microcystis*. The primary objective is to determine how photoperiod influences the growth and MC production. Specific objectives include evaluating whether short-term and long-term exposures lead to consistent outcomes, examining potential strain-specific responses, and assessing the potential relevance of photoperiod on toxin production for bloom management and risk prediction.

In this work, two unicellular strains of *Microcystis* capable of producing MC-LR, one of the most prevalent and toxic MC variants commonly detected in the environment, were selected for investigation. The strains were cultivated under LD cycles with light periods ranging from 8 h to 16 h, approximating the range of natural seasonal day lengths. Both short-term and long-term observations were designed to evaluate the direct influence and overall impact. Cell density and MC-LR content were measured and monitored. Statistical methods were used to analyze the differences and explain the variations in the monitored data. By clarifying the role of light duration on MC production, this study aims to provide a scientific basis for refining risk assessment frameworks and management protocols for harmful algal blooms.

2. Materials and Methods

2.1. Strains and Cultivation Conditions for the Experiments

Two unicellular *Microcystis* strains, *Microcystis* sp. DH and *Microcystis aeruginosa* FACHB-905 were employed in this study. The *Microcystis* sp. DH strain was isolated from East Lake in Wuhan (China). This strain produces MC-LR, and no other MC variants (such as MC-RR or MC-YR) were detected. The *M. aeruginosa* FACHB-905 strain was purchased from the Freshwater Algae Culture Collection at the Institute of Hydrobiology (FACHB), Chinese Academy of Sciences. To obtain the exponential cultures and facilitate cell adaptation to culture conditions, the strains were grown in a 500 mL Erlenmeyer flask each with 200 mL of BG11 medium at $25\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$. The cultures were placed under a light intensity of $25\text{ }\mu\text{mol of photons m}^{-2}\text{ s}^{-1}$ provided by the cool white fluorescent tubes in a 12 h light–12 h dark (12L:12D) cycle before the start of the experiments.

2.2. Experiment Design

A 9-day experiment was designed to observe the dynamic changes in cell growth and MC-LR production of the two strains, *Microcystis* sp. DH and *M. aeruginosa* FACHB-905. Five LD cycle treatments, each with triplicate cultures, were arranged as follows: 8L:16D cycle, 10L:14D cycle, 12L:12D cycle, 14L:10D cycle and 16L:8D cycle. In accordance with these arrangements, the exponential cells of the strains were separately divided and inoculated into fifteen 500 mL Erlenmeyer flasks with 220 mL of BG11 medium. The initial densities were $4.8 \times 10^5/\text{mL}$ and $7.8 \times 10^5/\text{mL}$ at the beginning of the experiment for the strain *Microcystis* sp. DH and *M. aeruginosa* FACHB-905, respectively. The inoculated strains were grown at $25\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ under $25\text{ }\mu\text{mol of photons m}^{-2}\text{ s}^{-1}$. This light intensity is within the common range used for *Microcystis* cultivation [38,39] and was chosen to represent a sub-saturating, non-stressful condition while maintaining consistency with the pre-culture conditions to minimize acclimation artifacts. Ecologically, this level is relevant to the light conditions found within dense surface scums or at the deeper parts of the water column in turbid, bloom-affected lakes. The light phase was initiated at the same time every day for all the test groups. All culture flasks were fully randomized on the incubator shelves, and their positions were re-randomized every 24 h throughout the experiment to minimize potential shelf-level effects. Cell density and MC-LR content were monitored over 9 days, whereby a total of 4.5 mL of culture medium was collected in three replicates of 1.5 mL every two days for cell counting and MC-LR analysis. The sampling time was set uniformly at 3 h after the start of the light phase.

To evaluate the long-term effects of photoperiod on cell growth and MC-LR production, the two strains (*Microcystis* sp. DH and *M. aeruginosa* FACHB-905) were monitored for 27 days under five LD cycles with photoperiods ranging from 8 to 16 h, each performed in triplicate. The exponential cells of the two strains were separately divided and inoculated into fifteen 1 L Erlenmeyer flasks with 450 mL of BG11 medium. The light intensity, the synchronized start of the light phase, and the randomization procedures were identical to those in the 9-day experiment. Cell density was monitored every two days, and the MC-LR level was detected on the 27th day. To exclude the interference of the extracellular MCs in the possible stationary phase, cells were collected by means of centrifugation (at $8500 \times g$ for 10 min) on the 27th day for MC-LR detection.

2.3. Growth Observation

Cell densities were detected with three replicates through optical density at 450 nm (optical path 0.5 cm) and by means of cell counting, using a hemocytometer. Samples were analyzed immediately after collection. Growth curves were plotted as the logarithm of cell density versus culture time (days). The specific growth rate (μ) was calculated using

the following formula: $\mu = (\ln N_2 - \ln N_1) / (t_2 - t_1)$, where N_1 and N_2 represent the cell densities at time points t_1 and t_2 , respectively. The bar graphs of growth rates were produced based on the specific growth rate (μ) and the time of culture in days.

2.4. MC-LR Analysis

To detect the MC-LR content, three repeated freeze and thaw cycles were performed to break the cells to release the MC. The resulting solution was filtered through a 0.22 μm filter membrane for subsequent high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS–MS) analysis. The quantified MC-LR concentrations were normalized to cell count to express values as fg/cell. For the 9-day experiment, culture samples were directly processed, measuring the total MC-LR (intracellular + extracellular) content and reported as total MC-LR per cell, which was termed “cellular MC-LR”. For the long-term experiment (day 27), pelleted cells (collected as described in Section 2.2) were resuspended and processed, specifically measuring the intracellular MC-LR content and reported as intracellular MC-LR per cell, which was termed “intracellular MC-LR”.

MC quantification was performed with an HPLC–MS–MS consisting of an SHIMADZU SIL-30AC HPLC system (Shimadzu Corporation, Kyoto, Japan) and a Sciex API 4000+ LC/MS/MS system (Sciex, Framingham, MA, USA) with a Waters Atlantis C18 column (150 mm $\times$ 2.1 mm i.d., 3.5 μm particle sizes; Waters Corporation, Milford, MA, USA). Mobile phase A consisted of 0.1% formic acid in water, and mobile phase B comprised acetonitrile. The gradient program was initiated at 10% B for 2 min, increased to 60% B over 3 min, maintained at 100% B for 4 min and then reduced to 10% B for 6 min prior to the next injection at a flow rate of 0.25 mL/min. The injection volume was 20 μL , and the column temperature was maintained at 30 $^{\circ}\text{C}$. The main MS parameters were set as follows: desolvation temperature of 500 $^{\circ}\text{C}$, ion spray voltage (IS) of 5500 V and positive electrospray ionization mode. The monitored signals in multiple reaction monitoring mode were precursor $[\text{M}+\text{H}]^+$ m/z 995.3 and product ion m/z 135.1. Quantification of MC was based on the standard curves of the MC-LR standard (Enzo Life Sciences, Lausen, Switzerland).

The analytical method was rigorously validated. The average recovery rate of MC-LR from spiked samples was 107% $\pm$ 11%. The intra-day precision, expressed as relative standard deviation (RSD), was 10.45%. The limit of detection (LOD) and limit of quantification (LOQ), determined at signal-to-noise ratios of 3 and 10, were 0.0079 $\mu\text{g/L}$ and 0.0263 $\mu\text{g/L}$, respectively.

2.5. Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics 26.0 (IBM Corp., Armonk, NY, USA), with statistical significance defined as $p < 0.05$ for all two-tailed tests. Figures were generated using OriginPro 2025b (OriginLab Corporation, Northampton, MA, USA).

For the analysis of growth curves from day 0 to 9, linear mixed models (LMMs) were used to account for the repeated measurements design. The model included Strain, Photoperiod, Time (Day), and their interactions as fixed effects, with a random intercept for Culture ID to model the variability among individual culture units. The specific growth rates among the five groups on the 3rd day were compared using a one-way analysis of variance (ANOVA) for each strain (with homogeneity of variance assessed through Levene’s test and post hoc pairwise comparisons conducted using Bonferroni-corrected tests).

For the analysis of MC-LR production during 0–9 days, the initial LMM was fitted with Strain, Photoperiod, Day, and all of their two-way and three-way interactions, with a random intercept for Culture ID to model the variability among individual culture units. Thereafter, Cell Density was added as a covariate, and the Cell Density $\times$ Photoperiod interaction term was added to the initial model. After removing non-significant variables,

the final model was derived. The assumptions of all LMMs were verified by examining Q-Q plots (normality) and plots of residuals versus fitted values (homoscedasticity). The absence of influential outliers was confirmed by calculating Cook's distance, with all values below the conservative threshold of 1. For significant main effects involving more than two levels, post hoc pairwise comparisons were conducted using Bonferroni correction, with the total number of comparisons explicitly stated in the results. Effect sizes for LMMs are reported as marginal and conditional R^2 values.

The toxin content on day 27 was analyzed using a general linear model (GLM) with Strain and Photoperiod as fixed factors and Cell Density as the covariate. The assumptions of normality, homoscedasticity, and absence of influential outliers were verified as described for the LMMs above. Post hoc pairwise comparisons were conducted using Bonferroni correction. Effect sizes are reported as partial eta-squared (η^2_p).

3. Results

3.1. Short-Term Effects of Light Duration on the Growth of *Microcystis* Species

The growth curves of both *Microcystis* strains (*Microcystis* sp. DH and *M. aeruginosa* FACHB-905) exhibited an upward trend with increasing light duration throughout the 9-day experiment (Figure 1a,b). This trend was corroborated by the analysis of specific growth rates (Figure 1c,d). For both strains on day 3, growth rates under 12L:12D, 14L:10D, and 16L:8D cycles were significantly higher than under the 8L:16D cycle (one-way ANOVA, $p < 0.05$). Notably, *M. aeruginosa* FACHB-905 also showed a graded response, with the growth rate in the 16L:8D group being significantly higher than in the 12L:12D group (one-way ANOVA, $p = 0.001$). After reaching a peak, the growth rates began to decline from day 5 for *Microcystis* sp. DH and from day 7 for *M. aeruginosa* FACHB-905. Results from the LMM for cell density showed that the main effects of strain, photoperiod, time and their interactions were all statistically significant ($p < 0.001$, marginal $R^2 = 0.991$, conditional $R^2 = 0.992$).

3.2. Effect of Different LD Cycles on Cellular MC-LR in *Microcystis* Species over Night Days

The monitoring data are presented in Table 1. To comprehend the general trend, one-way ANOVA was first employed to analyze differences within each day. The five LD cycle groups showed no significant differences in MC-LR production on day 3 or day 5 for either strain ($p > 0.05$ for all). However, significant inter-group differences emerged starting from day 7 for *Microcystis* sp. DH and from day 9 for *M. aeruginosa* FACHB-905. Furthermore, the cellular MC-LR in the 16L:8D groups on day 9 was significantly higher than that on day 3 in *Microcystis* sp. DH and *M. aeruginosa* FACHB-905 ($p < 0.05$). Overall, the monitoring data reveal a clear trend of increasing toxin levels over time and with extended light exposure.

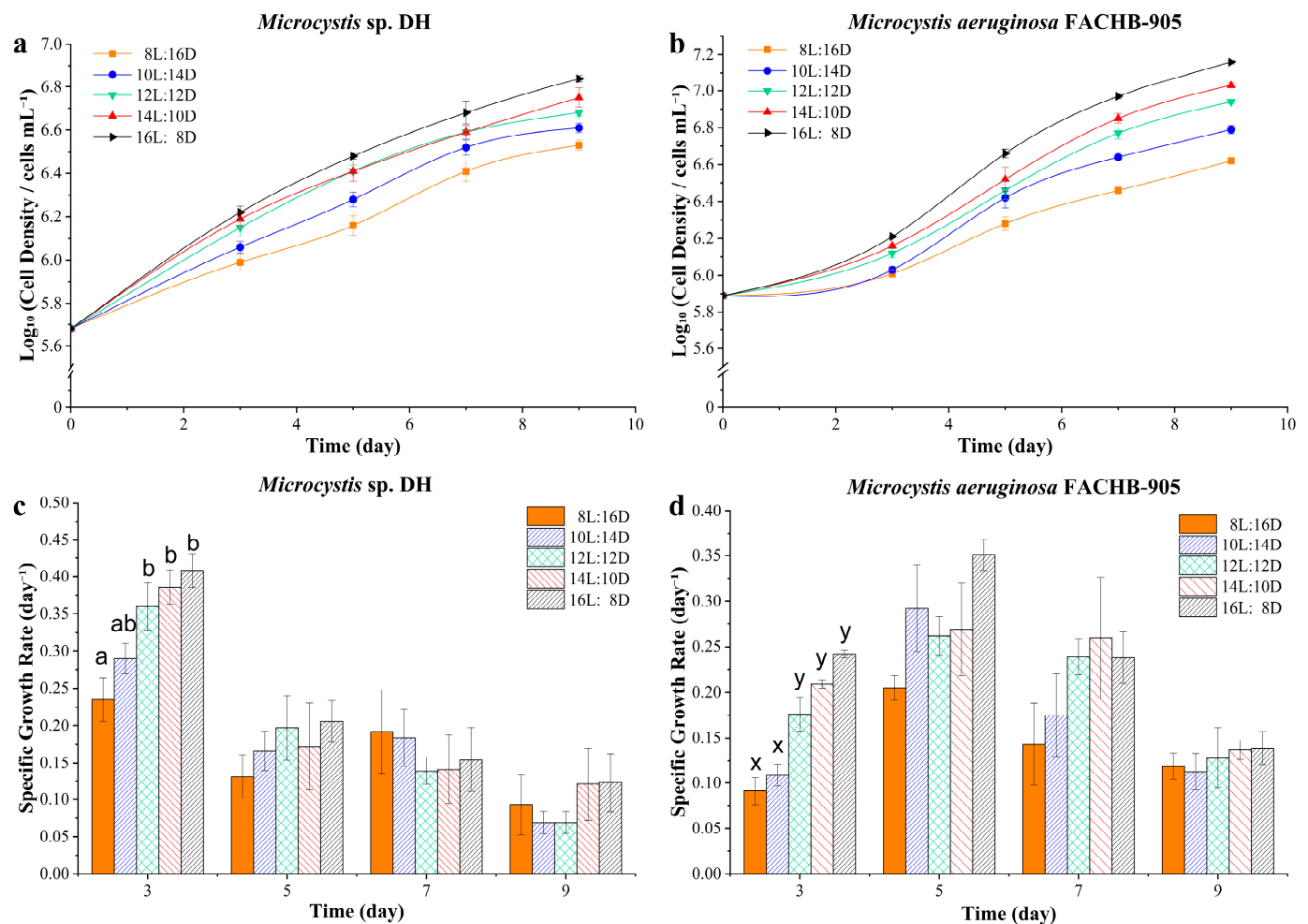


Figure 1. Growth of *Microcystis* species under different light (L)–dark (D) cycles. (a,b) Growth curves for strains *Microcystis* sp. DH and *Microcystis aeruginosa* FACHB-905, respectively. (c,d) Specific growth rates for strains *Microcystis* sp. DH and *M. aeruginosa* FACHB-905, respectively. Error bars represent as mean ± SD (n = 3). Different lowercase letters (a, b for *Microcystis* sp. DH; x, y for *M. aeruginosa* FACHB-905) indicate significant differences among photoperiod groups within the same time point (p < 0.05, Bonferroni-corrected for 10 comparisons).

Table 1. Total microcystin-LR (MC-LR) per cell in *Microcystis* species under different photoperiods (fg/cell).

Microcystis Strain	Time (Day)	Light (L)–Dark (D) Cycles in 24 h				
		8L:16D	10L:14D	12L:12D	14L:10D	16L:8D
<i>Microcystis</i> sp. DH	3	1.70 ± 0.218	1.71 ± 0.473	1.53 ± 0.179	1.61 ± 0.314	1.49 ± 0.074 _A
	5	2.05 ± 0.349	1.83 ± 0.971	2.06 ± 0.431	2.38 ± 0.583	2.95 ± 0.277 _C
	7	1.71 ± 0.286 _a	2.31 ± 0.213 _{ab}	2.85 ± 0.254 _{ab}	3.80 ± 0.449 _b	4.23 ± 0.670 _{b_B}
	9	3.15 ± 0.522 _a	4.09 ± 0.128 _{ab}	4.84 ± 0.341 _b	4.57 ± 0.670 _b	4.89 ± 0.378 _{b_B}
<i>Microcystis aeruginosa</i> FACHB-905	3	6.02 ± 0.162	7.42 ± 0.971	7.64 ± 2.137	8.09 ± 0.730	7.07 ± 1.177 _x
	5	5.20 ± 1.374	5.96 ± 0.376	7.47 ± 0.586	7.43 ± 2.944	8.71 ± 1.810 _x
	7	8.70 ± 0.079	7.24 ± 0.933	6.73 ± 0.852	10.52 ± 1.605	9.50 ± 1.286 _x
	9	5.74 ± 1.315 _x	6.62 ± 0.240 _{xy}	9.16 ± 0.761 _{xy}	11.82 ± 3.447 _y	14.17 ± 0.776 _{y_Y}

Note. Values are monitoring data shown as mean ± SD (n = 3). Statistical significance was determined by one-way ANOVA followed by Bonferroni's post hoc test. Within each row, means with different superscript letters (a, b for *Microcystis* sp. DH; x, y for *M. aeruginosa* FACHB-905) are significantly different (p < 0.05, corrected for 10 comparisons per strain). Within each column, means with different subscript letters (A, B, C for *Microcystis* sp. DH; X, Y for *M. aeruginosa* FACHB-905) are significantly different (p < 0.05, corrected for 8 comparisons per strain).

To identify the factors affecting MC-LR production, LMMs were employed. The LMMs were fitted using data from both *Microcystis* strains, encompassing all five photoperiod

groups per strain. The analysis incorporated 120 data points obtained from $n = 3$ independent cultures per group across four timepoints. Initially, a model incorporating the fixed effects of Strain, Photoperiod, Day, and their full factorial interactions was constructed. This initial model revealed significant main effects of all three factors, in addition to a significant three-way interaction (Strain $\times$ Photoperiod $\times$ Day). Subsequently, cell density was added to the model as a covariate, together with its interaction with Photoperiod. However, the Cell Density $\times$ Photoperiod interaction was not significant ($p = 0.898$) and was consequently removed. The final model thus included the fixed effects of Strain, Photoperiod, Day, Cell Density, and all two-way and three-way interactions among the three primary factors. The variance inflation factors (VIFs) for light treatment groups and cell density were 1.205. The overall effect of Strain $\times$ Photoperiod $\times$ Day interaction (without controlling for cell density) is illustrated in Figure 2, and the complete statistical results of the initial and final models are presented in Table 2.

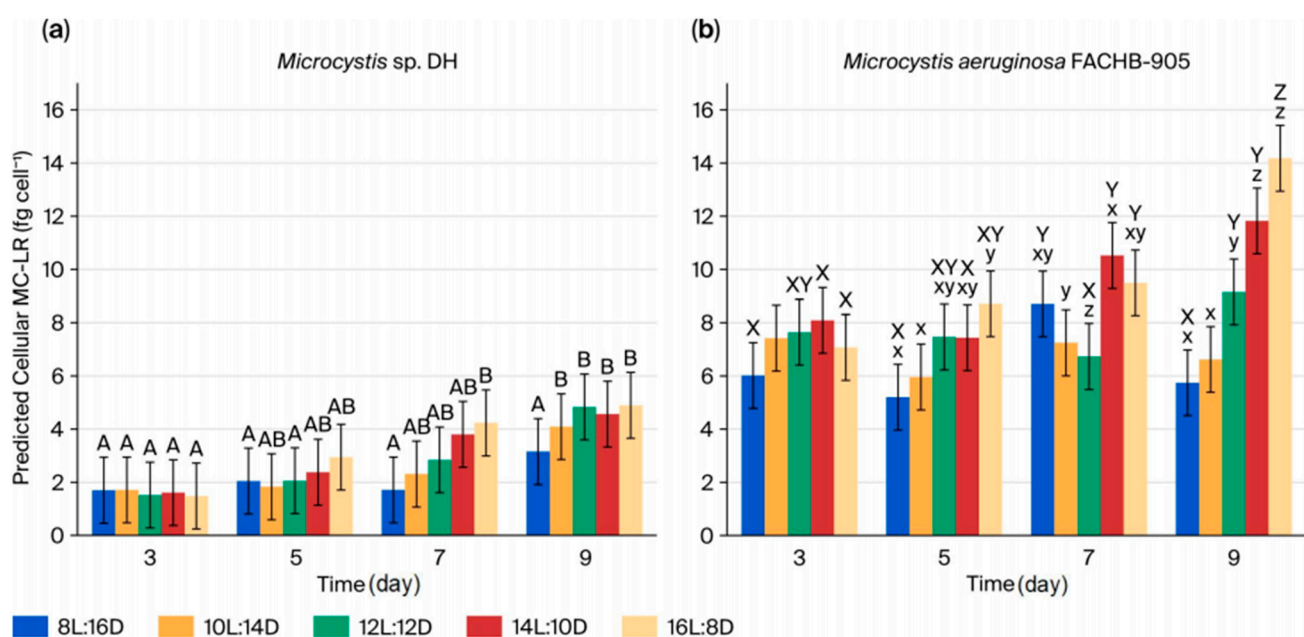


Figure 2. Model-predicted marginal means of cellular MC (total MC-LR per cell, fg cell⁻¹), grouped by strain, photoperiod, and day. Panel (a) shows *Microcystis* sp. DH and panel (b) shows *M. aeruginosa* FACHB-905. Data points and vertical bars represent estimated marginal means and their 95% confidence intervals from the linear mixed-effects model. Different lowercase letters (x, y, z for *M. aeruginosa* FACHB-905) indicate significant differences among photoperiod groups within the same time point ($p < 0.05$, Bonferroni-corrected for 40 comparisons per strain). Different uppercase letters (A, B for *Microcystis* sp. DH; X, Y, Z for *M. aeruginosa* FACHB-905) indicate significant differences across time points within the same photoperiod group ($p < 0.05$, Bonferroni-corrected for 30 comparisons per strain).

Table 2. Statistical analysis of linear mixed-effects models for total MC-LR per cell in *Microcystis* species.

Fixed Effects	Model with Cell Density	Model without Cell Density
Strain	$F(1, 78.8) = 104.21, p < 0.001$	$F(1, 80) = 720.57, p < 0.001$
Photoperiod	$F(4, 24.11) = 7.36, p = 0.001$	$F(4, 80) = 21.26, p < 0.001$
Day	$F(3, 63.94) = 7.64, p < 0.001$	$F(3, 80) = 34.34, p < 0.001$
Cell density	$F(1, 76.65) = 10.99, p = 0.001$	
Strain $\times$ Photoperiod	$F(4, 24.42) = 6.85, p = 0.001$	$F(4, 80) = 6.11, p < 0.001$
Strain $\times$ Day	$F(3, 63.8) = 4.70, p = 0.005$	$F(3, 80) = 1.19, p = 0.320$
Photoperiod $\times$ Day	$F(12, 60.24) = 3.55, p = 0.001$	$F(12, 80) = 4.19, p < 0.001$
Strain $\times$ Photoperiod $\times$ Day	$F(12, 60.24) = 4.61, p < 0.001$	$F(12, 80) = 3.31, p = 0.001$
marginal R^2 /conditional R^2	0.906/0.909	0.894/0.894

Note: Values from two linear mixed-effects models analyzing factors affecting total MC-LR per cell in *Microcystis* sp. DH and *M. aeruginosa* FACHB-905. The left column shows results from the initial model, including Strain, Photoperiod, Day, and their interactions as fixed effects. The right column shows results from the final model that additionally included Cell Density as a covariate. Denominator degrees of freedom were estimated using the Satterthwaite approximation. Significant effects were considered at $p < 0.05$.

As shown in Table 2, the main effects of Photoperiod, Strain, and Day were all significant regardless of controlling for Cell Density, confirming that each factor independently affects MC-LR production. Beyond these independent effects, a significant three-way interaction (Strain $\times$ Photoperiod $\times$ Day) was consistently robust, demonstrating that the influence of light is contingent upon both the strain and the day of measurement. When cell density was added to the model, it itself emerged as a significant predictor, exhibiting a clear main effect. The inclusion of this covariate led to a slight attenuation in the strength of the photoperiod main effect, though it remained highly significant (p -value changed from < 0.001 to 0.001).

Figure 2 illustrates the predicted marginal means of cellular MC-LR levels from the LMM, elucidating the distinct temporal responses of the two *Microcystis* strains to photoperiod. A fundamental strain-specific disparity in MC-LR regulation is evident. Strain *Microcystis* sp. DH presented a photoperiod-dependent manner of temporal response. While MC-LR levels in the 8L:16D group remained stable over time without significant variation, the 10L:14D to 16L:8D groups exhibited significant differences between day 3 and day 9. In stark contrast, Strain *M. aeruginosa* FACHB-905 exhibited a pronounced dual-axis response pattern, characterized by significant differences across both time and photoperiod dimensions. MC-LR levels displayed a marked temporal induction within the same photoperiod and exhibited a clear photoperiod-dependent gradient at the same time point from day 5.

3.3. Long-Term Impacts of the LD Cycle on the Growth of *Microcystis* Species

The results presented in Figure 3 show that the growth of *Microcystis* sp. DH increased in line with the photoperiod duration. In contrast, *M. aeruginosa* FACHB-905 initially followed this trend for the first 15 days. Thereafter, the 14L:10D group surpassed the 16L:8D group to achieve the highest density ($p = 0.014$ on day 24 and 0.038 on day 27); in comparison, the original density ranking among the shorter photoperiod groups (8L:16D to 12L:12D) was maintained. Analysis of the specific growth rates showed an initial consistency with the 0–9 day experimental data. Both strains reached their peak rates on day 3 (*Microcystis* sp. DH) and day 6 (*M. aeruginosa* FACHB-905), respectively, after which a general decline ensued. The growth rates of *Microcystis* sp. DH dropped below 0.1 from day 15; in comparison, those of *M. aeruginosa* FACHB-905 fell below this threshold from day 18. The 16L:8D group in both strains exhibited a marked decline, transitioning from the highest rate initially to a relatively lower level in the later stage.

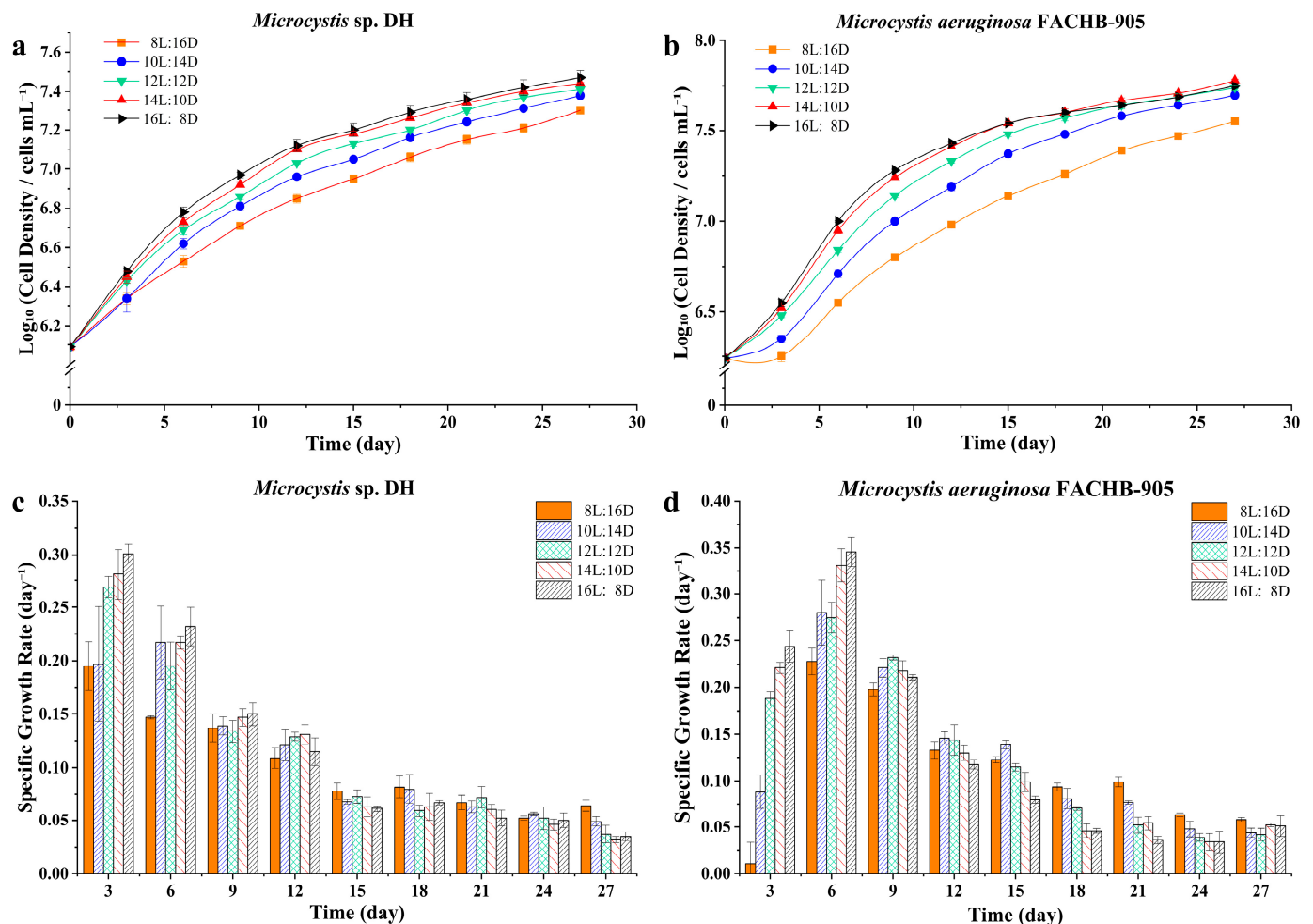


Figure 3. Growth of *Microcystis* species under different LD cycles over a 27 day period. (a,b) Growth curves for strains *Microcystis* sp. DH and *M. aeruginosa* FACHB-905, respectively. (c,d) Specific growth rates for strains *Microcystis* sp. DH and *M. aeruginosa* FACHB-905, respectively. Error bars represent mean $\pm$ SD (n = 3).

3.4. Twenty-Seven-Day Effects of the LD Cycle on the MC-LR Production of *Microcystis* Species

The test data are presented in Table 3. The GLMs were applied to analyze the intracellular MC-LR on day 27. Initially, a model incorporating the fixed effects of Strain, Photoperiod, and their interaction was constructed. Since the Strain $\times$ Photoperiod interaction was not statistically significant ($p = 0.704$), it was removed from the model. Subsequently, Cell Density was introduced as a covariate. The interaction between Photoperiod and Cell Density was also found to be non-significant ($p = 0.140$) and was therefore excluded. The final model included the main fixed effects of Strain, Photoperiod, and Cell Density. The final model revealed significant main effects of Strain ($F(1) = 26.306$, $p < 0.001$, $\eta^2_p = 0.534$), Photoperiod ($F(4) = 9.357$, $p < 0.001$, $\eta^2_p = 0.619$) and Cell Density ($F(1) = 4.84$, $p = 0.038$, $\eta^2_p = 0.174$), indicating that each factor had an independent effect on intracellular MC-LR contents in this growth phase. VIF for light treatment groups and cell density was 1.105. The model explained a significant proportion of the variance (Adjusted $R^2 = 0.886$).

Table 3. Intracellular MC-LR per cell (fg/cell) of *Microcystis* species under different photoperiods on the 27th day.

Light–Dark (L:D)	<i>Microcystis</i> sp. DH	<i>Microcystis aeruginosa</i> FACHB-905
8L:16D	7.67 ± 0.971 ^a _A	15.31 ± 1.251 ^x _X
10L:14D	9.57 ± 1.415 ^a _{AC}	15.55 ± 0.803 ^x _{XZ}
12L:12D	9.36 ± 1.421 ^a _{AC}	17.06 ± 0.897 ^x _{XZ}
14L:10D	10.37 ± 1.828 ^{ab} _{BC}	17.56 ± 0.842 ^{xy} _{YZ}
16L:8D	13.53 ± 3.056 ^b _B	19.26 ± 1.632 ^y _Y

Note: Values are monitoring data shown as the mean ± SD (n = 3). Statistical significance was determined by general linear models followed by Bonferroni's post hoc test. Within each column, means with different subscript letters (A, B, C for *Microcystis* sp. DH; X, Y, Z for *M. aeruginosa* FACHB-905) are significantly different ($p < 0.05$, corrected for 10 comparisons per strain) in the model with cell density as a covariate. Within each column, means with different superscript letters (a, b for *Microcystis* sp. DH; x, y for *M. aeruginosa* FACHB905) are significantly different ($p < 0.05$, corrected for 10 comparisons per strain) in the model without cell density as a covariate. Means sharing a common letter within a column are not statistically different.

Post hoc analyses with Bonferroni correction were conducted to delineate the specific differences among the photoperiod groups. In the initial model (incorporating Strain and Photoperiod without controlling for Cell Density), the pairwise comparisons revealed that the 16L:8D group in both strains exhibited statistically significant differences compared to their respective 8L:16D, 10L:14D, and 12L:12D groups ($p < 0.05$ for all comparisons). When Cell Density was included as a covariate in the model, a broader pattern of group differences emerged. In the final model, in addition to the previously observed differences involving the 16L:8D group, significant differences were also identified between the 14L:10D and 8L:16D groups in both strains ($p < 0.05$). Notably, the MC-LR contents in both strains on day 27 were increased relative to the contents observed during the initial 7-day period of the short-term experiment.

4. Discussion

4.1. Short-Term Photoperiod Extension Promotes *Microcystis* Growth

Light is the critical factor for cyanobacterial survival and growth. Light intensity and light period are the important components of light. In the present study, the effects of different light periods in the LD cycle on two *Microcystis* strains were explored. The results presented in Figure 1 show that extended light exposure enhanced cell proliferation in both strains, as indicated by a duration-dependent increase in cell density throughout the 9-day period. The statistically significant differences in specific growth rates among the five LD cycles further confirmed that light duration enhances cell proliferation. Results from the linear mixed-effects model provide strong statistical evidence that prolonging light time in the LD cycle promotes cell density increase. This trend of growth is consistent with the results of Yuan's study, in which the authors describe the growth trend of a *Microcystis aeruginosa* strain under different LD cycles [40]. The effect of extended photoperiod was similar to that of light intensity, whereby increasing the total light energy input stimulates cell growth [39]. According to the specific growth rate data, day 3 represented the detected logarithmic growth phase for the strain *Microcystis* sp. DH, characterized by nutrient-replete conditions. From day 5 onwards, the specific growth rate decreased, suggesting the emergence of nutrient limitation due to the batch culture. Similarly, strain *M. aeruginosa* FACHB-905 began to experience nutrient limitation starting on day 7 of cultivation. This finding indicated that, from day 5 (for strain *Microcystis* sp. DH) and day 7 (for strain *M. aeruginosa* FACHB-905) onwards, the influencing factors on cells were not only light duration but also changes in the culture environment caused by the increasing cell biomass. The rapid biomass increase under the extended light period during the initial stage suggests its potential as an early warning signal for bloom risk. Therefore, the priority of management strategies at this stage should be to control algal population growth, particularly in summer when the natural photoperiod is long.

4.2. Direct and Indirect Regulation of MC-LR Production by Light During the Initial Phase

In previous studies, it has been reported that the initiation of the light phase can trigger an increase in *mcy* gene expression in algal cells [37,41,42]. To avoid this potential confounding effect, light exposure was synchronized across all experimental groups, and sampling was consistently performed three hours after light onset. Based on the initial analysis of the overall monitoring data (Table 1), a general trend was observed wherein toxin levels in both strains tended to increase with time and longer photoperiods. Given that light clearly promoted early growth, it remained to be determined whether this trend was due to the direct effect of light or indirectly mediated by time and the increasing biomass in the batch culture. To address this question, LMMs were applied to analyze the early-phase toxin data. The results demonstrated that the Strain $\times$ Photoperiod $\times$ Day interaction was statistically significant, regardless of whether Cell Density was controlled for. These findings indicate that the effect of photoperiod on MC-LR production is subject to time-dependent and strain-specific modulation. Furthermore, the significant main effects of strain, photoperiod, day, and cell density confirm that each of these factors is a core variable influencing MC-LR production.

Building on the evidence for a direct photoperiod effect, the persistent and highly significant main effect of photoperiod, which remained even after controlling for cell density, provides compelling evidence for its direct regulatory role. This finding indicates that the direct influence of photoperiod on cellular MC-LR content is, to a substantial degree, independent of its well-established effect on biomass accumulation. The significant Strain $\times$ Photoperiod $\times$ Day interaction, persistent across both models, reveals that the temporal dynamics of MC-LR production in response to photoperiod are fundamentally strain-specific. This complex interaction is clearly visualized in Figure 2, which delineates two distinct response patterns. For *Microcystis* sp. DH, the effect of photoperiod unfolded primarily as a time-dependent process. This pattern suggests a delayed, cumulative response to extended light exposure in this strain. In contrast, *M. aeruginosa* FACHB-905 exhibited an immediate and pronounced dual-axis response. Significant differences emerged as early as day 5, manifesting both across time within the same photoperiod and across different photoperiods at the same time point, establishing a clear photoperiod-dependent gradient. These findings indicate that *M. aeruginosa* FACHB-905 is not only more sensitive to the duration of light but also translates this signal into toxin production through a different regulatory mechanism and on a different timeline than *Microcystis* sp. DH.

In parallel to the direct effect of photoperiod, the LMMs also revealed a significant main effect of Cell Density, underscoring its role as a key predictor of intracellular MC-LR content. Cell density was included as a controlling variable to account for the confounding effects of batch culture dynamics, as the characteristic S-shaped microbial growth curve arises primarily from nutrient depletion driven by increasing biomass [43]. The absence of a significant interaction between Cell Density and Photoperiod ($p = 0.898$) suggests that the manner in which biomass influences toxin levels is consistent across different light regimes. Moreover, the magnitude of the direct effect of Photoperiod on MC-LR was attenuated (from $p < 0.001$ to $p = 0.001$) when Cell Density was added to the model. Coupled with the well-documented promotional effect of prolonged illumination on cell proliferation, these results support the existence of a fundamental indirect pathway whereby photoperiod stimulates growth, and the resulting increase in biomass subsequently affects MC-LR production through mechanisms that become particularly pronounced in dense cultures. Collectively, these results highlight the importance of considering photoperiod not only as a factor influencing growth but also as a direct modulator of toxin production. This understanding suggests that future strategies for bloom and toxin management may benefit from an integrated assessment that accounts for the light environment.

4.3. Long-Term Impact of Extended Photoperiod on *Microcystis* Biomass

Similar to the short-term experiment, cell densities in both strains increased with longer light periods during the first 15 days of the long-term experiment. These growth trends provide evidence that prolonging the light duration could promote cell growth, identical to the 9-day experiment. However, the cell biomass of *M. aeruginosa* FACHB-905 in the longest light period groups failed to maintain the highest level among the five groups on the subsequent days, which indicated that the duration of light exposure in the 16L:8D group was no longer the best condition for biomass accumulation during this growth stage, but instead hinders it directly or indirectly. In addition, the 16L:8D group of *Microcystis* sp. DH maintained the highest biomass among the five test groups after 15 days. Although both strains entered a similar slow-growth phase, as indicated by their specific growth rates falling below 0.1, *M. aeruginosa* FACHB-905 achieved a substantially higher biomass than *Microcystis* sp. DH under identical culturing volumes. Comparing the growth of the two strains, it was found that the cell density of *M. aeruginosa* FACHB-905 is much higher than that of *Microcystis* sp. DH at the same time point. This disparity in yield underscores the existence of strain-specific traits. The difference in the biomass of the two strains, cultivated in media of equal volume, indicates that the higher-biomass strain FACHB-905 experienced more stressful growth conditions. This stress likely arose from the increased demand for limited nutrients within the denser culture, leading to intensified resource competition and autocatalytic effects such as self-shading [44]. For intra-strain comparisons among the five groups, the growth curve showed that the extended light exposure (16L:8D) prompted an initially rapid growth in strain *M. aeruginosa* FACHB-905. However, this could have led to the premature depletion of nutrients in the culture medium. At the same time, the increasing cell density exacerbated the effect of self-shading, reducing light availability per cell. Consequently, with the combined limitation of nutrients and light in the later phase, the final cell density of the 16L:8D group in *M. aeruginosa* FACHB-905 was surpassed by that of the 14L:10D group cultivated under a more balanced light regime. The growth curves of the 27-day experiment indicate that while a prolonged light period can accelerate cell growth, it may ultimately limit the final biomass yield in a finite nutrient supply. These results offer a critical caveat to appropriate parties regarding the risk that overreliance on shading could inadvertently favor peak biomass, underscoring the need for strategies that are context-specific.

4.4. The Evolving Roles of Photoperiod and Biomass in Long-Term Toxin Accumulation

The MC-LR analysis on day 27 further refined the regulatory patterns observed earlier. The results from GLM affirmed that photoperiod continued to exert a significant direct effect on intracellular MC-LR content, independent of cell density. Notably, when cell density was included as a non-interacting covariate in the final model, post hoc pairwise comparisons revealed a broader pattern of significant differences among photoperiod groups, including newly identified contrasts such as that between the 14L:10D and 8L:16D groups in both strains (Table 3). These findings suggest that the direct effect of light was partially obscured by biomass variation in the simpler model, and controlling for this factor enabled a clearer resolution of photoperiod-specific influences on late-phase toxin levels. The decline in specific growth rates below 0.1 supports the presence of a sustained stressful environment during this stage. This led to a hypothesis that a transition in the dominant regulatory mode may occur, from a biomass-driven process in the early phase to a stress-driven process in the late phase. In this context, cell density may have shifted to a confounding background variable, while retaining its own significant main effect—possibly through self-shading, which reduces the average light availability per cell. This hypothesized stress-driven phase could be characterized by several physiological responses not directly measured in this study. For

instance, the nutrient-limited environment likely triggered metabolic adjustments [45,46], which may in turn influence MC synthesis. It is plausible that photoinhibition or oxidative stress, potentially measurable as a decrease in the maximum quantum efficiency of photosystem II (Fv/Fm) or an increase in reactive oxygen species (ROS), accompanied the extended light exposure in the nutrient-depleted medium. Furthermore, upregulation of *mcy* gene expression could be a direct transcriptional response underlying the sustained toxin production. The statistically significant differences in MC-LR content on day 27 may thus represent the outcome of such integrated physiological adjustments during this sustained stress phase, consistent with previously reported stress-induced MC production under nutrient limitation [47,48] or metal stress [49–51].

It is important to note that the measurement for the 27th day specifically quantified intracellular MC-LR. This approach was chosen to provide a clear metric of ongoing toxin synthesis potential by minimizing the confounding influence of differential cell lysis between photoperiod treatments. While this approach does not capture the total toxin load (intracellular + extracellular) and may thus underestimate the absolute environmental risk, its strong positive response to longer photoperiods robustly indicates an overall stimulation of MC production. It is a reasonable inference that a culture maintaining higher intracellular MC-LR per cell would also be likely to contribute more toxin to the extracellular pool. This hypothesis is further supported by the growth dynamics, where longer photoperiods promoted faster growth and likely earlier onset of the stationary phase, suggesting a potentially greater historical accumulation of extracellular MC-LR from cell turnover in these treatments. Consequently, the reported photoperiod-dependent increase in intracellular MC-LR likely represents a conservative estimate of the total toxin-related risk, affirming the significance of photoperiod as a key regulatory factor.

These findings suggest several prudent considerations for bloom management. During the sustained stage of a bloom, reliance solely on biomass monitoring may be insufficient to fully assess toxin risk, given that the direct effect of photoperiod and self-generated stressful conditions appears to contribute to maintaining or elevating intracellular MC-LR levels. Moreover, the observed strain-specificity in toxin response indicates that the dominant species composition should be considered when assessing potential toxin risk. Therefore, management strategies may need to extend beyond biomass control to include monitoring of the in situ light environment and the identification of dominant cyanobacterial populations. An integrated approach that acknowledges these multiple influencing factors could support the development of more tailored and effective mitigation strategies.

4.5. Insights from Controlled Cultures to Natural Contexts

The findings from this batch-culture study clarify the potential role of photoperiod in MC-LR regulation, though their translation to field management requires careful consideration of environmental complexity. It is acknowledged that natural aquatic ecosystems involve hydrodynamic processes, diverse microbial communities, and fluctuating multi-nutrient regimes. While this study utilized a simplified batch culture system, it isolates and elucidates the fundamental effect of light duration, thereby providing crucial insights into the dynamics of natural blooms. This is particularly relevant for turbid, deeply mixed, or high-biomass water bodies, where self-shading and light attenuation create a predominantly low-light environment analogous to the experimental condition ($25 \mu\text{mol m}^{-2} \text{s}^{-1}$). In such systems, relying solely on biomass as a proxy for risk may be inadequate, as the photoperiod-driven shifts in toxin production per cell that are observed could lead to unexpected toxicity.

However, it is important to note that this study employed a single light intensity, resulting in the co-variation in photoperiod and total daily light integral. Thus, the responses

reported herein represent the integrated effect of both variables under controlled settings. Extrapolation to natural environments should be made with caution, as widely fluctuating irradiance levels, variable mixing depths, and turbidity can significantly alter the in situ light climate and interact with photoperiod in complex manners. Despite these complexities, seasonal photoperiod forecasts and diel fluorescence patterns may serve as feasible proxies for light-driven physiological stress in management contexts, while extracellular MC monitoring could complement biomass-based risk assessments. Ultimately, applying these insights in the field will require integrating site-specific hydro-optical conditions with physiological proxies. Future studies incorporating multiple light intensities will be valuable to uncover the effects of photoperiod from total light dose, further refining the management implications proposed herein.

5. Conclusions

This study systematically explored the role of photoperiod in *Microcystis* growth and MC-LR production, finding that photoperiod significantly influenced the growth of the strains. Extended light exposure with suitable intensity significantly promoted early-stage cell growth. However, prolonging the light phase of the LD cycle may limit the maximum biomass under long-term cultivation. During the early growth stage, photoperiod exerted a direct regulatory role on intracellular MC-LR, with effects that were both time-dependent and strain-specific. Concurrently, cell density exhibited a significant independent main effect, supporting the presence of an indirect pathway whereby light regulated toxin production via stimulated growth. In the late growth phase, photoperiod continued to significantly affect MC-LR content, with cell density transitioning into a confounding background factor, though still retaining an independent influence. These findings collectively indicate that the toxicity risk of cyanobacterial blooms is not static but dynamically regulated by light-driven physiological mechanisms. Consequently, in addition to biomass monitoring, risk assessment frameworks should consider incorporating photoperiod conditions and strain variability where feasible, to enable more accurate prediction of toxin dynamics.

Author Contributions: Conceptualization, W.X.; methodology, W.X.; software, L.W. and X.W.; validation, W.X. and X.W.; formal analysis, W.X.; investigation, W.X., X.W. and L.W.; data curation, W.X.; writing—original draft, W.X.; writing—review and editing, W.X.; visualization, L.W.; project administration, W.X.; funding acquisition, W.X. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Jiangxi University of Science and Technology. Grant number: 205200100580.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

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

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