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Influence of wavelength photoperiods and N/P ratio on wastewater treatment with microalgae–bacteria

Juan S. Arcila [a,*], Daniela Céspedes [a,b] and Germán Buitrón [b]

[a] Research Group on Technological and Environmental Development (GIDTA), Universidad Católica de Manizales, Carrera 23 No 60-63, Manizales, Caldas, Colombia

[b] Laboratory for Research on Advanced Processes for Water Treatment, Unidad Académica Juriquilla, Instituto de Ingeniería, Universidad Nacional Autónoma de México, Blvd. Juriquilla 3001, Querétaro 76230, México

*Corresponding author. E-mail: jarcila@ucm.edu.co

JSA, 0000-0002-8890-472X; GB, 0000-0003-3975-7644

ABSTRACT

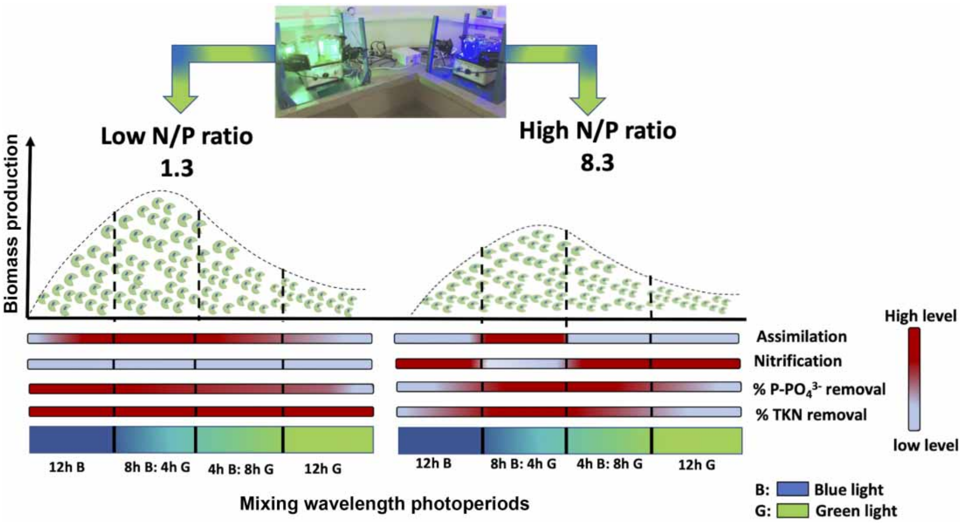
This research investigates the effect of mixing wavelength light photoperiods (12 h blue, 8 h blue: 4 h green, 4 h blue: 8 h green, and 12 h green) and N/P ratios (1.3 to 8.3) on the growth microalgae–bacteria systems, organic matter, and nutrient removals. The highest microalgae–bacteria growth performance ($\mu = 0.2\ d^{-1}$, 481.1 ± 15.3 mg DW L^{-1}) was observed when a 8 h blue: 4 h green mixed wavelength and a low N/P ratio were used. For both N/P ratios, biomass productivity was favored when using the blue light dominated at longer time periods. Mechanisms for nitrogen removal by assimilation depend on the N/P ratio, achieving assimilation between 49 and 65% at a low N/P ratio. High nitrogen removal ($>$50%) showed a strong relation with alkalinity culture conditions (pH $>$ 8.5). The mixing of wavelength photoperiods seems to be a promising strategy to achieve high biomass productivity and nutrient removal. However, for optimal conditions, N/P ratios in the wastewater should be considered.

Key words: biomass productivity, microalgae–bacteria, mixing wavelength photoperiods, nutrients removal, wastewater

HIGHLIGHTS

- Mixing wavelength photoperiods and N/P ratio for microalgae–bacteria grown in wastewater was investigated.
- The highest biomass productivities were obtained by mixing blue (8 h) and green (4 h) LED wavelengths.
- High nitrogen removal and biomass assimilation was observed at a low N/P ratio.
- P limitation affects microalgae–bacteria wastewater treatment performance at the high N/P ratio.

GRAPHICAL ABSTRACT



1. INTRODUCTION

The current trends in wastewater management demand new advanced technologies based on a cost-effective approach and sustainable financing models. The microalgae–bacteria system has been widely recognized as an eco-friendly and sustainable alternative for treating wastewater due to the excellent nutrient removal performances and the potential use of their biomass as a 3G biofuel feedstock and bio-fertilizer (Hussain *et al.* 2021). Microalgae growth uses autotrophic metabolism as the primary method of inorganic carbon fixation (CO_2, HCO_3^-) by utilizing the ATP and NADPH of the light reaction through the Calvin–Bensen cycle (Su 2021). In the photosynthesis light reaction, sunlight is the most cost-efficient energy source used by the microalgal system. However, the low photosynthetic solar light conversion efficiency (between 1.3 and 2.4%) by microalgae causes high degrees of variation in the environmental conditions associated with light photoperiods (night: light) cycle, seasonal changes, and weather conditions (Park & Craggs 2011). High sunlight levels above saturation trigger photoinhibition due to photo-oxidative damage that occurs in Photo-System II. In contrast, low sunlight levels decrease biomass growth and accumulation of bioproducts (Wang *et al.* 2014). These characteristics ultimately affect nutrient removal and stability of the wastewater treatment process. During the last decade, artificial LED light has gained relevance due to its ability to mitigate the adverse effects of sunlight fluctuation and regulate photosynthetic photon flux density (PPFD), optimizing biomass growth and biochemical composition of the microalgae (Barreira *et al.* 2014; Duarte & Costa 2018).

Additionally, high durability (>50.000 h), absence of toxic compounds such as mercury (Duarte & Costa 2018), and low energy consumption make LED light the ideal artificial photon supply source for microalgae growth at full scale (Yan *et al.* 2013a). White LED light has been utilized as a conventional light source for microalgae growth due to its wide spectrum that significatively covers the absorption bands of the chlorophyll pigment (450–475 nm and 630–675 nm). However, narrow-band light spectrums such as blue (420–450 nm) and red (660–700 nm) in wastewater have been demonstrated to have the capability not only to achieve high biomass productivity but also to increase the production of metabolites with high added values such as carotenoids (Xu *et al.* 2019) and lipids (He *et al.* 2021).

Comparative studies have evidenced that the low wavelength of blue light promotes higher energy efficiency than red light (Gatamaneni *et al.* 2020; He *et al.* 2021). In addition, blue light helps to regulate gene transcription and pigment production that favor microalgae acclimation under light stress conditions (Tanno *et al.* 2020). Previous studies have evaluated biomass productivity and nutrient removal at different wavelengths. Chlorophyte species such as *Chlorella* sp. and *Scenedesmus* sp. are predominant in several types of wastewater sources and in the structure of granular microalgae–bacteria aggregates (MABAs) (Arcila & Buitrón 2017) and have presented an excellent growth rate and pigment synthesis under LED blue wavelengths (Kang *et al.* 2015). However, this photosynthetic activity presented photoinhibition at high light intensity (>2,000 µmol m^{-2} s^{-1}). In contrast, green light did not show inhibition under the same light intensity conditions (Kang *et al.* 2019). Although green light is not an appropriate light source for chlorophyte growth due to the absence of phycobilins;

the presence of a green wavelength band in the photosynthetically active radiation (PAR) range enhances microalga productivity and pigment production in *Chlorella vulgaris* and *G. membranacea* (Mohsenpour *et al.* 2012). The characteristics mentioned above make the interaction between green and blue light an attractive strategy for a microalgae–bacteria wastewater treatment approach with biotechnological perspectives. A strategy based on mixing wavelengths demonstrated that blue: green light ratio (50:50) promotes higher nitrogen removal than single blue light (Xu *et al.* 2019). Furthermore, Bredda *et al.* (2019) demonstrated that a mixing wavelength ratio of 65% blue and 35% green light led to a high *Dunaliella salina* biomass productivity (105.06 mg/L d) coupled with outstanding lipid production (51%). Similarly, Jung *et al.* (2019) proposed a two-phase culture process based on growing phases carried out under blue light conditions (12 days) followed by a green wavelength stress condition (3 days). This strategy achieved high *Phaeodactylum tricornutum* and *Isochrysis galbana* biomass productivity ranges from 66 to 77 mg L^{-1} d^{-1} with lipid production up to 60%. Despite the mixing wavelength light strategy that seems to be applied to different pure microalgae species, their behavior still is uncertain in terms of the microalgae–bacteria treatment process.

Apart from the light wavelength, the microalgae–bacteria system scaling-up shows a strong dependency on the C/N/P ratio of the wastewater. The stoichiometric Redfield ratio of 106:16:1 (mass N/P ratio 7.2) has been considered an optimal nutrients supply ratio in the medium for microalgae growth. However, this mass ratio may fluctuate in wastewaters from 5.1 to 10, depending on the type of wastewater (Wang *et al.* 2010; de Godos *et al.* 2016). In a conventional municipal wastewater system, the two main streams, primary settled wastewater (PSW) and secondary treatment effluent (STE) show an average C/N/P ratio of 100/19/3 and 100/34/7 (Mohsenpour *et al.* 2021). Comparative studies of microalgae–bacteria treatment confirm that a C/N/P ratio close to the Redfield ratio observed by the PSW streams shows a higher nutrient removal capacity (68.5% TN and 90.6% TP) and growth rate (0.22 to 0.42 d^{-1}) than STE (Wang *et al.* 2010; Cabanelas *et al.* 2013). Similar trends were observed in original piggery effluent (OPE: N/P = 15.4:1, molar ratio) and anaerobically-digested piggery effluent (DPE: N/P = 21.8:1, molar ratio), in which the highest removal rates for both N-NH_4^+ P-PO_4^{3-} and biomass concentration of 0.33–0.39 g L^{-1} were detected in DPE streams (Li *et al.* 2021).

In contrast, wastewater streams from central lines after sludge dry in municipal wastewater (Wang *et al.* 2010; Cabanelas *et al.* 2013) had a high concentration of nitrogen and phosphorus (>130 mg L^{-1} and >55 mg L^{-1}, respectively) and no evidenced significant differences in the nutrients removal rate and biomass productivity even though their N/P showed a substantial deviation from the Redfield N/P ratio. These facts demonstrate that the N/P ratio in wastewater is a key factor for evaluating the microalgae–bacteria performance. However, these variables interactions (wavelength light and N/P) and their effect on photosynthetic activity relative to growth rate and nutrients removal efficiency remain primarily unknown. This research focused on the response of biomass growth rate and nutrient removal in microalgae–bacteria systems exposed to a novel mixing wavelength light strategy based on variation in the blue and green photoperiods under two different N/P ratios utilizing synthetic wastewater.

2. MATERIALS AND METHODS

2.1. Microalgae–bacteria inoculum and synthetic wastewater

A mixture of microalgal biomass was collected from a lake reservoir located at Chinchina, Caldas, Colombia (4°59′24,585″N 72°36′240″W). The microalgal mixture was grown and maintained in a 2 L working volume photobioreactor made of acrylic, containing Synth medium composed of a mixture of synthetic wastewater and a minor percentage (10% v/v) of domestic wastewater taken out of a discharge from a residential area. The synthetic wastewater is a modification of Nopens *et al.* (2001) comprise 91.7 mg L^{-1} of CH_4N_2O, 12.75 mg L^{-1} of NH_4Cl, 79.3 mg L^{-1} of $C_2H_3NaO_2$, 17.4 mg L^{-1} peptone, 23.4 mg L^{-1} of KH_2PO_4, 23.72 mg L^{-1} of NaH_2PO_4, 14 mg L^{-1} of MgCl, 5.8 mg L^{-1} of $FeSO_4.7H_2O$, 122 mg L^{-1} of starch, 116 mg L^{-1} of milk powder, 52,24 mg L^{-1} of yeast, 29,02 mg L^{-1} of soy oil, 0.1 mg L^{-1} of $Pb(NO_3)_2$ 0.1 mg L^{-1}, 0.57 mg L^{-1} of $CuSO_4$, 0.108 mg L^{-1} of $MnSO_4.H_2O$, 0.208 mg L^{-1} of $ZnCl_2$, 0.336 mg L^{-1} of $NiSO_4.6H_2O$ and 0.77 mg L^{-1} of $Cr(NO_3)_3$. Active sludge collected from a domestic wastewater treatment plant and the microalgae consortium was inoculated in to the systems under a ratio of 1:1 (w/w), achieving an initial biomass concentration of 200 mg VSS L^{-1}. The culture was constantly stirred at 150 rpm in batch mode for eight days (corresponding to the exponential growing period) at an average temperature of 22 °C with light:dark illumination photoperiods (12 h:12 h) using white LED lamps of 10 W under a light intensity of 120 μmol m^{-2} s^{-1}. At the end of every batch cycle, 90% of the culture was extracted and recharged with a fresh Syntho medium. Injection of CO_2 into the culture was not considered to control the pH. *Chlorella* sp. was microscopically identified as the dominant genus.

2.2. Experimental set-up

A 500 mL cylindrical glass bottle containing 400 mL Synth wastewater was used for microalgae–bacteria growth. Two Synth wastewater levels with a low inorganic N/P of 1.3 and high N/P ratio of 8.3 were prepared through the adjustment of inorganic nitrogen (N-NH_4^+) and phosphorus (P-PO_4^{3-}) concentrations of the synthetic medium (Table 1), ensuring initial nutrient conditions without phosphorus limitation. The N/P ratios were exposed to a mixture of blue (B) and green (G) light under different exposition times: 12 h B, 8 h B: 4 h G, 4 h B: 8 h G, and 12 h G, all in triplicate. LED RGB lamps of 10 W (85 mm wide and 115 mm long; GR-TGD-10 W) installed around the glass bottle provided a light irradiance of 136 ± 11 μmol m^{-2} s^{-1}. The B and G photoperiods were controlled using an Arduino ONE with light sensor software -TSL2561 (Figure 1). A white box covered the experimental set-up to avoid undesirable wavelengths. The settings used in each experiment lasting 14 days operated at a stirring speed of 150 rpm, an average temperature of 22 °C, and initial pH of 6.8. The typical light photoperiod of 12 h:12 h light:dark observed in the equatorial zone was considered during the experimental process.

2.3. Microalgae growth and kinetic growth

The biomass growth was measured by optical density (OD) utilizing a UV-VIS Pharo 300 Spectroquant at 680 nm. Linear regression between OD_{680} and dry weight of microalgae–bacteria (DW, mg/L) was used as a calibration curve (Equation (1)). Biomass dry weight was measured by filtering an aliquot of the culture suspension through a glass microfiber filter

Table 1 | Physicochemical characteristics of synthetic wastewater under two N/P ratios, Low N/P (L-N/P) and High N/P (H-N/P)

Parameters	L-N/P	H-N/P
COD (mg L^{-1})	179 ± 33	183 ± 25
TKN (mg L^{-1})	35.9 ± 7.5	52.3 ± 9.8
N-NH_4^+ (mg L^{-1})	15.1 ± 3.1	35.1 ± 6.2
P-PO_4^{3-} (mg L^{-1})	11.5 ± 4.6	4.2 ± 0.4
Inorganic N/P ratio	1.3	8.3
VSS (mg L^{-1})	185 ± 18	211 ± 21
pH	6.8 ± 0.3	6.7 ± 0.2

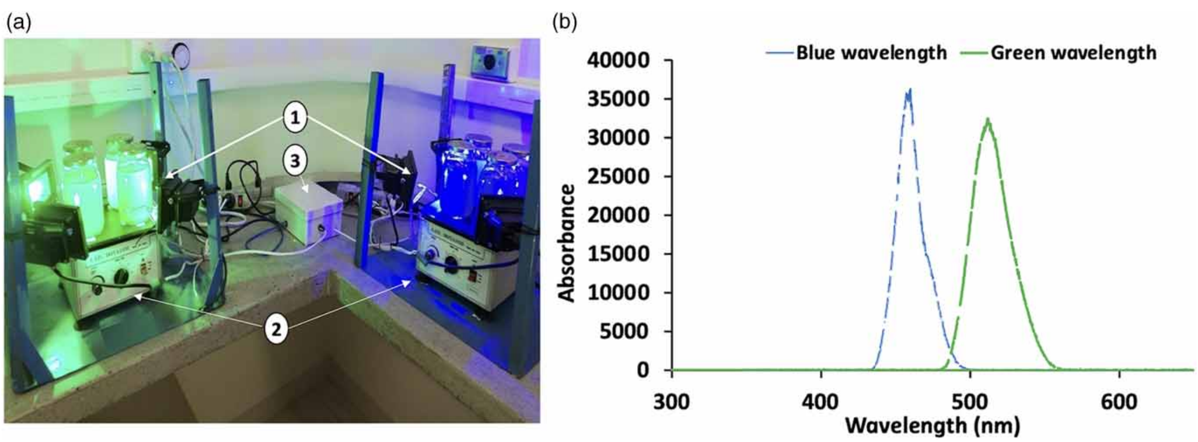


Figure 1 | (a) Microalgae–bacteria experimental set-up: (1) Light configurations with a constant light intensity of 120 μmol m^{-2} s^{-1}, (2) orbital shaker systems, (3) Arduino ONE control system with light sensor software -TSL2561. (b) Spectral composition of blue and green lighting systems employed during the experimental process.

with a pore size of 0.45 μm. After that, the filter was dried at 105 °C for 12 h.

$$DW(mg\,L^{-1}) = 479\,OD_{680} + 64\,R^2 = 0.992 \quad (1)$$

The specific growth rate is a measure of the biomass increase over time and it was determined through a first-order kinetic model (Equation (2)). Where μ is the specific growth rate (d^{-1}), X_1 and X_2 are the cell dry weight concentration (mg L^{-1}) at time t_1 and t_2, respectively, t is the number of days.

$$\mu = \frac{\ln \frac{X_2}{X_1}}{t_2 - t_1} \quad (2)$$

2.4. Nitrogen balance

The mechanisms of nitrogen removal were defined based on a mass balance considering the following measure nitrogen soluble sources. Total kjeldahl nitrogen (TKN: organic nitrogen + $N\text{-}NH_4^+$), $N\text{-}NO_x^-$ ($N\text{-}NO_2^- + N\text{-}NO_3^-$) at the beginning and ending of the experimental process. The mechanisms of nitrogen uptake by microalgae–bacteria (TN_{upkate}), nitrification ($TN_{nitrification}$), and nitrogen loss associated with stripping (TN_{loss}) were considered for the nitrogen balance. Denitrification was negligible because dissolved oxygen, throughout the experimental test, was maintained above 1.0 mg O_2/L. Typical nitrogen microalgae–bacteria content in wastewater ranged from 0.05 to 0.092 mgN mgVSS^{-1} (stoichiometric fraction in microalgae biomass) (Mara 2004; de Godos *et al.* 2016). An average of 0.071 mgN mgVSS^{-1} was considered to calculate the TN_{uptake}. The N_{loss} was determined from Equation (3).

$$N_{Loss} = TN_{intel} - TKN_{outlet} - TN_{uptake} - TN_{nitrification} \quad (3)$$

2.5. Analytic methods

Daily monitoring parameters such as pH were measured with pH (Thermo Scientific ORION 3 star), while the dissolved oxygen (DO) and temperature were recorded by the DO sensor (HANNA HI98193). The filtrate was used to evaluate the soluble organic and nutrients removals for each experiment condition. $N\text{-}NH_4^+$, COD, and $P\text{-}PO_4^{3-}$ were determined through the 8,000 HACH, 10,031 HACH, and 10,127 HACH colorimetric methods, and nitrate and nitrite were determined by the 91,865 and 91,867 Macherey-Nagel NANOCOLOR method, respectively. Total Suspended Solid (TSS), Volatile Suspended Solid (VSS), and total Kjeldahl nitrogen concentration were analyzed according to Standard Methods (APHA 2005). For morphological identification of microalgal species, light microscopy OLYMPUS CH microscopic, equipped with a camera Amscope MD500 for image acquisition, was used. The microalgae genera were identified according to Wehr & Sheath (2003). The light irradiance was measured in units of W/m^2 utilizing a Pyranometer apogee Model MP-100 and convert to photon flux (μmol/m^2 s) according to Taiz *et al.* (2015).

2.6. Statistical analyses

All the statistical analyses were conducted by R software. Two-way ANOVA analyzed the effect of the mixing wavelength photoperiods, N/P, and their interaction with a significance level of $p < 0.05$. Significant differences were evaluated through Tukey honest significant differences (HSD) test.

3. RESULTS AND DISCUSSION

3.1. Biomass production

The interaction impact of the mixture of the different blue and green light photoperiods on the microalgae–bacteria growth for 14 days was evaluated. Two key growth phases were analyzed. During the first phase from 0 to 7 days (Figure 2), the maximum specific growth was achieved for all the experimental conditions. At a low N/P ratio (L-N/P), a maximum specific growth rate of 0.2 d^{-1} was observed under light photoperiods of 8 h B: 4 h G and 12 h G. However, 12 h G evidenced a lag phase for three days coupled with a sharp decrease in the specific growth rate which caused a lower biomass concentration (417.6 ± 14.3 mg DW L^{-1}) compared to 8 h B: 4 h G conditions (481.1 ± 15.3 mg DW L^{-1}) at the end of this first

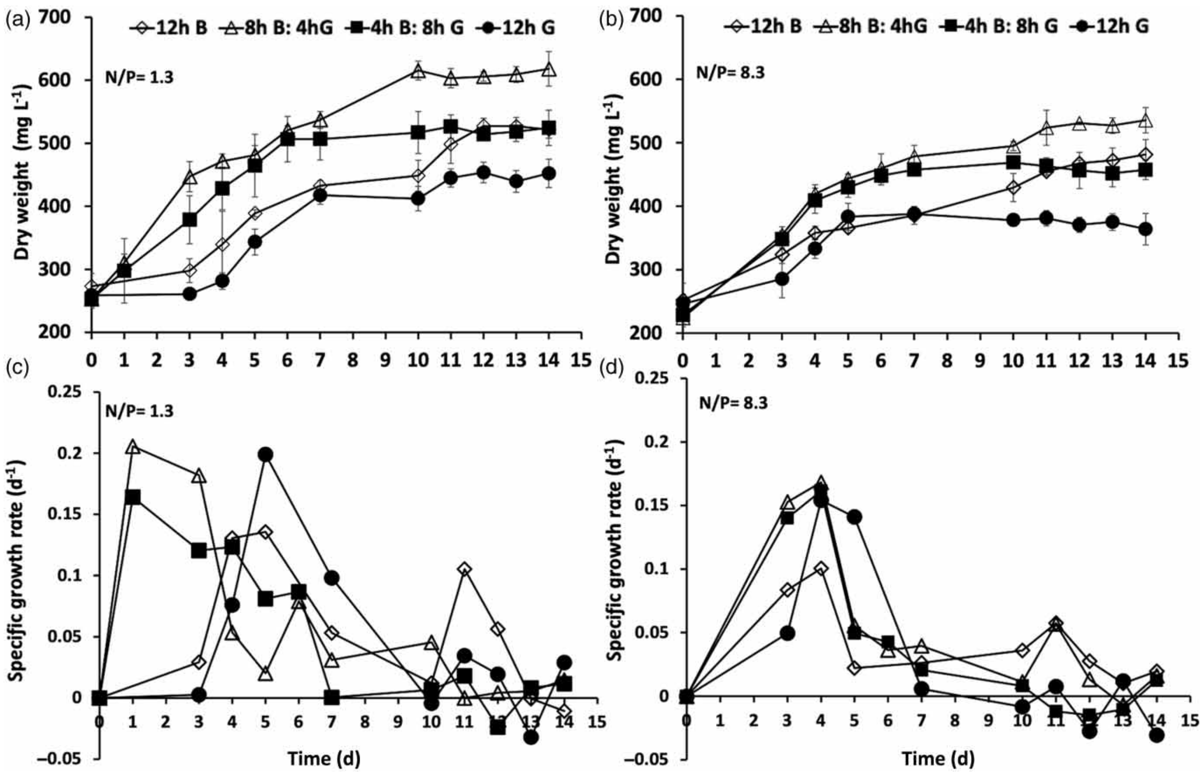


Figure 2 | Biomass density (dry weight) and specific growth rate in microalgae–bacteria system cultivated at two N/P ratios, L-N/$P = 1.3$ (a, c) and H-N/$P = 8.3$ (b, d), under different mixing wavelength photoperiods of 12 h G, 8 h G: 4 h B, 4 h G: 8 h B and 12 h B. Data are expressed as mean $\pm$ SD, $n = 3$.

phases. As for the high N/P ratio (H-N/P), all light photoperiod conditions reached a similar specific growth rate (ranges of 0.15 d^{-1} to 0.17 d^{-1}) at four days, except at 12 h B, which evidenced the lowest specific growth rate of 0.1 d^{-1}. In terms of biomass concentration, in both N/P ratios, the mixed wavelength photoperiod (8 h B: 4 h G and 4 h B: 8 h G) was higher than the single wavelength photoperiod (12 h G: 12 h B). However, no significant differences were observed between the two light photoperiods conditions of 8 h B: 4 h G and 4 h B: 8 h G, achieving an average concentration of 521 mg DW L^{-1} and 467 mg DW L^{-1} at N/P ratios of 1.3 and 8.3, respectively. In contrast, 12 h B and 12 h G light photoperiods presented a low biomass performance with an average concentration of 425 mg DW L^{-1} and 386 mg DW L^{-1} at N/P ratios of 1.3 and 8.3, respectively. These trends could be supported by the analysis of the specific growth rate (Figure 2(c) and 2(d)), in which 12 h B light evidenced the lower value (0.1 to 0.13 d^{-1}), while 12 h G light, although achieving higher values (0.15 to 0.2 d^{-1}) than 12 h B, its sharp, specific growth rate decreased (at H-N/P) ratio and long lag phase (at L-N/P) did not favor the final biomass concentration at seven days. These poor biomass productivity performances at blue and green lights agree with Yan *et al.* (2013a, 2013b), who reported that in synthetic high-strength wastewater (N/P ranges from 10 to 28), the blue and green wavelengths under light photoperiods of 12 h:12 h (light:dark) and intensity of 2,000 μmol m^{-2} s^{-1} are not a feasible light condition for *Chlorella* sp. genus growth. Nevertheless, Kang *et al.* (2015) suggested that 24 h of blue wavelength illumination (light intensity of 50 μmol m^{-2} s^{-1}) in municipal wastewater promoted optimal growth of *Chlorella* sp. and *Scenedesmus* sp. compared to a green wavelength. In concordance with this research, our results seem to indicate that during the short growth phases ($<$7 days), the single green and blue light under photoperiods of 12 h:12 h (light:dark) is not an optimal condition for microalgae–bacteria growth.

Conversely, the outstanding performance observed at 8 h B: 4 h G and 4 h G: 8 h B could be attributed to the presence of the green wavelength. Mohsenpour *et al.* (2012) observed that a green light wavelength band into the photosynthetic active radiation band (PAR, $\lambda \approx$ 400–700 nm) is essential to promoting a high *Chlorella vulgaris* growing performance, achieving a specific growth rate above 0.1 d^{-1}, which is slightly lower than that obtained in our study. In this sense, our results suggested a quick adaptation of the photosynthetic apparatus of *Chlorellaied* sp. at the perturbation of the wavelength photoperiods of blue and green light. Nevertheless, the stability of the adaptation for long time operative periods in continuous mode should be evaluated and compared with its performance with conventional white light spectrum.

At the second phase (8 to 14 days), the green and blue light's exposition time evidenced significant variability in the biomass growth. Contrary to the results during the first phase, 8 h B: 4 h G showed an average biomass increase of 14% concerning 4 h B: 8 h G under both N/P ratio, leading to maximum biomass concentration of 618 $\pm$ 27 mg DW L^{-1} and 535 $\pm$ 27 mg DW L^{-1} at L-N/P and H-N/P, respectively. Additionally, 12 h B and 4 h B: 8 h G conditions did not show significant differences in the biomass concentration. However, using 12 h G, the lowest biomass performances (452 $\pm$ 22 mg DW L^{-1} and 363 $\pm$ 24 mg DW L^{-1} at $N/P = 1.3$ and $N/P = 8.3$) were obtained. The explanation could be the changes in the microalgae metabolism toward the accumulation of lipid, as was detected by Nie *et al.* (2020), growing *Golenkinia* SDEC-16 (Chlorophyta species) in domestic wastewater. These results implied that the microalgal production for a long-term reaction is favored when the blue light photoperiod is the dominant spectrum in the wavelength mixture. Previous studies have shown the potential increase of lipid content (up to 50%) in microalgae monocultures such as *Dunaliella salina* and *Isochyrsis galbana* applying a mixed blue:green light strategy (Bredda *et al.* 2019; Jung *et al.* 2019). The novel strategy of mixed photoperiod presented in our work could be further evaluated considering a biofuel perspective.

Table 2 shows the average physicochemical parameters and specific microalgal growth rate after 14 days. In both N/P ratios, high dissolved oxygen is observed under mixing light conditions of 8 h B: 4 h G and 4 h B: 8 h G (range from 3.9 to 4.5 mg O_2 L^{-1}), while a marginal decrease of dissolved oxygen (<3.6 mg O_2 L^{-1}) is detected under 12 h G and 12 h B conditions. Therefore, all the experimental conditions meet the minimum oxygen requirement for the aerobic heterotrophic microorganisms ($O_2 > 1.5$ mg O_2 L^{-1}). Before finishing the dark photoperiods, the DO concentration was monitoring periodically, observing in all batch tests a DO concentration above 1.0 mg L^{-1} (data not shown), indicating no presence of the culture's anoxic condition.

A high N/P ratio evidenced control near pH 7, except under light conditions of 8 h B: 4 h G, which generated an average pH of 8.5. This increasing pH was also observed throughout the experiments carried out at low N/P, promoting alkalinity conditions in the wastewater system. High pH levels (pH >9) suggest total CO_2 fixation via photosynthesis. Previous studies have demonstrated that alkalinity conditions encourage the *Chlorella* photosynthetic activity (Ihnken *et al.* 2014), and attributed this behavior to the *Chlorella* capacity to regulate the CO_2 concentrating mechanisms (CCMs), leading to acquiring both CO_2 and HCO_3^- effectively. The findings support the highest biomass growth detected in our systems under L-N/P (pH> 9), dominated by the *Chlorella* genus. Conversely, fluctuation in pH has been associated with the carbon sources used for microalgal cell synthesis. According to Kim *et al.* (2013), pH increase is caused by the dominance of autotrophic mechanisms, while neutral pH is associated with mixotrophic mechanisms. It could suggest that L-N/P promoted autotrophic carbon consumption, while H-N/P leads to mixotrophic growing. Figure 3 depicts the dominance of *Chlorella* sp. throughout the experimental process in terms of the microalgal community. N/P ratios influenced microalgae community diversity. L-N/P ratio did not show a significant variation in both morphology and microalgae diversity. The presence of single-cell growth is prevalent under all wavelength photoperiods (Figure 3(a)–3(d)). In contrast, H-N/P conditions demonstrated filamentous communities' presence when blue light was the dominant photoperiod (12 h B, 8 h B: 4 h G). Under these conditions, small aggregates are formed in the system (Figure 3(e) and 3(f)). According to Arcila & Buitrón 2017, the presence of filamentous species promotes the formation of microalgae–bacteria aggregates (MABAs), due to their ability to produce hydrophobic proteins that

Table 2 | Physicochemical parameters and maximum specific growth rate during the microalgal growth dynamic at different photoperiods of blue (B) and green (G) light wavelengths

	pH		**Dissolved oxygen (DO) (mg/L)**		**Specific growth rate (μ) (d^{-1})**	
Light condition	**L-N/P**	**H-N/P**	**L-N/P**	**H-N/P**	**L-N/P**	**H-N/P**
12 h B	9.5 $\pm$ 0.8	6.9 $\pm$ 0.4	3.8 $\pm$ 0.8	3.4 $\pm$ 0.6	0.13	0.1
8 h B: 4 h G	9.3 $\pm$ 0.7	8.9 $\pm$ 0.5	4.6 $\pm$ 0.5	3.8 $\pm$ 0.5	0.20	0.17
4 h B: 8 h G	8.7 $\pm$ 1.1	6.8 $\pm$ 0.6	4.2 $\pm$ 0.4	3.8 $\pm$ 0.4	0.16	0.16
12 h G	9.2 $\pm$ 1.0	6.8 $\pm$ 0.4	3.5 $\pm$ 0.6	3.5 $\pm$ 0.6	0.20	0.15

pH and DO records in the table only considered the measures during the second growth phase (8–14 days).

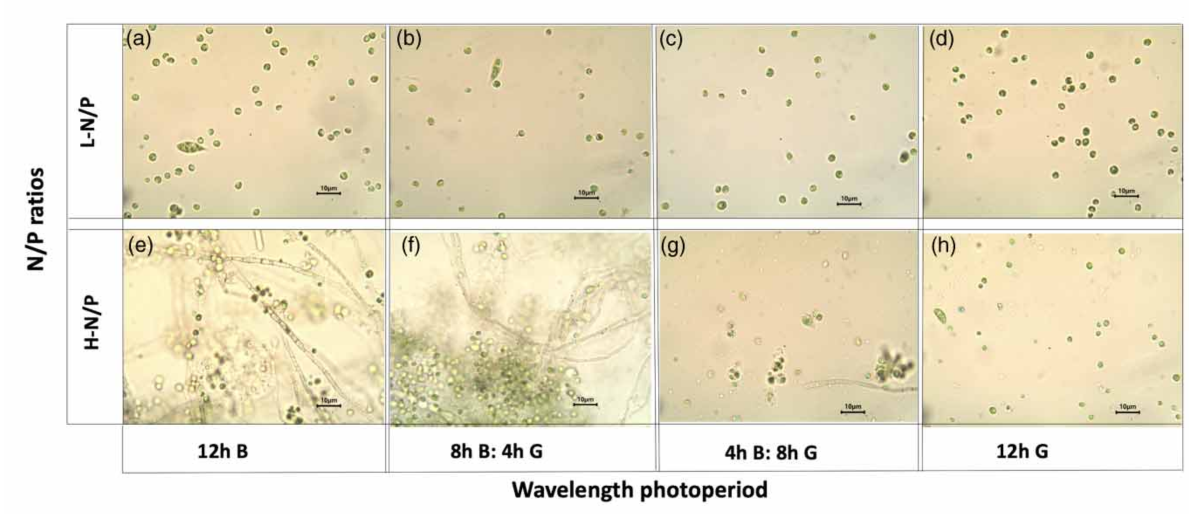


Figure 3 | Microscope images of the microalgae–bacteria system. Micrographs from (a to h) represent the microalgal community observed at 10 μm. (a–d): Microalgae growth under L-N/P ratio utilizing wavelength photoperiod conditions of 12 B, 8 h G:4 h B, 4 h B:8 h G, 12 G. (e–h): H-N/P ratio at the same wavelength photoperiods mentioned above.

favor the attachment of microalgae and bacteria. In this context, limiting P conditions in our system favor the filamentous species growth. However, the predominance of filamentous species and symbiotic interaction with bacteria and microalgae to form large-sized aggregates under wavelength photoperiods dominated by blue light should be evaluated.

3.2. Evaluation of organic matter removal

Figure 4 shows the COD removal at two N/P ratios and four mixing wavelength light photoperiods after 14 days of batch operation. The medium mixing wavelength of 8 h B:4 h G and 4 h B:8 h G achieved the best performance with an average COD removal of 57 and 65% at low N/P and high N/P ratios. Similar COD removal (range from 57 to 83%) has been reported in wastewater with an N/P ratio near to 5 (Wang *et al.* 2010). In contrast, single wavelength light exposition evidenced a low COD removal (<40%). Similar low-efficiency removal below 12% under green wavelength was detected under a light intensity of 2,000 μmol m^{-2} s^{-1}. In terms of N/P variations, COD removal did not show significative differences (p-value >0.05). Consistent with this low COD removal obtained at 12 h B and 12 h G, studies by Lee *et al.* (2016) and Lima *et al.* (2020) showed that the microalgae treatment alone is not effective in reducing the COD, even finding negative COD removal performances. This trend has been attributed to the excretion of secondary metabolites such as glycolic acid

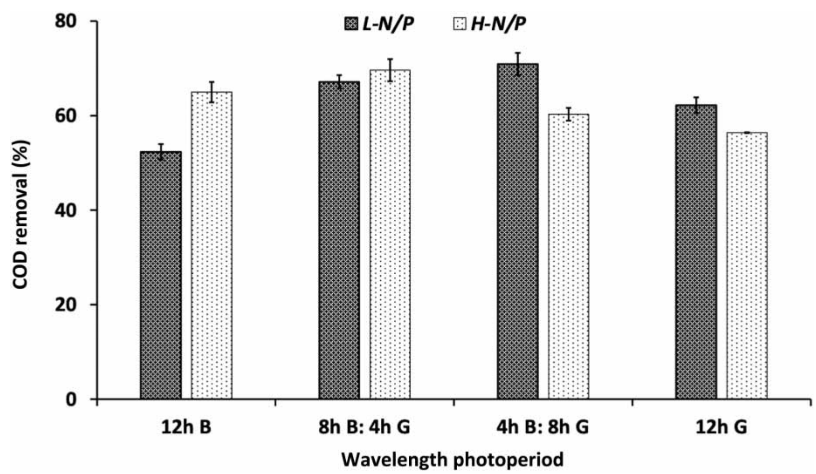


Figure 4 | COD removal profile under four different blue (B): green (G) mixing wavelength under L-N/P and H-N/P conditions. Data are expressed as mean ± SD, $n = 3$.

(Wang *et al.* 2010) and toxic bactericidal compounds (Leflaive & Ten-Hage 2007) that limited the COD removal efficiency of the microalgae–bacteria system. Concomitantly with the outstanding biomass growth performance (Figure 2), the high COD removal under mixing wavelength photoperiods (8 h B:4 h G and 4 h B:4 h G) seems a reasonable condition to promote an increase in the heterotrophic activity of the microalgae–bacteria system. However, this should be analyzed deeper in further studies to elucidate the carbon consumption mechanisms under different wavelength photoperiod conditions.

3.3. Evaluation of nutrients removal

Figure 5 depicts the kjeldahl nitrogen (A), ammonia (B), and phosphorus (C) removal efficiency after 14 days of a batch process. The best nitrogen removal performances were observed at an L-N/P ratio, achieving a TKN and N-NH_4^+ concentration at the end of the batch experiment below 10 mg L^{-1}. In terms of removal efficiency, the highest N-NH_4^+ removal of 68% was detected at 12 h B photoperiods, followed by constant removal of 58% under the remaining wavelength photoperiod conditions (Figure 5(b)). As for the TKN, the removal remained invariant throughout the mixing wavelength with an average percentage of 80% (Figure 5(a)). Conversely, H-N/P showed a wide removal variation of the N-NH_4^+ and TKN throughout the mixed wavelength conditions. The maximum nitrogen removal efficiency was observed with the 8 h B:4 h G condition, followed by 4 h B:8 h G, 12 h B, and 12 h G. The best conditions (8 h B:4 h G) presented an average N-NH_4^+ and TKN removal of 55 and 60%, corresponding a final average concentration of 19 mg N-NH_4^+ L^{-1} and 23 mg TKN L^{-1}. Monochromatic lights 12 h B and 12 h G evidenced the lowest performances with a final average TKN and N-NH_4^+ concentrations of

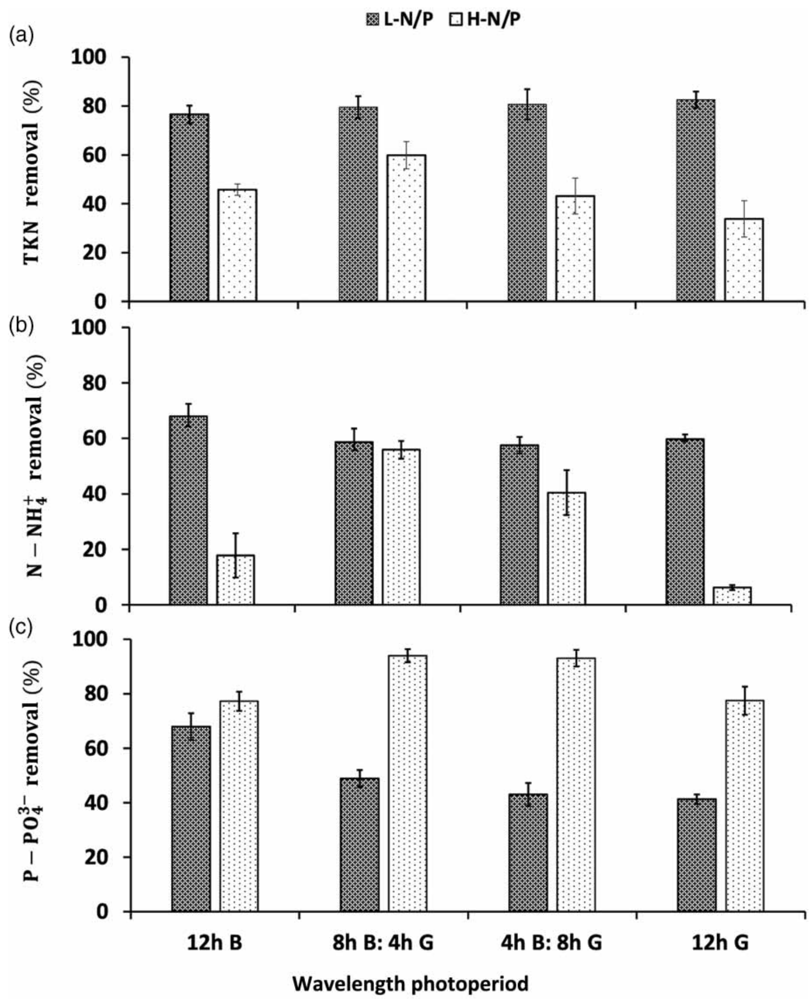


Figure 5 | (a) TKN, (b) N-NH_4^+ and (c) P-PO_4^{3-} removal profile under four different blue (B): green (G) mixing wavelengths under two N/P conditions of 1.3 and 8.3. Data are expressed as mean ± SD, $n = 3$.

28 mg N-NH_4^+ L^{-1} and 32.4 mg TKN L^{-1}, associated with an inadequate removal N-NH_4^+ efficiency below 20% and maximum TKN of 46%. As for P-PO_4^{3-} removal, opposite behavior was detected. H-N/P promotes higher P-PO_4^3 removal for all B:G mixing conditions (Figure 5(c)). Under H-N/P, intermedia light conditions (8 h B:4 h G; 4 h B:8 h B) showed almost a complete phosphorus depletion (<0.1 mg P-PO_4^{3-} L^{-1}, 98 ± 2%), In contrast, at L-N/P, the maximum have removal efficiency of 67% was reached under 12 h B light conditions. Additionally, the green light's presence seems to negatively affect the P-PO_4^{3-} removal, decreasing its efficiency up to 41%, corresponding to 12 h G conditions. Previous works have reported that the optimal inorganic N/P ratio for new microalgal growth and nutrients removal ranges from 6.8 to 10 (Wang *et al.* 2010). The analysis of N/P at the end of the batch test reveals a drastic increase above 25 under H-N/P conditions, while L-N/P no significant variation was observed compared with the initial N/P ratio. The reason behind this trend is associated with severe phosphorus limitation, hampering the microalgae growth and N-NH_4^+ removal, as observed in our results at H-N/P conditions (Figure 5(b)). Studies in synthetic wastewater at different N loading (N/P range from 4 to 29) evidenced low nitrogen removal (<46%) utilizing *Chlorella vulgaris* in both blue and green wavelength light (intensity of 2,000 μmol/m^2 s) (Yan *et al.* 2013a, 2013b). Contrary to our experiment, Yan *et al.* (2013b) did not detect significant variation in the nutrients and phosphorus removal at different N loading. This finding could be associated with the pH increase detected at L-N/P (Table 2) compared with the constant pH maintained in the studies previously mentioned under different N/P ratios. Mohamed *et al.* (2021) indicated that the high presence of HCO_3^- (inorganic carbon species dominate in pH above 9) and low levels of N-NH_4^+ as detected at L-N/P conditions promote the microalgal growth, limiting the P removal due to the shortage of N-NH_4^+ or polyphosphate accumulating organisms (PAOs) growth. This theory might validate the trends observed in all mixing light photoperiods at L-N/P and the unusual behavior observed at 8 h B:4 h G under H-N/P conditions. These findings suggest that pH control seems not to be an optimal strategy to achieve high removal of nutrients. However, the effect of this variable should be analyzed under different operative conditions.

3.4. Nitrogen removal mechanisms

Figure 6 shows the nitrogen removal mechanisms under L- N/P (Figure 6(a)), H-N/P (Figure 6(b)). Mechanisms of assimilation (TN_{uptake}), nitrification ($TN_{nitrification}$), and nitrogen loss (TN_{loss}) were considered in the nitrogen balance. TN_{loss} only considered volatilization mechanisms because the dissolved oxygen during the batch process maintained values above 1 mg O_2/L, making negligible the denitrification mechanism. Additionally, the assimilation mechanisms considered a microalgal biomass nitrogen content of 7.1%. L-N/P ratio promoted average nitrogen assimilation above 45%, followed by an average percentage of $TKN_{residual}$ and N_{loss} of 25.5% and 17.4%, respectively. Similar high nitrogen assimilation of up to 96% has been detected in microalgae–bacteria systems dominated by *Chlorella* sp. (Nguyen *et al.* 2020). Nitrification was not detected in the batch test. The absence of nitrate might be associate with the high pH (>9) observed in the system, which inhibits the proliferation of nitrifying bacteria. Although these alkalinity conditions promoted the volatilization upon assimilation mechanism, in our case, the pH increase to 9 was detected after 4 days. At that time, the maximum N-NH_4^+ removal was achieved (Supplementary material, Figure S1). As a consequence, is plausible that the microalgae utilized the ammonium nitrogen source for growth. According to the theoretical microalgae yield coefficient per ammonium consumption ($Y_{algae/N\text{-}NH_4^+} = 10.83$ mgVSS/mg NH_4^+) obtained from the stoichiometric expression defined by Mara (2004),

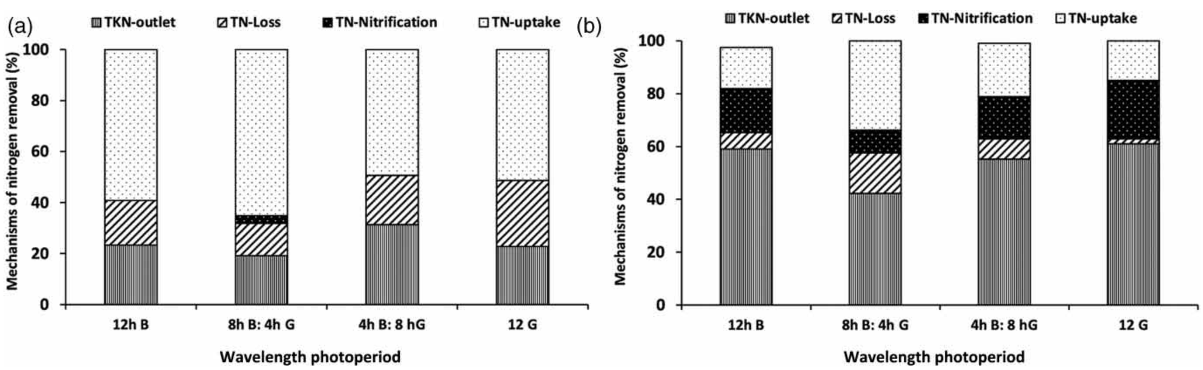


Figure 6 | Mechanisms of nitrogen consumption under L-N/P (a) and H-N/P (b) under four mixing wavelength photoperiods 12 h B, 8 h B:4 h G, 4 h B:8 h G, and 12 h G at the end of the batch period.

the experimental $Y_{algae/N\text{-}NH_4^+}$ at L-N/P ratio achieved values above 20. These differences imply that the microalgae might employ the organic nitrogen source for their growth. Some *Chlorella* spp. utilized urea as suitable nitrogen sources (Li *et al.* 2013; Khalili *et al.* 2015) in low concentration or absence of NH_4^+. Morris (1974) established that their consumption is through of urea amidolyase (UALse) enzymatic pathway, followed by the hydrolysis of allophanate to ammonia and bicarbonate (Su 2021). As for the H-N/P ratio, a high percentage of nitrogen was not oxidized (average TKN_{outlet} of 54%), followed by the assimilation mechanism does not exceed 20%, except at 8 h B:4 h G, which reached 34%. Nitrification gains relevance in the nitrogen removal mechanisms, achieving values above 19% under mixing wavelength of 12 h B, 4 h B:8 h G, and 12 h G, corresponding to a final average concentration of 10, 8.8 and 11.6 mg N-NO_3^-, respectively. A significant difference in the nitrification at 8 h B:4 h G (4.5 mg N-NO_3^-) related to a pH increase from 6.8 to 8.9, suggested a nitrification inhibition under L-N/P conditions. This lower assimilation could be associated with a phosphorus limitation. According to the results, the nitrogen consumption profile is not affected by the wavelength mixing, while the N/P ratio plays an essential role in the nitrogen mechanisms. Additionally, the pH increment seems to be a key factor in increasing assimilation mechanisms in microalgal systems dominated by *Chlorella* sp. However, in future studies, elementary biomass analysis (C, N,P) and total TKN need to be conducted to estimate the microalgae–bacteria nitrogen uptake better.

4. CONCLUSIONS

This study confirmed that mixing wavelength photoperiods (blue:green) of 8 h B:4 h G and 4 h B:8 h G is a suitable strategy to increase biomass production, organic and nutrients removals, in comparison with exposure to a single blue or green light. Under the best conditions, an average removal of COD (68%), N-NH_4^+ (53%) for both N/P ratios. However, P-PO_4^{3-} removal performances were affected by the N/P ratio and the wavelength interaction. The microalgae–bacteria growth dynamic is influenced by the wavelength applied. Microalgae–bacteria growth phase (<7 days) is promoted by mixing wavelength photoperiods (8 h B:4 h G and 4 h B:8 h G), while for long operative times (10–14 days) biomass growth is favored under wavelengths where blue light predominates (12 h B, 8 h B:4 h G). An increase in pH under L-N/P conditions favors the mechanisms of nitrogen assimilation of the microalgae–bacteria systems dominated by *Chlorella* sp. The interaction of mixing wavelength photoperiods and N/P ratios plays a pivotal role in the optimization of the microalgae–bacteria systems.

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CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

Daniela Céspedes: investigation, data curation, and writing – original draft. Germán Buitrón: writing – original draft, review & editing. Juan S Arcila: Investigation, conceptualization, writing – original draft, review & editing, project administration, funding acquisition. The funding sources were not involved in the research and/or preparation of the article in terms of study design, the collection, analysis, interpretation of data, and the writing of the report. The founding source contributed to the research through financial support and student scholarship.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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