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Performance assessment of microalgae-based wastewater treatment (MBWT) system in response to operation modes, hydraulic retention time (HRT) and cyclical light

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ABSTRACT

The performance of the microalgae-based wastewater treatment (MBWT) process was investigated with a main focus on two key operational parameters: operation modes and hydraulic retention time (HRT). Compared with continuous mode, consistent semi-continuous cultivation exhibited comparatively sustainable algal growth due to the well acclimatization effect. When operated semi-continuously with an HRT of 5 d, a *Chlorella*-based MBWT system obtained the highest average algal growth rate at 0.054 d^{-1}, with subsequent biomass accumulation at 0.21 g L^{-1}. Average total nitrogen, NH_4^+–N and chemical oxygen demand removal was 64.00% ± 6.48%, 73.52% ± 3.96% and 44.66% ± 13.29%, respectively, while total phosphorus removal remained stable at >99% despite different operational parameters applied. Co-influence of cyclical light of a natural light/dark cycle on semi-continuous MBWT was also observed, and the result indicated a relationship between possible biomass harvesting time and diel patterns of algal cells. Moreover, the techno-economic analysis displayed the nutrients recovery advantage of microalgae, as approximately 5.29 g nitrogen and 10.08 g phosphorus could be recovered per ton treated wastewater, which equals 193.2 t nitrogen and 36.8 t phosphorus for a scaled-up MBWT plant with a capacity of 3.65×10^7 m^3. This also creates potential economic benefits of 0.8 M Chinese Yuan (CNY) for the MBWT plant annually.

Keywords: Microalgae; Municipal wastewater; Operation mode; Hydraulic retention time; Algal biomass; Nutrient removal

1. Introduction

In China, the activated sludge-based process is the most widely-used technology for pollutant removal of sewage in wastewater treatment plants (WWTPs). With the promotion of stringent primary A discharged standards across the country, WWTPs are required to cut down effluent nutrient concentrations of total phosphorus (TP), total nitrogen (TN) and NH_4^+–N to 0.5, 15 and 5 mg L^{-1}, respectively. This could hardly be achieved with the biological sludge process alone, especially for the elimination of phosphorus [1]. Additional chemical dosing for phosphate sedimentation is usually compulsory in order to meet the strict discharge demand, which results in high operational costs and undesirable sludge discharge [2,3]. As the future wastewater treatment industry focusing more on enhanced pollutant removal with simultaneous nutrients and energy recovery from the sewage [4], conventional processes with high energy costs, inefficient nutrients removal and resource wash-off would be incapable of a couple with the tightening discharge standards and resource recovery trend.

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Microalgae-based wastewater treatment (MBWT), an emerging technology that attracts lots of research interest in recent years, provides a new opportunity for wastewater treatment revolution [4]. Several studies of algae-based systems have unleashed the feasibility of microalgal nutrients and organics removal. Gouveia et al. [5] reported maximum removal of nitrogen, phosphorus and chemical oxygen demand (COD) of a fed-batch *Chlorella* photobioreactor (PBR) system integrated into a WWTP by 84%, 95% and 36% respectively during steady operation. Shayan et al. [6] demonstrated nutrients removal of 80% and 79% for TN and TP in an outdoor algal reactor operating with a hydraulic retention time (HRT) of 6 d [6]. Unlike heterotrophic activated sludge, which requires intensive aeration and external carbon sources as energy input, microalgae are photosynthetic microorganisms that can use solar energy for cell growth [7]. Through the photosynthesis process, algae absorb CO_2 and generate O_2 into the air, while pollutants such as nitrogen and phosphorus in the wastewater are utilized as a nutrient source to support algal biomass accumulation. Algae also display high effectiveness for removing heavy metals through biosorption, which could be used as a simple alternative for enhanced water purification [8,9]. In this way, wastewater is treated under low operation cost and low energy consumption, with a simultaneous considerable amount of algal biomass production. The biomass obtained in the MBWT system can be further converted into several high-value products, such as biofertilizer, animal feed, biodiesel and biogas [10,11], bringing significant economic benefits to the wastewater treatment industry.

When running an algae-based system, the operation mode is a fundamental parameter. During the past few years, batch cultivation remains the most popular way for algae biomass production [12,13]. Despite easier control for maintenance, batch mode lacks the feasibility in real WWTP applications due to its large land demand and unrealistic long processing time. Most batch trials also over-simplified the observation of algal physiological behavior due to the limitation of steady hydraulic conditions [14]. With light as another influencing factor, the mechanistic understanding of algae cultivated in a fluctuated environment is much more complicated and requires a comprehensive study of the relevant factors involved. Considering the scaling-up of MBWT, a system with dynamic water flow serves more feasibility regarding its lower capital costs and higher processing load [15]. Periodical outflow also allows consistent harvesting of biomass, which provides great potential to obtain satisfying nutrients resources recovery. Nevertheless, it was estimated that only ~4% of algae studies involved (semi-) continuous culture, while most other research still stagnated at traditional batch cultivation. The continuous algal reactors, especially operated with cyclical light, is still relatively uncommon in algal research [14].

Apart from operation mode, hydraulic retention time is another key operational parameter. HRT represents the volume of substrate replaced per day of the reactor and could be indirectly linked to biomass production and pollutants removal performance. Proper HRT control could serve as a simple, economical-friendly but sustainable method for system efficiency optimization [6]. It has been proved that HRT could pose selection pressure on algal culture [16], and could be critical in terms of enhancing the nutrients removal capacity of an algal reactor [17]. However, HRT observation in most studies is only conducted after a long time of batch algal cultivation [18,19], which neglects the HRT impact on the initial algal growth pattern in the wastewater environment. While the optimal HRT of a certain algal-based system may range from different algal species and cultivation conditions, understanding HRT influence on microalgal growth interpreted from operation modes may offer new insights on algal species selection and acclimatization despite variations of environmental conditions. To our best knowledge, HRT study combined with the consistent continuous culture that focuses on algal acclimatization and growth performance is relatively rare and demands more research.

The objectives of this study were to investigate the effects of hydraulic retention time (HRT) and operation modes on *Chlorella vulgaris* grown in municipal wastewater. Algal biomass and pollutants removal were further analyzed to determine suitable parameters of a MBWT system. Besides, the co-influence of the light/dark (L/D) cycle under semi-continuous cultivation was also accessed to inform microalgal diel patterns for proper harvesting and light control of the integrated system.

2. Materials and methods

2.1. Microalgae and substrate

The microalgae used in this research was *C. vulgaris* (FACHB-8), obtained from the Freshwater Algae Culture Collection at the Institute of Hydrobiology, Chinese Academy of Science. Algal cells were inoculated and developed in 300 mL conical flasks containing 200 mL Blue-Green (BG-11) culture medium that consisted of the following chemicals per liter: 1.5 g $NaNO_3$, 0.04 g K_2HPO_4, 0.075 g $MgSO_4·7H_2O$, 0.036 g $CaCl_2·2H_2O$, 0.006 g citric acid, 0.006 g ammonium ferric citrate, 0.001 g $EDTANa_2$, 0.02 g Na_2CO_3 and 1 mL A5 trace elements solution. The A5 trace elements solution contained chemicals per liter: 2.86 g H_3BO_3, 1.86 g $MnCl_2·4H_2O$, 0.22 g $ZnSO_4·7H_2O$, 0.39 g $Na_2MoO_4·2H_2O$, 0.08 g $CuSO_4·5H_2O$ and 0.05 g $Co(NO_3)_2·6H_2O$ [20]. The initial incubation ratio of algae and BG-11 medium was 1:4, and the inoculum flasks were preserved in a 12 h-illumination incubator with a light intensity of approximately 3,000 lux and temperature maintained at 25°C. Sterile Parafilm was applied on the flasks to avoid bacteria pollution during the cultivation. After 12 d of growth, as microalgae reached its stationary stage (determined by monitoring algal cell growth), the culture was used as inoculants for the wastewater experiments.

Artificial wastewater used in the experiment was simulated on the influent from a real municipal wastewater treatment plant in Guangdong Province, China [21]. The composition of the wastewater per litre was as followed: 0.03 g peptone, 0.03 g urea, 0.15 g glucose, 0.15 g CH_3COONa, 0.063 g NH_4Cl, 0.022 g $KHSO_4$, 0.013 g KH_2PO_4, 0.106 g $CaCl_2$, 0.026 g $MgSO_4$, 0.014 g $FeCl_3·6H_2O$ and 0.026 g $Al_2(SO_4)_3·18H_2O$. The initial pollutant concentration of the artificial wastewater substrate was 3.0 ± 0.01 mg L^{-1} TP, 37.4 ± 1.47 mg L^{-1} TN, 16.6 ± 1.30 mg L^{-1} NH_4^+–N and 410.0 ± 4.42 mg L^{-1} COD.

2.2. Experimental set-up and operation

Three beakers of 1 L volume were used to examine the influence of different HRTs of semi-continuous mode on *C. vulgaris*. Each beaker had a working volume of 300 mL and was inoculated with algae culture at an algae cell density of 1.4 × 10^9 cell mL^{-1}. Experiments were carried out in the incubator under the same environmental conditions as those for primary inoculum cultivation. During the first 6 d, 50 mL of liquid was replaced with wastewater every day as acclimatization. From day 7, the replacing volume of wastewater was exchanged to 60, 80 and 100 mL in order to obtain an HRT of 5 d (SC-5d), 3.75 d (SC-3.75d) and 3 d (SC-3d) respectively (Fig. 1). The beakers were agitated manually four times a day for the complete mixture.

A polymethacrylate flat panel photobioreactor (PBR) measuring 0.25 m × 0.13 m × 0.13 m (length × width × height) with a working volume of 3.6 L was used to access the influence of continuous cultivation as well as the transitions between different operation modes (Fig. 2). A stirrer was set at the middle of the reactor for constant mixing of the culture and to avoid algae settling. Two peristaltic pumps were used to control the volume of influent and effluent accurately. Illumination during the light period was provided by two sets of the LED light modules on both sides across the reactor with a light intensity of 3,000 ± 100 lux. A portable digital lux meter was used to measure light intensity around the photobioreactor to ensure constant illumination throughout the experiment. L/D cycle was controlled at 12:12 automatically by several digital timers. The temperature was maintained at 25°C ± 1°C to match the same environmental conditions as the HRT experiments. Deionized (DI) water was added to the reactor every day for evaporation offset.

Prior to the PBR operation, *C. vulgaris* was transferred from batch cultivation to semi-continuous cultivation with an HRT of 5 d (Fig. 3). For semi-continuous cultivation, there was no inflow or outflow during most of the time, despite a fixed time period of ~15 min d^{-1} for influent and effluent exchange. After 12 d of cultivation, when *C. vulgaris* reached stable growth, 0.36 L acclimatized algal culture was collected and mixed with 3.24 L artificial wastewater as inoculum for the continuous PBR start-up. The initial algal density of the mixture was approximately 1.6 × 10^9 cells mL^{-1}. To sustain an equivalent HRT of 5 d, 0.72 L volume of culture had to be replaced with the 3.6 L flat PBR each day. As for continuous operation, it was realized by delivering artificial wastewater into the PBR consistently at a flow rate of 0.5 mL min^{-1} and pumping out effluent at the same rate simultaneously.

2.3. Determination of microalgal growth and biomass

Effluent samples were collected and measured the optical density at 680 nm (OD_{680}) using a UV spectrophotometer (Persee, T9, China). The cell density of *C. vulgaris* was determined under the microscope (Nikon, ECLIPSE E100, Japan) with a hemocytometer. Samples were diluted when necessary. A correlation curve between OD_{680} and algal cell density is shown as Eq. (1):

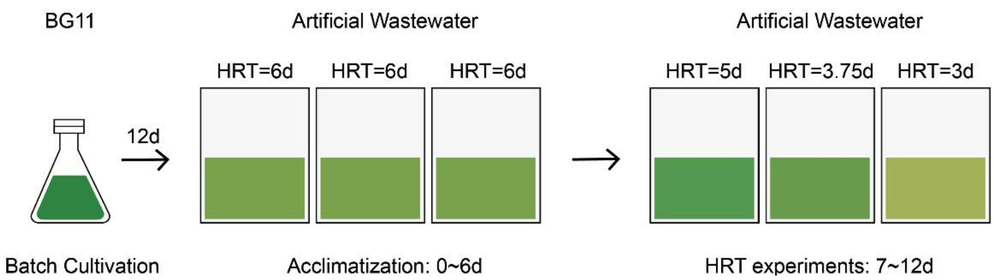

Fig. 1. Process flow of the HRT experiments.

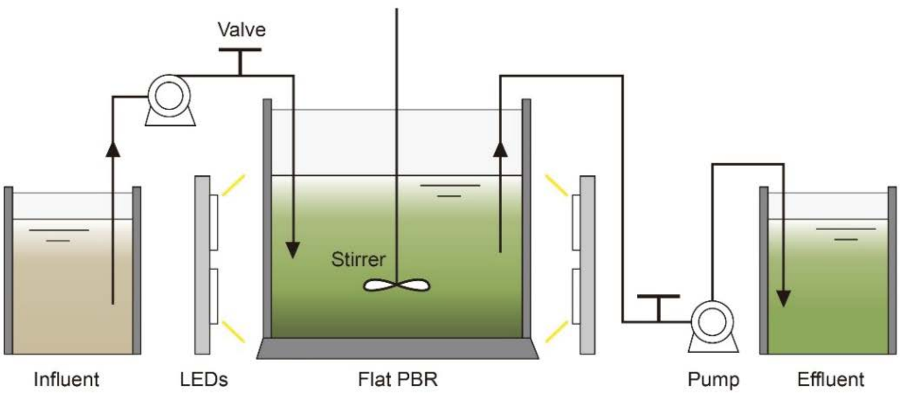

Fig. 2. Schematic diagram of the flat PBR system.

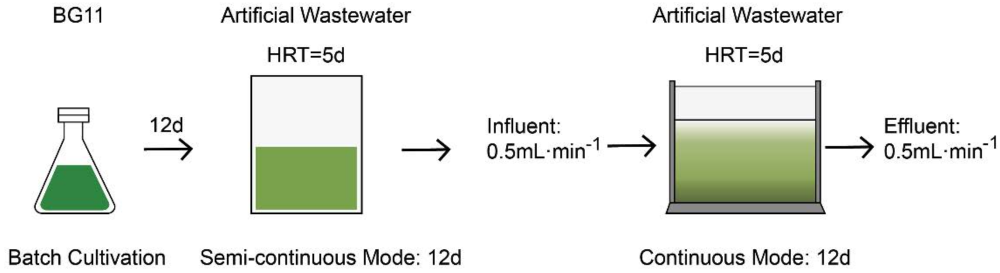

Fig. 3. Process flow of the operation mode experiments.

$$\text{Cell density}\left(10^9\,\text{cells/mL}\right) = 12.271 \times \text{OD}_{680} + 0.0194 \quad \left(R^2 = 0.9988\right) \tag{1}$$

Specific growth rate (d^{-1}) was calculated as Eq. (2), where X_2 and X_1 represent algal cell density (10^9 cells mL^{-1}) at time t_2 (d) and t_1 (d), respectively.

$$\mu\left(d^{-1}\right) = \frac{\ln X_2 - \ln X_1}{t_2 - t_1} \tag{2}$$

Algal harvesting was realized through the filtration method. Biomass was collected using the following process: a 10 mL liquid sample was filtered through a pre-weighted filter (W_1) with a pore size of 0.45 μm and rinsed twice with DI water. The filter was dried at 65°C in an oven to constant weight (W_2). Algal biomass concentration was defined by dry weight (DW) per liter (g L^{-1}) according to Eq. (3), and average biomass productivity was determined as Eq. (4), where HRT represented the hydraulic retention time of the sampled system and was equivalent to the microalgae retention time in the well-mixed reactor.

$$\text{DW}\left(\text{g L}^{-1}\right) = \frac{W_2 - W_1}{0.01} \tag{3}$$

$$\text{Biomass productivity}\left(\text{g L}^{-1}\,\text{d}^{-1}\right) = \frac{\text{DW}}{\text{HRT}} \tag{4}$$

2.4. Determination of effluent water quality

Effluent samples were collected every 12 h during the HRT experiment and every 24 h during the PBR experiment. All samples were filtered through a 0.45 μm filter to remove algae particles, and the filtrate was used for water quality analysis. TP and ammonia (NH_4^+–N) were determined spectrophotometrically (Hach, DR 2800, USA) based on ammonium molybdate and salicylic acid methods, respectively. TN was determined spectrophotometrically (Persee, T9, China) after alkaline potassium persulfate oxidation. COD was determined after digestion (Hach, DRB200, USA) followed by dichromate titration. All measurements were performed according to the Chinese State Environmental Protection Agency Standard Methods [22].

The removal capacity for each pollutant was calculated as Eq. (5), where C_0 (mg L^{-1}) and C (mg L^{-1}) represent the influent and effluent concentration of the pollutant. Q (L) represents the volume of the effluent. V (L) represents the volume of the whole system. HRT (d) represents the hydraulic retention time of the mixture in the system.

$$\text{Removal capacity}\left(\text{mg L}^{-1}\,\text{d}^{-1}\right) = \frac{C_0 - C}{Q / V} = \frac{C_0 - C}{\text{HRT}} \tag{5}$$

2.5. Statistical analysis

All results are presented in the form of mean values ± standard deviation (SD) from at least two independent experiments. Visualization of data was conducted in origin 9.0. Analysis of variance was conducted in SPSS 20.0 Statistical Software (IBM). For the HRT experiment, a one-way analysis of variance (ANOVA) followed by a LSD multiple comparisons was applied to determine the difference. For operation modes and diel variations statistics, independent-samples *T*-test and paired-samples *T*-test were performed respectively to access the difference. The significance level was at $p < 0.05$ for all analyses.

3. Results and discussion

3.1. Algal growth and wastewater treatment performance under different HRTs

Variations of HRT had a direct impact on algal growth and biomass, as shown in Table 1 and Fig. 4. Compared with SC-3d and SC-3.75d, SC-5d with an increasing HRT generally demonstrated higher algal cell density (6.08×10^9 cell mL^{-1}), specific growth rate (0.054 d^{-1}) and biomass concentration (0.14 ± 0.03 g L^{-1}). This can be mainly attributed to the wash-out effect of shorter HRT [23], that algal growth rate fails to match the biomass flow rate of the withdrawn. A notable decrease of algal cells was observed for all the systems, when HRTs were switched to 3 d, 3.75 d and 5 d after the first 6 d (data not shown). Algal density started to bounce back since day 9 and ultimately reached 8.24×10^9, 7.75×10^9 and 7.01×10^9 cell mL^{-1} for SC-5d, SC-3.75d and SC-3d respectively, indicating strong resilience and adaptability of *C. vulgaris* in response to hydraulic volume changes under consistent semi-continuous cultivation.

Regarding biomass productivity, significant difference was observed between SC-5d and SC-3d ($p = 0.035 < 0.05$).

When applying shorter HRT, average biomass productivity has seen an increase from 0.03 ± 0.01 g L^{-1} d^{-1} (SC-5d) to 0.04 ± 0.01 g L^{-1} d^{-1} (SC-3d). However, shorter HRT was incapable of sustaining biomass accumulation (Fig. 4). SC-5d, despite relatively lower biomass productivity, achieved maximum biomass accumulation (0.21 g L^{-1}) amongst three HRT systems after 12 d of cultivation. The results hinted at the influence of HRT on the light conversion efficiency of the algal cells, with the consequent effects on overall biomass concentration and productivity. Shorter HRT with lower cell density is more likely to suffer from photoinhibition triggered by excessive light intensity and impedes algal growth, while longer HRT that achieves high biomass concentration may be influenced by light limitation due to self-shading and result in reduced productivity per unit [24,25].

Hence, the volumes withdraw per day play a critical role in algal biomass performance. Since biomass concentration is closely related to the stability of the algal system [26], a proper HRT should ensure long-term sustainable performance while keeping it as short as possible [27]. The ideal HRT ought to match the counterbalance between algae biomass concentration and biomass productivity to achieve the maximum outcome of the system.

Table 2 demonstrated the nutrients and carbon removal performance of the three HRT-based algal systems. At an influent concentration of ~3.0 mg L^{-1}, total phosphorus removal remained relatively high at >99% regardless of different HRTs (p = 0.916 > 0.05). Effluent TP concentrations were all far below 0.5 mg L^{-1} and met the wastewater discharge standards well. As an essential source for intracellular energy transforming, phosphorus is important for algal growth and metabolism [28]. Orthophosphate, such as HPO_4^-, $H_2PO_4^-$ and PO_4^{3-}, are prevalently existed in wastewater, and can be used preferably for algal cell assimilation [29]. According to previous research, luxury uptake of phosphorus is ubiquitous for most microalgae species, which allows algal cells to uptake excessive amounts of phosphorus from the environment [30,31]. This can explain the great capability of *C. vulgaris* for phosphorus removal in this study despite HRT changes. Considering the existence of Ca^{2+} in the artificial wastewater, the chemical precipitation of PO_4^{3-} may also contribute to a portion of phosphorus removal in the algae culture [32].

There was no significant difference between the three systems regarding total nitrogen removal (p = 0.287 > 0.05), while ammonia removal varied notably (p = 0< 0.05). At an

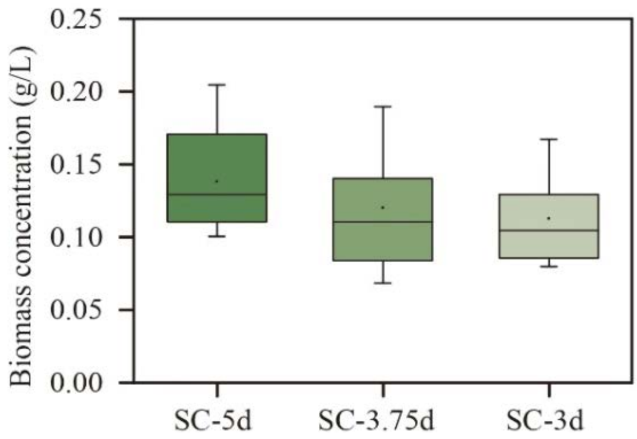

Fig. 4. Average biomass concentration of the system under different HRTs.

Table 1
Algal growth and biomass performance of different HRTs under semi-continuous mode

System	Day 0	Day 7–12*				Day 12	
	Cell density (cells mL^{-1})	Specific growth rate (d^{-1})	Cell density (cells mL^{-1})	Biomass concentration (g L^{-1})	Biomass productivity (g L^{-1} d^{-1})	Cell density (cells mL^{-1})	Biomass concentration (g L^{-1})
SC-5d	1.37×10^9	0.054	6.08×10^9	0.14 ± 0.03	0.03 ± 0.01	8.24×10^9	0.21
SC-3.75d	1.37×10^9	0.029	5.49×10^9	0.12 ± 0.04	0.03 ± 0.01	7.75×10^9	0.19
SC-3d	1.37×10^9	0.021	5.26×10^9	0.11 ± 0.03	0.04 ± 0.01	7.01×10^9	0.17

*Values of Day 7–12 are presented as the average measurements across the 6 d of cultivation

Table 2
Pollutant removal and effluent performance of different HRTs under semi-continuous mode (day 7–12), mean values ± standard errors

System	TP		TN		NH_4^+–N		COD	
	Removal rate (%)	Effluent concentration (mg L^{-1})	Removal rate (%)	Effluent concentration (mg L^{-1})	Removal rate (%)	Effluent concentration (mg L^{-1})	Removal rate (%)	Effluent concentration (mg L^{-1})
SC-5d	99.02 ± 0.80	0.03 ± 0.02	64.00 ± 6.48	13.46 ± 2.42	73.52 ± 3.96	4.40 ± 0.66	44.66 ± 13.29	226.87 ± 54.49
SC-3.75d	99.13 ± 0.49	0.03 ± 0.01	61.00 ± 6.75	14.59 ± 2.52	64.16 ± 5.11	5.95 ± 0.85	50.79 ± 9.05	201.76 ± 37.11
SC-3d	99.15 ± 0.85	0.03 ± 0.02	58.83 ± 7.20	15.40 ± 2.69	57.97 ± 4.79	6.98 ± 0.80	47.27 ± 11.09	216.21 ± 45.49

influent concentration of ~37.4 mg L^{-1}, effluent TN concentrations subsequently reduced to 13.46–15.40 mg L^{-1}, which refers to TN removal rates of 58.8%–64.0%. Meanwhile, under an influent ammonia nitrogen concentration of ~16.6 mg L^{-1}, average effluent NH_4^+–N concentrations ranged from 4.4–7.0 mg L^{-1}, with NH_4^+–N removal rates fluctuated between 58.0%–73.5%. Generally, the SC-5d system obtained the highest nutrients removal efficiencies and was the only one being able to meet the nitrogen discharge requirement. Amongst all kinds of nitrogen, NH_4^+–N is the primary-used nitrogen source for microalgae, as it requires the least energy and shortest transfer pathway to be assimilated by algal cells, while other kinds of nitrogen such as NO_2^-–N and NO_3^-–N, have to be reduced to ammonium before being used intracellularly [28,33]. Although shorter HRT features relatively higher volumetric removal capacity, it lacks substantial nitrogen removal efficiency. Longer HRT, on the other hand, allows more sufficient time for nitrogen conversion and NH_4^+–N utilization in the algae cells [6], which leads to improved TN and NH_4^+–N removal performance.

Regarding COD removal, all systems showed similar removal efficiencies at 44.7%–50.8% with no significant difference (p = 0.521 > 0.05). Influent COD was ~410 mg L^{-1} in the substrate, and effluent COD concentrations were reduced to 201.76~226.87 mg L^{-1}. Overall, algal COD removal seems to be less influenced by HRTs changes, and this finding was in consistent with Shayan et al. [6] and Takabe et al. [17]. Compared to the activated sludge system and algae-bacteria system, COD removals in this study were comparatively lower and failed to meet the discharge standard, indicating insufficient carbon removal of algae alone. As reported in previous research, inorganic carbon remains the predominant carbon source for algal autotrophic metabolism [34]. Higher CO_2 biofixation through the photosynthesis process could promote algal growth and increase N removal, whereas affect the overall COD removal rates [35]. Thus, when mixotrophic cultivation conditions were applied, the equilibrium between inorganic carbon and organic carbon assimilation would define the ultimate carbon removal capacity of the system.

In this study, co-influence of semi-continuous mode and HRT was determined. Compared with conventional bacteria systems, microalgae demonstrated great competence in phosphorus removal, while lacks sufficiency for removing nitrogen and carbon under short HRTs that requires further improvement [36,37]. It is worth mentioning that higher pollutant removal capacities (in mg L^{-1} d^{-1}) for almost all parameters were observed at shorter HRT (Table 3). However, when removal capacity is estimated by mg pollutant per g biomass, there was no significant difference between SC-5d, SC-3.75d and SC-3d [TP (p = 0.222 > 0.05), TN (p = 0.453 > 0.05), NH_4^+–N (p = 0.894 > 0.05), COD (p = 0.241 > 0.05)]. This informs stabilized removal capacity per algal cell, regardless of HRT changes. Thus, the overall pollutant removal capacity of an algal system is mainly attributed to its biomass concentration. To ensure satisfactory effluent performance and biomass production, sufficient HRT is pivotal for algal growth and biomass accumulation. In this study, HRT of 5 d is selected for subsequent research.

Table 3
Pollutant removal capacity of different HRTs under semi-continuous mode (day 7–12), mean values ± standard

System	TP		TN		NH_4^+–N		COD	
	Removal capacity (mg L^{-1} d^{-1})	Removal capacity (mg pollutant/g biomass)	Removal capacity (mg L^{-1} d^{-1})	Removal capacity (mg pollutant/g biomass)	Removal capacity (mg L^{-1} d^{-1})	Removal capacity (mg pollutant/g biomass)	Removal capacity (mg L^{-1} d^{-1})	Removal capacity (mg pollutant/g biomass)
SC-5d	0.59 ± 0.00	22.74 ± 5.15	4.79 ± 0.48	181.14 ± 38.19	2.44 ± 0.13	93.64 ± 22.82	36.63 ± 10.90	1,430.23 ± 608.67
SC-3.75d	0.79 ± 0.00	27.40 ± 8.55	6.08 ± 0.67	206.35 ± 58.14	2.84 ± 0.23	97.54 ± 30.04	55.53 ± 9.90	1,936.71 ± 717.87
SC-3d	0.99 ± 0.01	28.30 ± 7.16	7.33 ± 0.90	206.13 ± 46.01	3.21 ± 0.27	91.82 ± 25.03	64.60 ± 15.16	1,831.08 ± 631.02

3.2. Effect of operation modes and substrate on algal biomass

There were significant differences between semi-continuous and continuous mode in regard to algal biomass (Fig. 5). Cell density ($p = 0.0012 < 0.05$), biomass concentration ($p = 0.001 < 0.05$) and biomass productivity ($p = 0.001 < 0.05$) all varied across the period. Overall, semi-continuous mode demonstrated better performance for algae growth and biomass accumulation.

When cultivated in continuous wastewater flow (C-5d), *C. vulgaris* initially demonstrated fast growth on day 1 (3.82×10^9 cells mL^{-1}), with a remarkable specific growth rate at 0.839 d^{-1}. Cell density increased moderately until reached a climax on day 3 (4.88×10^9 cells mL^{-1}) but declined persistently for the rest of the period. The ultimate cell density of C-5d was 2.44×10^9 cells mL^{-1}, indicating relatively poor biomass accumulation in the continuous system. In contrast, *C. vulgaris* cultivated semi-continuously (SC-5d) showed stable growth throughout the 12 d. A 1 d lag phase followed by a 2 d exponential phase was observed during the initial period (day 1–3). The duration of the algal lag phase is comparably shorter than most batch studies [38,39], which unveils the possibility of applying semi-continuous mode for reducing the lag phase and promoting algae acclimatization in mass cultivation [40]. Cell density remained stationary at approximately 4.9×10^9 cells mL^{-1} for 5 d (day 4–9), suggesting that microalgae had well adapted to the semi-continuous flow. Constant growth was recorded for SC-5d since day 9, which eventually led to a triple cell density compared to C-5d. And there was no sign of biomass decline for SC-5d at the end of the experiment.

Biomass productivity of the two systems demonstrated similar trends. In spite of nearly the same initial inoculum, the distinction was observed in the average biomass productivity between C-5d (0.01 g L^{-1} d^{-1}) and SC-5d (0.04 g L^{-1} d^{-1}). Besides, physical observation of the color of microalgae in the semi-continuous and continuous systems also showed significant variations as the latter became brownish-green while the former maintained fresh green, suggesting a healthier state of microalgae cells in semi-continuous flow.

Possible reasons accounted for the declined algal biomass in the continuous system are as following: (1) Lower enzymatic activity limits the cellular activity of microalgae. Compared to batch cultivation (97%), it was found that continuous cultivation of algae tends to feature lower enzymatic activity (20%–40%) intracellularly [41]. As enzymes are closely related to photosynthesis and metabolism processes, the decrease of enzymatic activity may prompt algal cells to enter declined phase ahead of time, which explains the poor biomass productivity of C-5d. (2) Contaminations of algal grazers and bacteria impede algal growth. In open systems like flat PBR, contaminants such as protozoa and bacteria from the air are inevitable during microalgae cultivation [42]. As reported by Reichardt et al. [43], a known algal grazer *Poteriochromonas* was detected commonly in outdoor cultures of *C. vulgaris*, which was also observed in our C-5d system approaching the end of the continuous cultivation. These bacteria competitors can inhibit algal growth through grazing pressure and nutrients competition [15]. The instability of continuous flow may exaggerate such algae-bacteria competition and prompt a selection effect of bacteria over green algae *Chlorella*, which leads to the deteriorated growth in C-5d.

C. vulgaris cultivated in BG-11 culture medium under batch mode (B-CM), in wastewater under semi-continuous mode (SC-WW) and in wastewater under continuous mode (C-WW) respectively were used to evaluate the impact of cultivation modes and substrates. Mean values of biomass concentration varied notably regarding different substrates and operation modes (Fig. 6). *C. vulgaris* cultivated in BG-11 culture medium under batch mode (B-CW) obtained the highest biomass at a concentration of 0.91 ± 0.16 g L^{-1}. When culture medium was replaced by wastewater, significant decrease of algal biomass was observed (SC-WW: 0.14 ± 0.04 g L^{-1} and C-WW: 0.04 ± 0.02 g L^{-1}). This could likely be due to two reasons: (1) The disparity between wastewater nutrients components and algae nutrients demand limits algal growth. It is reported that green algae, such as *Chlorella*, demand more nutrients than other algae species during cultivation [44]. The depletion of phosphorus in this

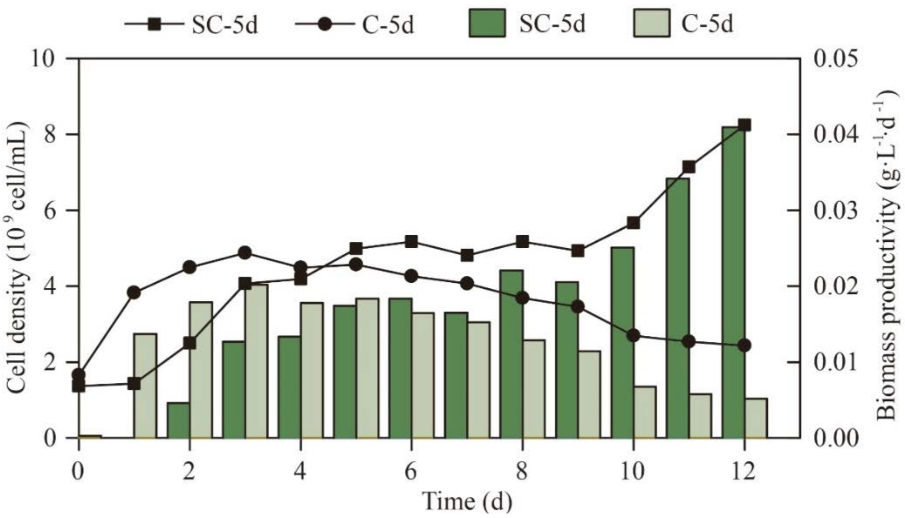

Fig. 5. Algal growth curve and biomass productivity in semi-continuous (SC-5d) and continuous (C-5d) MBWT system.

study may suppress algal growth in wastewater substrate. Besides, compared to a typical culture medium, wastewater also lacks several trace elements which are essential for optimal algae growth [4]. (2) The strategy of switching different operation modes induces varying degrees of biomass wash-out effect. In this study, B-CW was merely cultivated under stable batch mode with no culture flow-out. SC-WW and C-WW, however, underwent culture exchange every day and operation modes switching from batch-to-semi-continuous and semi-continuous-to-continuous, respectively.

Most previous algal cultivation was carried out under batch mode and tends to feature higher biomass concentration when comparing with (semi-) continuous mode due to less biomass wash-out [18,27,38]. However, it is noteworthy that batch mode lacks the feasibility in the large-scale application as it requires much longer time and higher land costs, which is impractical for real wastewater treatment. The flowing systems, when operating properly with either semi-continuous or continuous mode, are more promising for algae mass production in regards to the considerable advantages of higher nutrients recovery, lower operation cost and sustainability. Further research is needed to fully exploit the biomass potential of an algal-based (semi-) continuous wastewater systems. Proper screening, selection and acclimatization of algae species could be possible alternatives to improve algal adaptability to both wastewater substrate and dynamic water flow environment.

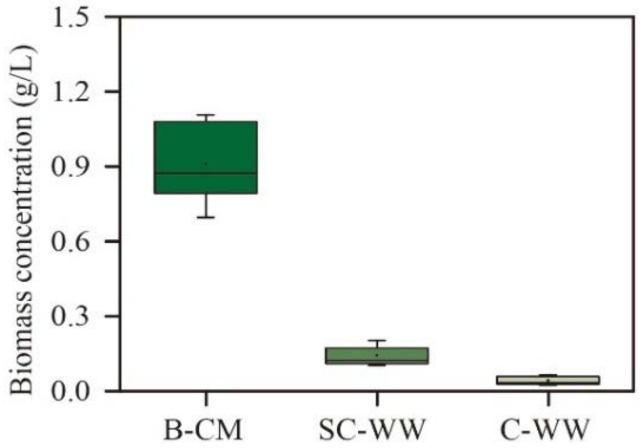

Fig. 6. Average biomass concentration of the system under different substrate and operation modes.

3.3. Effect of operation modes on wastewater treatment performance

The feasibility and stability of nutrients and carbon removal of the microalgal system was assessed under both semi-continuous and continuous mode (Fig. 7). Significant difference was noticed between the two operation modes in terms of ammonia nitrogen ($p = 0 < 0.05$) and total nitrogen ($p = 0.003 < 0.05$) removal. Initially, both systems saw a drop of NH_4^+–N from ~16.6 to ~9.5 mg L^{-1}, with NH_4^+–N removal reached approximately 43% (Fig. 7a). Since day 3, NH_4^+–N removal performance of the two operation modes started showing notable variations. The concentration of NH_4^+–N in C-5d gradually picked up and reached above 13 mg L^{-1} from day 6. Overall, the average effluent NH_4^+–N concentration was 14.48 ± 0.75 mg L^{-1} (C-5d), with a poor removal rate of less than 13%. In comparison to C-5d, NH_4^+–N concentration in SC-5d was consistently reduced to

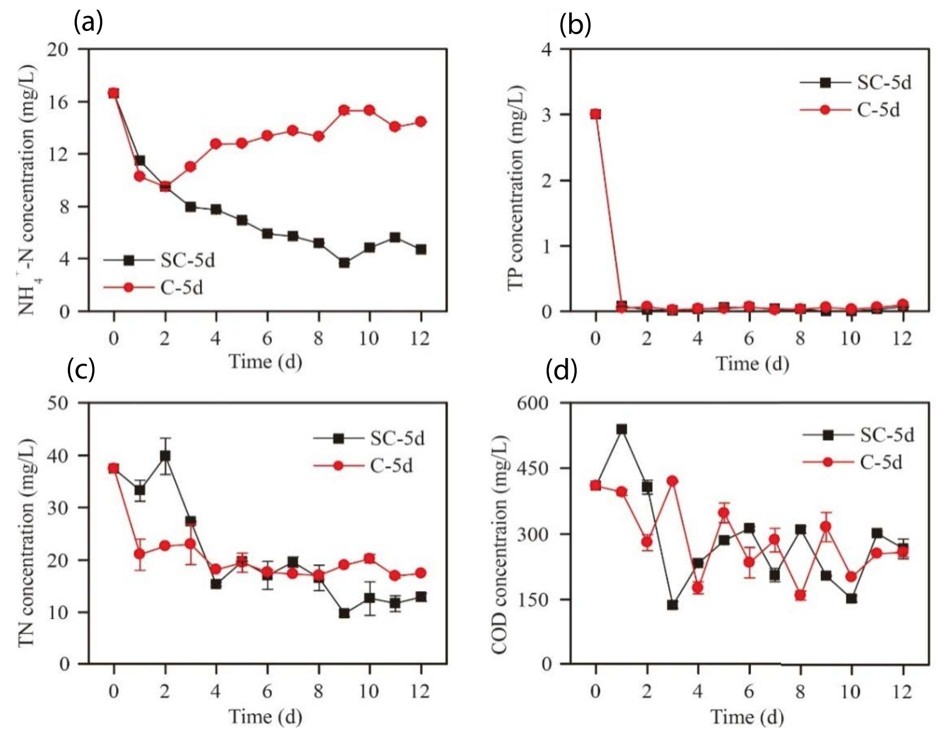

Fig. 7. Effluent concentrations of pollutants in semi-continuous (SC-5d) and continuous (C-5d) system.

an average effluent concentration of 4.80 ± 0.66 mg L^{-1}, with a stable removal of over 71%. A maximum NH_4^+–N removal was observed at day 9 (SC-5d) when NH_4^+–N removal rate reached up to ~80%. This exhibited the feasibility of semi-continuous mode over continuous mode for microalgal NH_4^+–N removal, especially for long-term operation.

Similarly, SC-5d demonstrated better TN removal performance than C-5d after 12 d of cultivation (Fig. 7c). There was a noticeable disparity of TN concentration between the two modes initially. SC-5d, with a TN influent of ~37.4 mg L^{-1}, surged to 39.80 ± 3.44 mg L^{-1} (day 2) then dropped to 27.36 ± 0.11 mg L^{-1} (day 3). C-5d, however, saw a dramatic TN decrease to 21.03 ± 2.97 mg L^{-1} on day 1, before it fully acclimatized to the wastewater substrate, then dropped consistently and remained stable at ~22 mg L^{-1}. Another disparity was observed near the end of the experiment, as the average TN concentration of C-5d set at 18.14 ± 1.3 mg L^{-1}, while TN concentration in SC-5d continually reduced to 12.74 ± 2.21 mg L^{-1}. In general, both TN removal rate and removal capacity were relatively higher for SC-5d (~66% and 4.93 ± 0.44 mg L^{-1} d^{-1}) than C-5d (~51% and 3.85 ± 0.26 mg L^{-1} d^{-1}), indicating stronger TN removal capacity of semi-continuous mode (Fig. 8). Nitrogen, which serves as an essential component of many important cellular products, such as proteins, nucleic acid and enzymes, is important for the functional and structural activity of algal growth [40,45]. The uptake rate of nitrogen in the substrate, hence indirectly reflects the growing status of algal cells. This was proved by the algal growth curve of the continuous-flow system (Fig. 5), where the decline of algal biomass was aligned with the upheaval of NH_4^+–N concentration in the C-5d effluent (Fig. 7a). The steady growth of *C. vulgaris* in SC-5d, on the other hand, explained the relatively stable nitrogen elimination of the wastewater.

It is noteworthy to see the comparable phosphorus removal efficiency despite different operation modes and withdrawn volumes (p = 0.101 > 0.05). Both modes saw significant decline of total phosphorus concentration from 3.00 ± 0.01 mg L^{-1} to almost zero after 24 h and remained steady across the 12 d (Fig. 7b). The overall TP removal for SC-5d and C-5d were 99.23% ± 0.79% and 98.23% ± 0.76%, equivalent to volumetric TP removal capacity as high as 0.60 and 0.59 mg L^{-1} d^{-1}, respectively (Fig. 8). McGinn et al. [38] reported an >99% phosphorus removal of *Scenedesmus* sp. AMDD cultivated under both batch and continuous mode, which would be in consistent with our results comparing semi-continuous and continuous mode in terms of phosphorus removal. With such considerable phosphorus uptake capacity, applying microalgae in real municipal wastewater treatment will be remarkably beneficial for stable and efficient phosphorus removal.

Regarding carbon removal, SC-5d and C-5d systems exhibited similar effluent concentrations (p = 0.829 > 0.05) (Fig. 7d). For SC-5d, effluent COD concentration went through an uncommon surge on day 1, possibly due to the overload of organic carbon in the substrate, then reduced to 137.20 ± 8.62 mg L^{-1} on day 3, with a maximum removal rate of ~67%. From day 8 to day 12, the eventual average COD removal rate of SC-5d was 39.99% ± 14.67%, referring to a removal capacity of 32.79 ± 12.03 mg L^{-1} d^{-1} per day (Fig. 8). Compared to SC-5d, COD concentration in C-5d was relatively less variable. Mean values of COD removal rate and daily capacity of C-5d were 42.19% ± 13.07% and 34.60 ± 10.72 mg L^{-1} d^{-1}, both of which were slightly higher than SC-5d. As the algae-bacteria system proved to have a higher carbon removal capacity [46], the nuance between the two operation modes could be due to some bacteria activity induced by the continuous flow.

Overall, Ge and Champagne [33] reported higher efficiency of *C. vulgaris* cultivated under batch and subsequent semi-continuous mode, whereas they observed COD removal dropped from 99% to 68% when COD loading increased from 30.5 to 235.3 mg L^{-1}. Considering COD influent in our study was up to 410 mg L^{-1}, the disparity of COD removals between different research could be attributed to the COD concentration of the substrate. As it is noted, the most organic carbon present in wastewaters is not suitable for microalgal autotrophic growth [47]. Chandra et al. [48] discovered a dramatic biomass decrease when COD increased from 500 to 2,000 mg L^{-1}, as a high concentration of organic carbon could induce physiological stress that impedes algal growth and negatively affect effluent treatment. In order to achieve sufficient COD removal, adding pre-treatment or dilute the influent may help decrease the organics loading and enhance microalgal COD removal [49].

3.4. Mechanism interpretation of cyclical light impact on semi-continuous MBWT system

LED lights were set at an illumination period of 12 h d^{-1} with a light intensity at approximately 3,000 lux to represent the natural diel cycle in outdoor cultivation. Wastewater influent was delivered into the semi-continuous algal system at 21:00 p.m. Lights turned on at 9:00 a.m. and switched off at 21:00 p.m. At the same time, samples were collected before and after 12 h of illumination to represent algae grown under dark cycle and light cycle, respectively. Cyclical light impact on algal growth and pollutant removal based on semi-continuous culture was then assessed accordingly.

As shown in Fig. 9a and b, there were significant difference between light and dark cycle in regard to algal

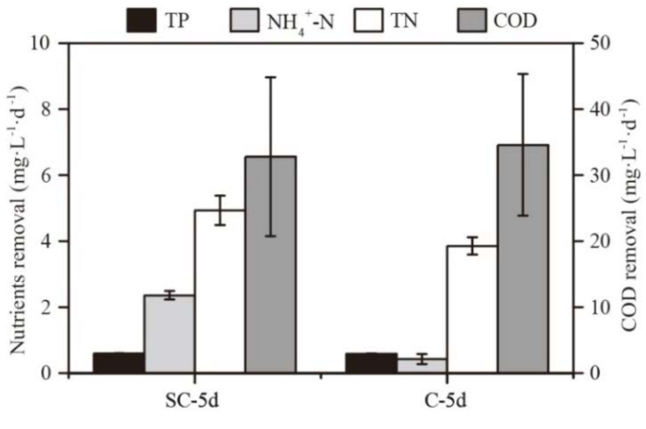

Fig. 8. Nutrients and COD removal capacity in semi-continuous (SC-5d) and continuous (C-5d) systems.

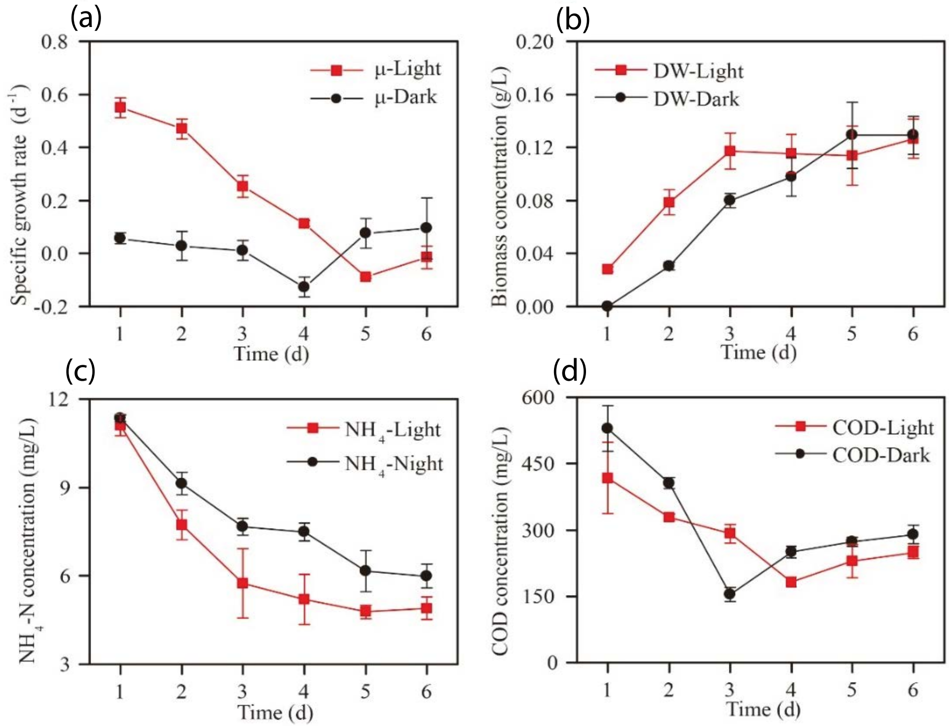

Fig. 9. Algal growth and wastewater treatment of *C. vulgaris* system performed under diel variations.

growth rate ($p = 0.013 < 0.05$) and biomass concentration ($p = 0.017 < 0.05$) from day 1–4. The light cycle, which provided indispensable illumination for photosynthetic activity, seemed to be more favorable for *C. vulgaris* to achieve faster growth and higher biomass accumulation initially. The dark cycle, on the other hand, demonstrated little growth. It has been proved that algal biomass is mainly derived from the solar energy attained through photosynthesis under solar radiance [50]. Artificial lights, such as LEDs used in our experiment, can provide 4%–6% conversion of electrical to chemical energy for algal photosynthesis [51]. Under illumination, autotrophic microalgae are able to reserve electron energy through photosynthesis. When light energy is unavailable during the night, microalgae would shift to heterotrophic condition, which requires internal energy consumption to support algal cells respiration and metabolism [52]. This results in the stagnated growth and biomass loss of algae in the dark.

The gap of algal growth under light and dark environments, however, started to narrow since day 2 and even switched conversely at day 5–6. The specific growth rate of *C. vulgaris* under illumination decreased from ~0.55 d^{-1} (day 1) to down below zero (day 6), indicating the declining growth of algae cells during the photoperiod. This could be explained by the self-shading effect when algal density increases to a certain point that induces light limitation along the photosynthesis process and impedes further algal growth at a high rate. In contrast, the dark cycle saw a moderate increase of specific growth rate from ~0.058 d^{-1} (day 1) to ~0.094 d^{-1} (day 6). Since microalgae require a dark period to compensate for chlorophyll-a depletion under high light intensity, the slight growth in the dark indicated enhanced algal functionality recovery after average irradiance per cell declined in the system [51]. Besides, the 'circadian clock' that internally controls algal cell activity may also contribute to such growth fluctuations [53]. As reported by Knutson et al. [52], there could be an internal synchronous cell cycle that regulates algal growth rate and maximum biomass production. When environmental conditions, such as light availability, vary over the cultivation period, it may affect the cell cycle of each algal cell and impact the whole culture [54].

Biomass concentration exhibited similar trends in response to diel changes. The difference value of dry weight biomass concentration under light (DW-Light) and dark (DW-Dark) environment, which corresponds to the additional biomass accumulation in the system, shrank gradually from day 4. At the end of the trial, DW-Dark reached 0.13 ± 0.01 g L^{-1}, which was nearly the same as DW-Light (0.13 ± 0.02 g L^{-1}). This shows that *C. vulgaris* had reached a steady growth state, and diel impact tends to decline after algae well adapted to the wastewater environment. For most algal research under continuous flow, continuous illumination is commonly preferred for simplified observation of algal cell physiology [14]. However, excessive exposure to light is against circadian rhythms that may cause cell damages and could be unrealistic for outdoor culture in long-term cultivation [53]. With the aim of application in real WWTPs, microalgal systems based on continuous culture operation would be crucially important to determine quasi-natural diel patterns of cell growth and physiological behavior [14]. To our best knowledge, our research is those rare ones that assess microalgal performance under both cyclical effluent and cyclical light irradiance. The results in this experiment suggest applying semi-continuous cultivation mode as a possible way for microalgae photo acclimatization. Besides, it also provides an insight into proper harvesting time in an algal system, that is when diel variations of biomass diminish and microalgal growth remains stable. This is helpful to ensure the sustainability of a microalgal system when scaling up.

The impact of diel light variations on microalgal wastewater treatment performance is shown in Fig. 9c and d. NH_4^+–N removal varied notably when environment turned illuminated and dark respectively ($p = 0.005 < 0.05$).Average NH_4^+–N concentration over the light cycle remained consistently lower than that of the dark cycle, suggesting better ammonia nitrogen removal of algal cells grown under sufficient illumination. The results could be interpreted by the physiological characteristics of microalgal nitrogen acquisition as sufficient light enables active photosynthesis that produces necessary energy for NH_4^+ transport. Besides, the coherent relationship between algal growth and nitrogen assimilation also serves as another explanation. As shown in Fig. 10, a linear relationship between algae cell amounts (10^{11} cell) and ammonia removal amount (mg) was discovered in the study ($y = 0.13932 + 0.15874$, $R^2 = 0.84653$). With higher algal growth, followed by higher N demand. The considerable performance of NH_4^+ removal in the algal system hinted at the acclimatization of *C. vulgaris* to both effluent and L/D cycles.

COD removal under diel variations showed significant difference ($p = 0.006 < 0.05$) except for day 3 (Fig. 7d). Generally, COD concentration over the light cycle was slightly lower, which means that organic carbon consumption during algal cultivation mostly occurred under light irradiance. The findings are consistent with [55], who proved that light enhancement could positively impact COD removal in municipal wastewater. In this state, *C. vulgaris* was grown under a mixotrophic condition, where carbon assimilation contains both inorganic and organic forms. During photoperiod, algae assimilate carbon dioxide through photosynthesis and store it as polysaccharides by activated dark reaction of Calvin cycle, while organic compounds could provide direct essential nutrients for the assimilation as well [33,56]. Hence, the exchange between carbon assimilation and respiration over the diel cycle determines the ultimate carbon equilibrium in the system. To further improved light-influenced algal COD removal, increasing light intensity while maintaining a suitable L/D ratio could be a possible method.

Understanding the general profile and mechanism of microalgae grown under dynamic flow and cyclical light is profound for the stable operation of an MBWT system. Apart from the results discussed, it is worth mentioning that microalgal removal of TN and TP demonstrated no significant difference in this study (data not shown), which implies that further attempt for algal nutrients removal enhancement deems other alternatives in addition to proper light control.

3.5. Implication for using microalgae as a secondary treatment for municipal wastewater

Scale-up feasibility, a critical issue that affects the real application of MBWT technology, was rarely discussed in most studies despite its great importance [4]. In this study, microalgal research was conducted based on the influent of a real A^2/O WWTP in southern China with an annual capacity of ~3.65×10^7 m^3. According to the results, *C. vulgaris* demonstrated maximum pollutants removal and biomass production performance under semi-continuous operation mode with an HRT of 5 d. A relevant techno-economic analysis was shown in Table 4.

Unlike traditional A^2/O systems, where chemicals are usually compulsory to assist further treatment due to poor nutrients removal of activated sludge, microalgae provide an ideal low-cost approach for enhanced municipal wastewater treatment. In this case, aluminum sulfate was used to remove residual phosphorus in the secondary effluent, and the annual dosage of the A^2/O WWTP was approximately 1,300 t. Taken general TP and TN removal as 99% and 60%, a microalgae system can remove around 819 t TP and 108 t TN annually. With residual nutrients in the effluent meeting

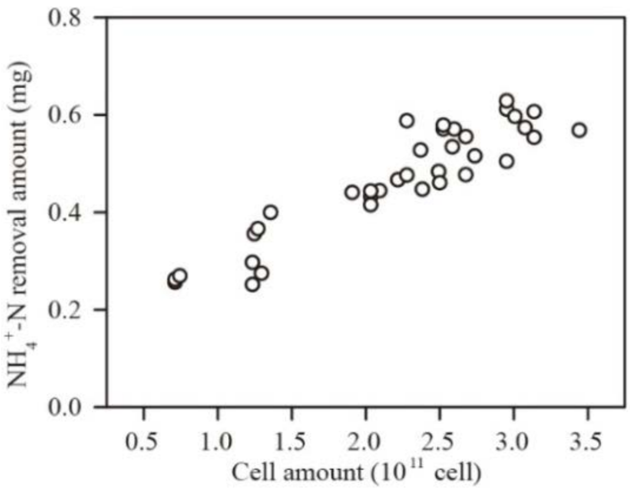

Fig. 10. A linear relationship between algae cell amounts and ammonia removal amount.

Table 4
Techno economic analysis of microalgae-based wastewater treatment (semi-continuous mode, HRT = 5 d)

	Removal rate (%)	Removal capacity (ton pollutant per year)	Influent concentration (mg L^{-1})	Effluent concentration (mg L^{-1})	Discharge standard (mg L^{-1})*
TP	99	819	3.0	<0.01	<0.1
TN	60	108	37.4	<15	<15

*Discharge standard refers to the primary A standard of pollutants concentration in WWTPs effluent of the "Discharge standard of pollutants for municipal wastewater treatment plant" (GB18918-2002) in China.

the discharge standard well. Considering aluminum sulfate market price at 410 CNY/t, replacing the activated sludge process with the microalgae process could therefore save at least 0.53 M CNY per year theoretically.

Another advantage of a microalgal WWTP is its considerable resource recovery potential. Algal biomass accumulated in wastewater is rich in nutrients and can be recovered through anaerobic digestion or hydrothermal methods [10]. Considering average N and P recovery efficiency at 60% and 80%, 5.29 g N and 10.08 g P could be recovered per ton treated wastewater based on nitrogen and phosphorus content of ~6.3% and 1% in algal biomass [57]. This forms up to an annual production of 193.2 t N and 36.8 t P for the aforementioned WWTP. As it is estimated, full recovery of phosphorus from municipal wastewater can save up to more than 5% of the chemical fertilizer annually used in China [1]. Nitrogen and phosphorus concentration in algal biomass that serves as fertilizer substitute theoretically bring the market value of more than 0.8 M CNY. Thus, the potential economic benefits of the MBWT system are tremendous, which would support the commercialization and scaling-up of microalgae-based municipal wastewater treatment in the future.

4. Conclusions

This study investigated the impact of hydraulic retention time, operation modes and cyclical light on microalgal growth, biomass production and wastewater remediation. An HRT of 5 d was managed to maintain the highest cell growth rate and stable biomass accumulation due to less wash-out, consequently with comparative pollutants removal efficiencies. In contrast to batch and continuous mode, semi-continuous cultivation showed a positive effect in terms of promoting microalgal acclimatization and ensuring sustainable biomass accumulation, which led to satisfying effluent that meets the stringent discharge standard well. Among all the observed pollutants, total phosphorus removal by *C. vulgaris* remained stable at >99% regardless of operational parameters changes, indicating the great potential of microalgae for enhancing phosphorus removal of municipal wastewater. Under cyclical light on a natural L/D basis, influence from both external light radiance changes and internal cell cycle on microalgal biomass, NH_4^+–N and COD performance was observed, and a possible criterion for biomass harvesting was proposed based on algal diel patterns variations. The findings of this study also shed light on the techno-economic feasibility of adapting microalgae for secondary wastewater treatment and unveiled the nutrients recovery potentials when scaling up the MBWT system.

Acknowledgments

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