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Role of hydraulic retention time in integration of microalgae and activated sludge process for nutrient recycle from diluted dairy liquid digestate

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ABSTRACT

The integration of microalgae and activated sludge (MAS) processes presents a promising method for recycling nutrients from animal digestates. However, the effects of operational factors such as hydraulic retention time (HRT) on this process remain unclear. Therefore, this study utilized semi-continuous photobioreactor systems with a working volume of 800 mL to investigate the impact of three different HRTs (4 d, 6 d, and 8 d) on the performance of MAS processes for nutrient recycling from diluted dairy liquid digestate (DLD). Results indicated that the 8 d HRT yielded the highest biomass concentration, ranging from 2.20 to 2.52 g/L. However, extending the HRT beyond 6 d decreased biomass productivity. The growth of MAS biomass favoured nutrient removal, with *C. vulgaris* the primary contributor. Longer HRTs improved nutrient removal, with the 8 d HRT achieved the highest total nitrogen (TN) removal of 87.68 ± 4.57 % and complete phosphorus elimination. Complete total ammonia nitrogen (TAN) removal occurred when HRT ≥ 6 d. All systems exhibited poor COD removal due to the poor biodegradability of DLD and the algal organic matter (AOM) produced by microalgae, with the highest COD removal of 16.43 ± 3.65 % (8 d HRT). Metagenomic analysis revealed that HRTs affected bacterial communities significantly, with the highest richness and diversity occurring at a 6 d HRT. Dominant phyla in all three systems were *Proteobacteria* and *Bacteroidetes*. Longer HRTs enhanced lipid accumulation and reduced carbohydrate contents but had minimal effect on higher heating value (HHV), which ranged from 20.63 to 21.72 MJ kg^{-1}. These findings have significant implications for developing efficient and sustainable MAS-based treatment systems.

1. Introduction

Economic growth and population expansion have led to a surge in demand for animal products, resulting an increase in animal wastewater and manure. Anaerobic digestion has become a common strategy for managing this waste through biogas production. However, liquid digestate, the by-product of anaerobic digestion, can pose threat to the eco-system due to its high organic and nutrient content [1–3]. Unfortunately, traditional methods of liquid digestate treatment such as ammonia stripping and aerobic biological treatment, are costly and treat the liquid digest as waste, leading to the production of unwanted by-products like CO_2 and activated sludge [4,5]. Therefore, it is essential to identify cost-effective and sustainable methods for treating liquid digestate as a valuable resource.

Recently, algal-bacterial symbiotic (ABS) system has gained popularity as a feasible alternative to improve wastewater treatment performance. In the ABS system, bacteria aid in COD biodegradation and release CO_2 for algal photosynthesis, while the oxygen produced by microalgae serves as an electron acceptor for bacteria to oxide organic compounds [6]. The microalgae and activated sludge (MAS) system has emerged as a viable alternative for wastewater treatment. In comparison to ABS systems, MAS systems are easier to construct and exhibit improved bioremediation abilities for chemical oxygen demand (COD), nitrogen, and phosphorus. They also have better sedimentation

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capabilities and emit lower levels of greenhouse gases [6–8]. Moreover, MAS systems are effective in removing metals, antibiotics, and other harmful pollutants [6,9,10]. Similar to the ABS system, MAS biomass can also be recovered for producing biofuel, fertilizer, and other by-products [11,12]. Therefore, the MAS system is a viable option for wastewater treatment.

The MAS process has been extensively researched for wastewater remediation and biomass recovery [7,13,14]. Performance of the MAS system is influenced by various factors such as illumination [15] and the inoculation ratio of microalgae and activated sludge [7]. Furthermore, immobilizing MAS has proven to be an effective approach for achieving good wastewater remediation efficiency [16]. Despite the extensive research on factors and measures affecting the MAS process, the hydraulic retention time (HRT), a critical factor that affects the performance of the MAS system, has received less attention. HRT is crucial in biological wastewater treatment as it determines the fate of microorganisms with distinct reproduction generations, especially autotrophic microbial communities. Therefore, it directly affects the economic feasibility of the treatment process [17]. Moreover, an appropriate HRT can significantly decrease processing time and enhance the effectiveness of biological treatment. During MAS processes, a short HRT leads to insufficient nutrient removal and a low biomass yield, while a long HRT results in nutrient starvation and a decreased wastewater treatment volume [6,18]. Additionally, the optimal HRT fosters a balanced relationship between microalgae and bacteria, promoting the formation of microalgae-bacteria symbiosis. This is conducive to establishing an efficient and continuous treatment system, and ultimately affects pollutant removal, nutrient recovery, and biomass production. Therefore, exploring optimal HRTs holds paramount importance for optimizing the MAS process. Although some studies have investigated the effects of HRT on MAS performance, their focus has mainly been on pollutant removal and the biomass potential of MAS [6,19]. Moreover, some researchers tend to use synthetic wastewater as a substrate [7,20], which is not conducive to practical applications due to the different biotic and abiotic characteristics between real and synthetic wastewater. Despite there are studies utilizing real wastewater as a substrate, they did not explore the effects of HRT on the relationships between microalgae and bacteria in semi-continuously operated systems [21–23].

The optimal HRT for MAS processes ranges from 2 to 8 days [6]. Therefore, this research aimed to employ the MAS process for treating real diary liquid digestate (DLD) under three different HRTs (4 d, 6 d, and 8 d) to (1) monitor the MAS growth condition; (2) evaluate pollutant removal and investigate possible mechanisms; (3) explore the relationships between microalgae and activated sludge bacteria during the operation period; and (4) assess the biofuel potential of the harvested MAS biomass.

2. Materials and methods

2.1. Experiment design

Activated sludge was collected from an aerobic tank at Da Tansha sewage plant, Guangzhou, Guangdong, China and stored at 4 ℃ for further use. DLD was collected from an anaerobic digester at a dairy farm (Kaiping, Guangdong, China), and it was then centrifuged and filtrated using Whatman glass microfiber filters (1.2 μm, Grade GF/C) to eliminate the adverse effects of large particles before being stored in a 4 °C refrigerator until further use. Our previous study found that 25 % DLD was optimal for *C. vulgaris* growth, nutrient recovery, and biofuel production [12]. Therefore, the DLD was diluted with deionized water at a 1:3 (v/v) ratio, resulting in a pH of 6.71 ± 0.01, COD of 360.0 ± 11.3 mg L^{-1}, total nitrogen (TN) of 151.3 ± 1.8 mg L^{-1}, total ammonia nitrogen (TAN) of 130.5 ± 5.7 mg L^{-1}, and total phosphorus (TP) of 59.7 ± 1.3 mg L^{-1}. Considering that the treated DLD could be reused to dilute the raw DLD, this dilution would not affect the industrial application.

The axenic strain of microalgae, *Chlorella vulgaris* NIES-227, was cultured in BG-11 medium. The compositions of BG-11 medium are shown in the supplementary materials. *C. vulgaris* was pre-cultured in a 5.0 cm diameter, 60 cm height air-lift photobioreactor (PBR) with a working volume of 800 mL. The pre-culture was conducted under continuous illumination (200 μmol m^{-2} s^{-1}) and room temperature, while aerating the mixed air containing 2 % CO_2 into the PBR with a flow rate of 0.3 gas volume per culture volume per min (vvm). The log-phase microalgae were used as inocula for the following experiment after centrifugation and washing twice with deionized water.

Microalgae and activated sludge were co-cultured in the same air-lift photobioreactors (PBRs) at a concentration of about 0.4 g/L, with a mass ratio of 1:1. The PBRs were aerated with mixed air containing 2 % CO_2 at 0.3 vvm and operated under continuous illumination (200 μmol m^{-2} s^{-1}) at room temperature [12]. The MAS was pre-cultured for 4 days to reach the log phase and accumulate biomass. Then, aeration was halted, and the systems were settled down 30 min to precipitate the MAS biomass. Subsequently, 50 % of the supernatant was replaced with diluted DLD every 2 days, 3 days, and 4 days to achieve 4 d, 6 d, and 8 d HRT, respectively. Each HRT was duplicated in separate PBRs, and four complete cycles were continuously conducted for each HRT (4 days, 6 days, and 8 days) without pH control. This resulted in a cumulative operation period of 8 days (4 d HRT), 12 days (6 d HRT), and 16 days (8 d HRT). After each cycle, 50 % of the culture was replaced with diluted DLD without settling, and the effluent was collected. Simultaneously, the mixed solution (influent) was collected from inside the PBRs. Additionally, samples were taken at specific time points during the half cycle: 1 day, 1.5 days, and 2 days after the replacement of the 4 d, 6 d, and 8 d HRTs, respectively. The sample volume was 15 mL. These samples were then stored at 4 °C for further analysis. The biomass and pollutant contents after pre-culture are presented in Table S1.

2.2. Chemical analysis

2.2.1. Biomass analyse

The dry weight of MAS was measured following the procedure described in APHA [24]. In brief, a 10 mL sample was filtered through 1.2 μm Whatman glass microfiber filters (Grade GF/C), and the filter with MAS biomass was dried in an oven at 105 ℃ for 4 h. The weight of the dried filter with biomass was determined by subtracting the weight of the filter alone. Microalgae growth was monitored by measuring the contents of chlorophyll (chl) a and b. To extract the pigments, 1 mL of the sample was centrifuged and the resulting pellet was resuspended in dimethyl sulfoxide (DMSO) and placed in a water bath at 55 °C following the method of Zhu et al. [25]. The absorption of the extract was measured at 649 (OD_{649}) and 665 nm (OD_{665}) using a spectrophotometer (EON, American BioTek Corporation). The contents of chl *a* and *b* (μg mL^{-1}) were calculated using the following equations:

$$Chl_a = 12.47 \times OD_{665} - 3.62 \times OD_{649} \quad (1)$$

$$Chl_b = 25.06 \times OD_{649} - 6.5 \times OD_{665} \quad (2)$$

2.2.2. Water quality analysis

The collected samples were centrifuged at 10,000 rpm for 5 min and then filtered through 0.22 μm nylon membranes [12,26]. COD, TN, TAN, nitrate (N-NO_3^-) and nitrite (N-NO_2^-), and TP were measured in the filtered samples using a water quality analyser (DR 2700 Hach Co., Loveland, CO, USA). Total organic nitrogen (TON) was calculated by subtracting TAN, N-NO_3^-, and N-NO_2^- from TN. Nitrogen assimilated by MAS was calculated using the equation (3), while nitrogen removed through denitrification was calculated by subtracting nitrogen assimilated by MAS from total removed nitrogen.

$$N_{assimilated} = 1000 \times \left(M_{effluent} - M_{influent}\right) \times N\% \quad (3)$$

where the $N_{assimilated}$ (mg L^{-1}) represents the nitrogen assimilated by MAS, the $M_{effluent}$ and $M_{influent}$ stand for the MAS dry biomass in the

effluent and influent, respectively (g L^{-1}). N% is the nitrogen element content of the MAS dry biomass.

2.2.3. Biomass potential of MAS

The freeze-dried MAS biomass collected from the effluent of the third cycle was analysed for its C, H, and N contents using an elemental analyser (Vario EL Cube, Elementar, Germany). The higher heating value (HHV) of the freeze-dried biomass was calculated using an empirical equation [27].

$$HHV(kJ/kg) = 3.55C^2 - 232C - 2230H + 51.2C \times H + 131N + 20600 \quad (4)$$

In this equation, C, H, and N represent the carbon, hydrogen, and nitrogen content (%) in the dry MAS biomass, respectively.

The protein content of the biomass was determined using Bradford Protein Assay Kit. The carbohydrate content was measured using the phenol–sulfuric acid method [28]. Specifically, about 30 mg of freeze-dried MAS biomass was reconstituted in 10 mL of deionized water. Aliquots of 2 mL of sample suspension were mixed with 5 mL of concentrated sulfuric acid (98 wt%) and 1 mL of phenol (6 %, w/v). The mixture was then placed in a boiling water bath for 20 min and subsequently cooled down to room temperature. After cooling, the mixture was measured at 490 nm. Samples were quantified by comparing them to a calibration curve made from glucose under the same conditions.

The total lipid content measurement method was revised from Zhu et al. [28]. Specifically, 30 mg of freeze-dried biomass was added to a mixture of chloroform, methanol, and deionized water (1:2:0.8, v/v/v) and stirred it with a magnetic stirrer at 40 ℃ for 30 min. The mixture was then centrifuged at 3500 rpm for 5 min, and the supernatant was collected. The mixed organic solvents mentioned previously were added to the residual and the process was repeated 3 to 5 times until the supernatant became colourless. Chloroform and deionized water were then added to the collected supernatant to achieve a volume ratio of chloroform: methanol: deionized water of 2:2:1.8. The mixture was allowed to stand for 30 min, after which the organic phase was combined, evaporated to dryness under a stream of N_2, and weighed.

2.3. Bacterial community analysis

Effluent samples from PBRs were collected after 4 days of pre-culture (original) and the third cycles. The samples were immediately centrifuged and stored at −80℃ for bacterial community analysis [12]. Genomic DNA of bacteria was extracted using an E.Z.N.A. Soil DNA Kit (Omega, US) following the manufacturers' instructions. The V3-V4 region of the 16S rRNA genes was amplified using the universal primers 341F (5′- CCTACGGGNGGCWGCAG − 3′) and 805R (5′- GACTACHVGGGTATCTAATCC − 3′) [29]. The PCR amplification method is described in detail in the Supplementary Materials. The resulting amplified PCR product was then subjected to sequencing to assess the richness and diversity of microbial communities, using measures such as the Chao1 estimator, ACE estimator, Shannon index, Simpson index, and Good's coverage. Further details about the bioinformatics analysis can be found in the Supplementary Materials.

2.4. Statistical analysis

The data showed the average measurements of collected samples with a standard deviation (mean ± sd). The figures were made using Origin 2021, and the data statistical analysis was conducted using IBM SPSS Statistics, with $p < 0.05$ considered statistically significant.

3. Results and discussion

3.1. Biomass growth

After four days of pre-culture, Fig. 1 illustrates the growth of MAS biomass and *C. vulgaris* during the four cultivation cycles under three HRTs. Both MAS biomass (Fig. 1A) and chl ($a + b$) (Fig. 1B) in all three HRTs showed a noticeable increase after four cycles' cultivation. The effluent MAS biomass stabilized within varying ranges, and the time required for stabilization differed across different HRTs. At 4 d and 6 d HRTs, stabilization occurred from the third cycle, resulting in

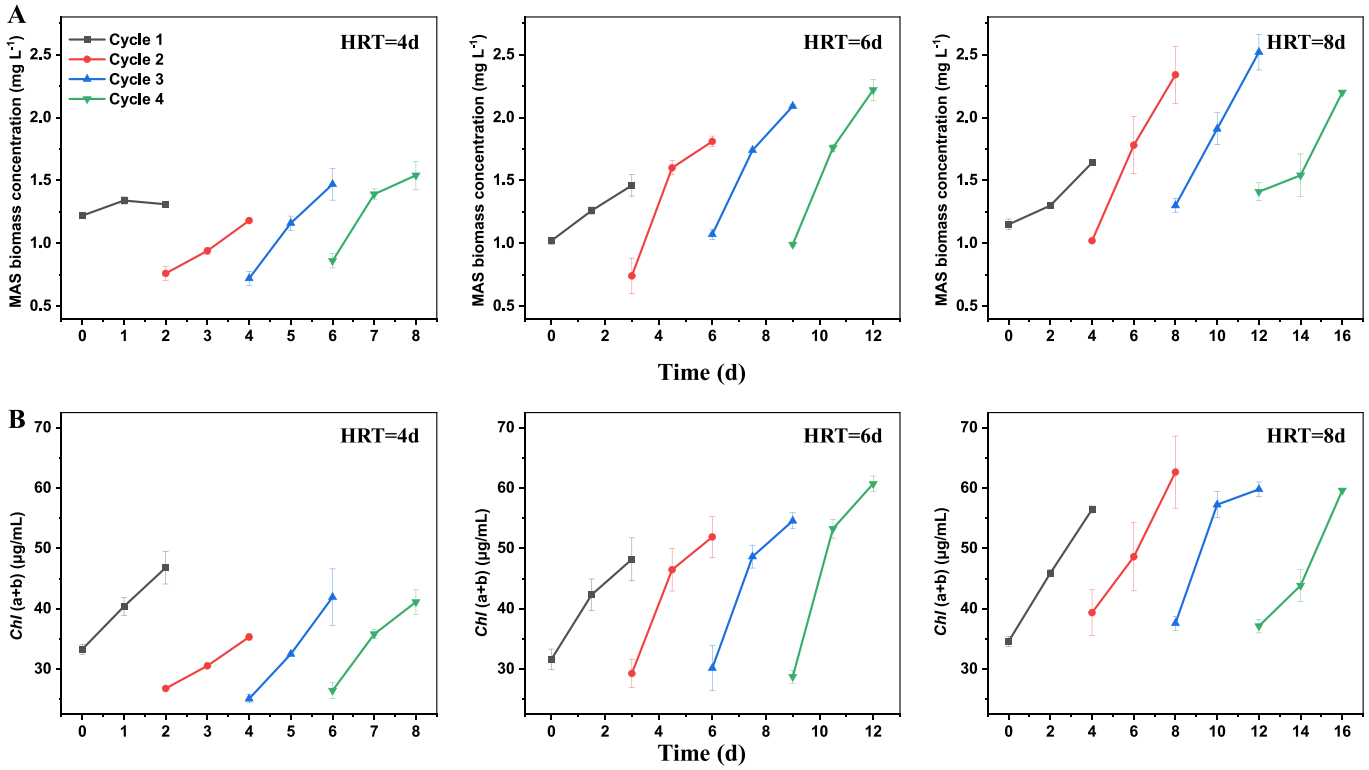

Fig. 1. MAS biomass concentration (A) and the content of chl ($a + b$) (B) during four cultivation cycles under three different HRTs.

concentrations of 1.47–1.54 g/L and 2.09–2.22 g/L, respectively ($p > 0.05$). Whereas 8 d HRT stabilized from the second cycle, yielding concentrations of 2.20–2.52 g/L ($p > 0.05$) (Fig. 1A). This suggests that extending HRT benefits the accumulation of MAS biomass, with a longer HRT achieving a higher stabilized MAS biomass concentration.

In this study, the variation trend of MAS biomass was consistent with the content of chl ($a + b$) (Fig. 1A and 1B). Our previous study found that both microalgae and bacteria contributed to MAS biomass growth when culturing the same *C. vulgaris* and activated sludge at an inoculum mass ratio of 1:1 in 25 % DLD for 8 days. The bacteria grew significantly in the first four days, then increased slower during the rest of cultivation, and the increased MAS biomass was primarily attributed to *C. vulgaris* [26]. Therefore, both microalgae and bacteria contributed to the increased MAS biomass in this study. Notably, bacteria made more contribution to MAS biomass in a shorter HRT. This finding is consistent with Anbalagan et al. [6], who reported that bacterial growth might dominant at lower HRTs when culturing MAS systems under 2 d, 4 d, and 6 d HRTs.

Fig. S1 demonstrates that the MAS biomass productivity initially

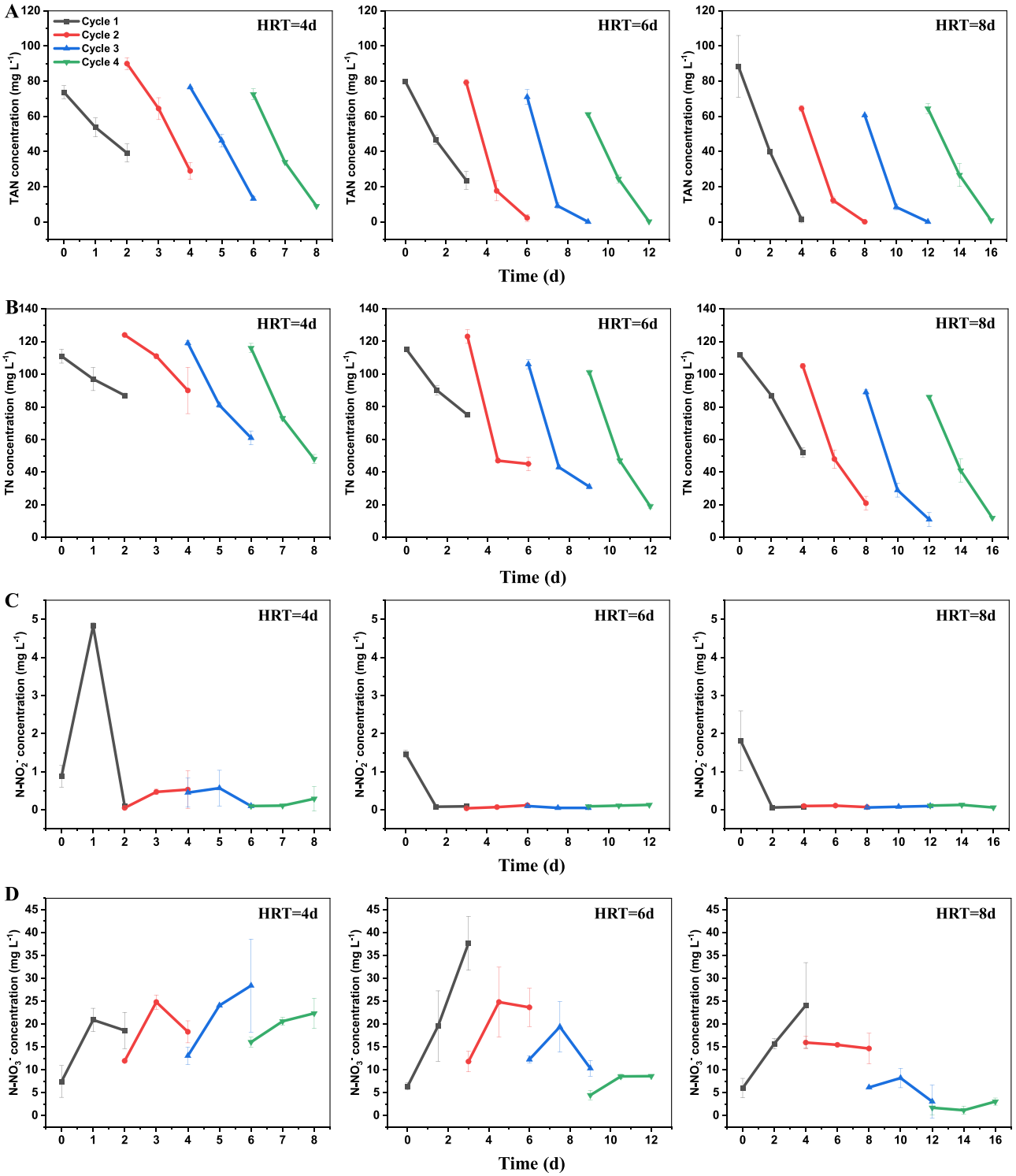

Fig. 2. The variation of TAN (A), TN (B), N - NO_2^- (C), and N - NO_3^- (D) at three different HRTs during the cultivation lasting four cycles.

increased in 4 d and 6 d HRT from 0.05 ± 0.01 and 0.15 ± 0.02 g/L d^{-1} and then stabilized at about 0.36 and 0.37 g/L d^{-1}, respectively. In contrast, for 8 d HRT, it increased from 0.12 ± 0.01 g/L d^{-1} to 0.31 ± 0.02 g/L d^{-1} and then decreased to 0.21 ± 0.00 g/L d^{-1}. It is worth noting that at the forth cycle, 8 d HRT had the highest MAS concentration compared to 4 d and 6 d HRT (Fig. 1A). Moreover, the nutrient starvation was observed from the third cycle at 8 d HRT (will be further discussed in Section 3.2). Therefore, the decreased MAS biomass productivity at 8 d HRT was owing to the high biomass concentration [30] and nutrient deficiency. From the third cycle onwards, 4 d and 6 d HRT exhibited similar MAS biomass productivity ($p > 0.05$), indicating that HRT ≤ 6 d is favorable for MAS biomass productivity. Therefore, in this study, longer HRT leads to increased biomass yield, but decreased biomass productivity when HRT > 6 d.

3.2. Pollutant removal

3.2.1. Nitrogen removal

Based on the results shown in Fig. 2A, longer HRTs benefited nitrogen removal. The TAN removal reached 87.58 ± 0.92 % at 4 d HRT, and complete removal was achieved at 6 d and 8 d HRT. A maximum TAN removal at 4 d, 6 d, and 8 d HRT occurred in the fourth, third, and second cycles, respectively. These findings indicate that longer HRTs require fewer cultivation cycles to achieve the highest TAN removal. Additionally, the elimination of TAN in the last two to three cycles at 6 d and 8 d HRT indicates the stability and reliability of the MAS system, which is significant for real applications. Regarding TN removal, it increased with each cultivation cycle for all three HRTs. The maximum TN removal achieved for 4 d and 6 d HRT occurred in the fourth cycle at 58.58 ± 3.45 % and 81.20 ± 1.14 %, respectively (Fig. 2B), while the 8 d HRT reached the highest TN removal of 87.68 ± 4.57 % in the third cycle. Notably, the effluent of TN in the third and the fourth cycles at 8 d HRT stabilized at about 12 mg L^{-1} (Fig. 2B), indicating that the TN removal at 8 d HRT stabilized from the third cycle. This suggests that longer HRTs promote TN removal because an extended HRT allows MAS more time to remove nitrogen.

In the MAS system, nitrogen could be removed via stripping, microalgae, and activated sludge. In this research, the pH levels in all three systems was lower than 8.00 (Fig. S2), indicating that stripping is unlikely as it typically occurs at pH levels above 9 [31]. A previous study reported that *C. vulgaris* could eliminate TAN under 120 mg L^{-1} within 2–3 days by microalgae assimilation when pH was between 6.50 and 7.50 [32]. In this study, the pH of the three cultivation systems was all lower than 6.60 from the second cycle (Fig. S2) and the initial TAN concentration was about 80 mg L^{-1}, demonstrating that microalgae made a substantial contribution to TAN removal. The accumulation of $N\text{-}NO_3^-$ (Fig. 2D) indicates that nitrifying bacteria also played a role in TAN removal. The nitrogen mass balance of the third cycle under all three HRTs is shown in Table 1. It can be seen from Table 1 that the nitrogen assimilated by MAS is all lower than the TN removal, indicating denitrification also contributed to nitrogen removal in the MAS system.

Table 1
Nitrogen mass balance of the third cycle in all three HRTs.

HRTs	4 d	6 d	8 d
N element content of the dry MAS biomass (%)	7.96 ± 0.07	8.18 ± 0.28	7.92 ± 0.42
Increased MAS biomass (g/L)	0.60 ± 0.12	0.82 ± 0.04	0.98 ± 0.06
Nitrogen assimilated by MAS (mg/L)	47.70 ± 9.50	66.75 ± 3.78	77.30 ± 5.98
Total nitrogen removal (mg/L)	58.0 ± 2.3	75.0 ± 3.5	78.0 ± 2.3
Nitrogen removal by denitrification (mg/L)	16.52 ± 0.42	13.87 ± 2.66	4.21 ± 2.30

Note: HRT means hydraulic retention time, while MAS represents microalgae and activated sludge.

The presence of denitrification bacteria will be discussed in Section 3.3. Therefore, the nitrogen removal observed in this research can be attributed to both microalgae and activated sludge.

In this study, 4 d HRT exhibited higher TN removal rate. The TN removal rate at 4 d HRT increased from 12.00 ± 1.41 (cycle 1) to 34.00 ± 2.83 (cycle 4) mg L^{-1} d^{-1} (Fig. S3). As TN removal was primarily attributed to MAS biomass, the fast MAS biomass productivity (Fig. 1S) elucidates the enhanced TN removal rate at 4 d HRT. The TN removal rate of 6 d HRT stabilized at about 26 mg L^{-1} d^{-1} ($p > 0.05$) during the last three cycles, indicating a stable TN removal rate in the system. At 8 d HRT, the TN removal rate peaked at the second cycle but decreased slightly at the fourth cycle. Since the effluent TN of the last two cycles at 8 d HRT stabilized at about 12 mg L^{-1}, the decreased TN removal rate was attributed to the insufficient nitrogen (Fig. 2B). Furthermore, it was noted that all three HRTs exhibited a similar TN removal rate in the initial two cycles ($p > 0.05$), but this rate decreased with the extension of HRT during the subsequent two cycles (Fig. S3). Despite 6 d and 8 d HRTs achieved a higher MAS biomass yield, the prolonged cultivation time and, specifically at 8 d HRT, insufficient nitrogen availability contributed to a slower TN removal rate.

The initial concentration of $N\text{-}NO_2^-$ and $N\text{-}NO_3^-$ in diluted DLD was 0.07 ± 0.01 and 0.6 ± 0.0 mg L^{-1}, respectively. However, during the first cultivation cycle, the TN removal for 4 d, 6 d, and 8 d HRT was 24.0 ± 2.8, 40.0 ± 2.8, and 60.0 ± 2.8 mg L^{-1}, while the TAN removal was 34.6 ± 1.4, 56.2 ± 5.9, and 74.6 ± 2.0 mg L^{-1}, respectively (Fig. 2A and 2B). The higher TAN removal indicates the conversion of ammonium into other forms of nitrogen. Notably, during the initial cultivation cycle, the $N\text{-}NO_2^-$ concentration in the 4 d HRT system increased first and then decreased, while the concentration of $N\text{-}NO_3^-$ increased significantly across all three systems (Fig. 2C and 2D). These results suggest the presence of ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). As shown in Fig. 2D, the concentration of $N\text{-}NO_3^-$ in the effluent of 4 d HRT ranged from 20 to 30 mg L^{-1} but decreased significantly at 6 d and 8 d HRT. These findings suggest that longer HRTs (≥6 d) promoted $N\text{-}NO_3^-$ removal, possibly due to the higher *C. vulgaris* biomass (Fig. 1B).

In this study, the production of nitrogen-containing algal organic matter (AOM) by *C. vulgaris* was observed. The initial TON content in diluted DLD was 20.08 ± 7.41 mg L^{-1}, which increased by approximately 15 mg L^{-1} after pre-cultivation in all three systems (Fig. 3), indicating the production of nitrogen-containing AOM during microalgal growth. This finding is supported by Chu et al. [33], who found the AOM produced by *C. vulgaris* during growth was rich in protein. During the last two cycles, the TON content in the effluent was consistently lower than that of the influent in all three systems (Fig. 3). Furthermore, the TON in the effluent of the forth cycle with 4 d, 6 d, and 8 d HRT were 16.36 ± 0.11, 10.07 ± 1.15, and 8.49 ± 0.21 mg L^{-1}, respectively, all of which were lower than the initial TON in diluted DLD (Fig. 3). These results suggest that the TON secreted by *C. vulgaris* and in diluted DLD can be removed by MAS, with longer HRTs facilitating TON removal. Similar findings have been reported in the literature for MAS systems capable of degrading TON in diluted DLD and landfill leachate [12,34]. However, in the last two cycles with 8 d HRT, TON concentration stabilized at around 8 mg L^{-1} (Fig. 3), indicating that MAS can only partially remove TON in diluted DLD produced by *C. vulgaris*. Further studies are required to identify the source of the remaining organic nitrogen, and post-treatment is necessary to eliminate the residual organic nitrogen.

3.2.2. Phosphorus and COD removal

The effectiveness of TP removal by MAS increased with longer HRTs. The highest TP removal was observed in the third and fourth cycles of 8 d HRT, with 100 ± 0 % removal, followed by 70.64 ± 1.65 % in the third cycle of 6 d HRT, and 52.03 ± 0.19 % in the fourth cycle of 4 d HRT (Fig. 4A). The thorough TP removal at the last two operating cycles of 8 d HRT suggests the stability and reliability of the MAS system in maintaining a high phosphorus removal over multiple cycles, which is

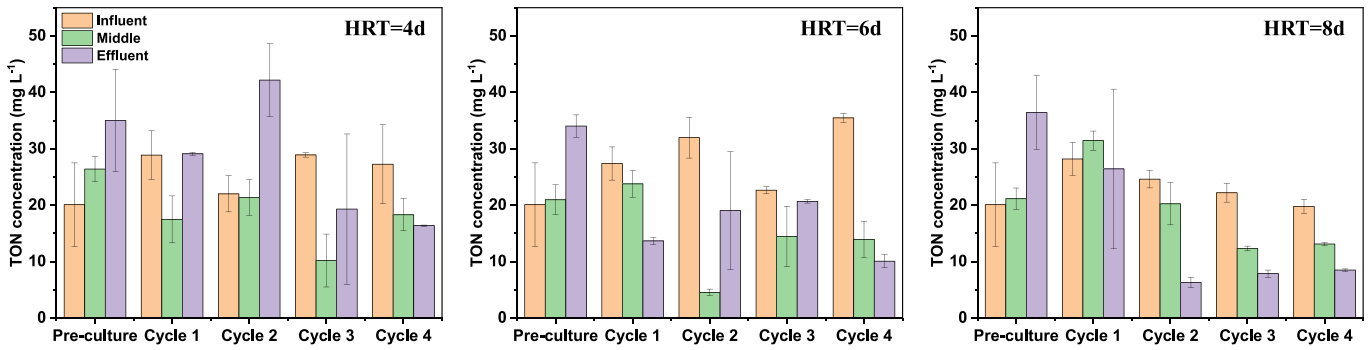

Fig. 3. The variation of total organic nitrogen (TON) during the entire cultivation period at 4 d, 6 d, and 8 d HRTs.

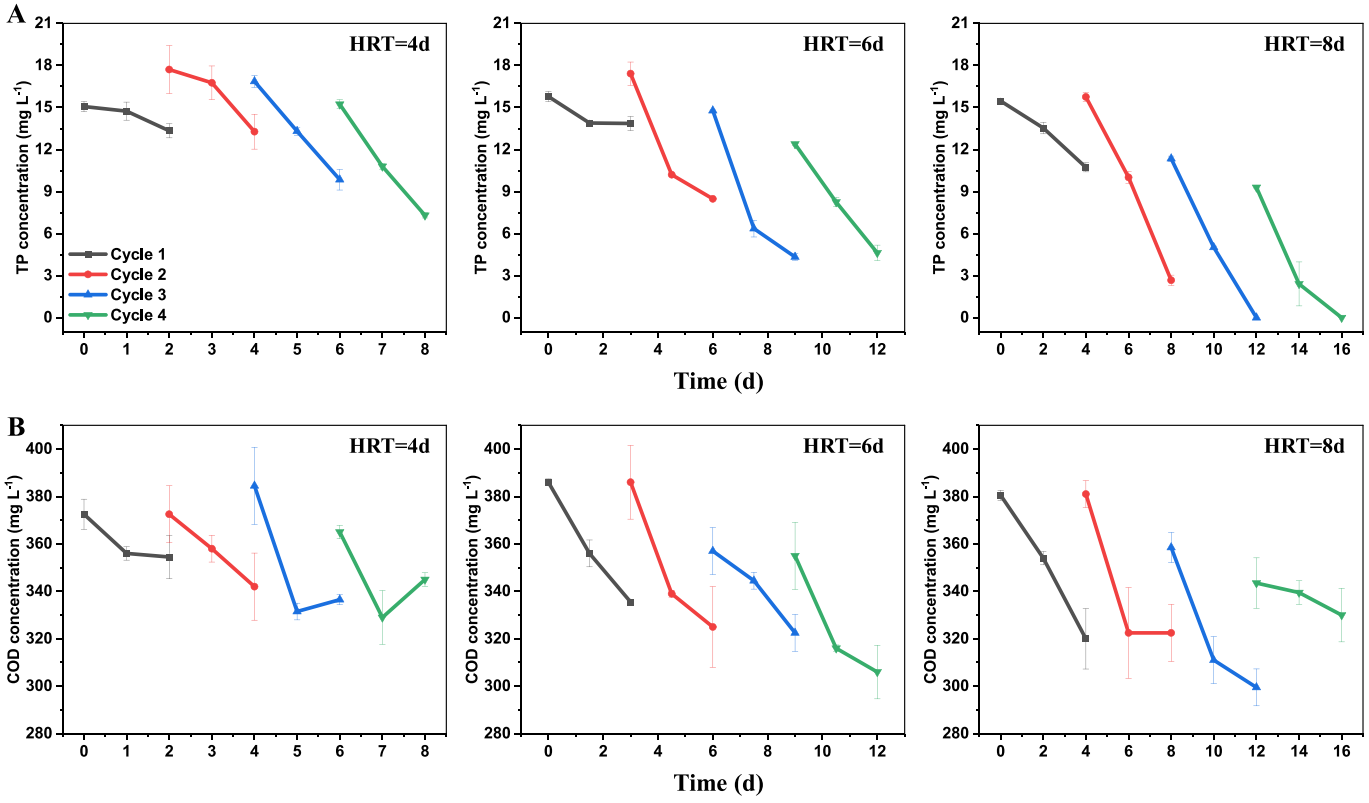

Fig. 4. TP (A) and COD (B) removal curves at three different HRTs during the whole cultivation.

crucial for practical wastewater treatment. The positive effects of longer HRTs on TP removal in the MAS system were reported previously [19].

In a MAS system, TP can be removed through microalgae uptake, precipitation, and phosphate accumulating organisms (PAOs). Previous studies have reported that phosphorus precipitation can occur at a pH exceeding 7.60 in 6 d HRT [35]. However, since the pH value was below 6.60 from the second cycle (Fig. S2), TP precipitation was unlikely to occur in this study. Notably, TP concentration slightly decreased during the first cycle in all three HRTs, but the reduction increased with prolonging cultivation (Fig. 4A), which coincided with the increase in MAS biomass (Fig. 1A). Therefore, TP removal was mainly attributed to MAS, with longer HRTs resulting in more MAS biomass and thus enhancing TP removal. PAOs in activated sludge could contribute to TP removal. In this study, PAOs were detected in all three systems (which will be further discussed in Section 3.3), indicating that activated sludge played a role in TP removal. It has been reported that microalgae can uptake phosphorus for various cellular processes in DLD [36], and *C. vulgaris* could eliminate phosphorus in 25 % DLD in 6 days [26], indicating microalgae contributed to TP removal in this study. The contributions of PAOs and microalgae on TP removal will be further discussed in Section 3.3.

The MAS system exhibited limited COD removal, as the effluents of all three systems still contained COD concentrations above 300 mg L^{-1} (Fig. 4B). While microalgae can utilize simple organics like glucose and acetate, it is not a reliable option for COD removal. Thus, the activated sludge in MAS was primarily responsible for COD removal. The unsatisfactory COD removal in this research was due to the poor biodegradability of DLD and the issue of AOM secretion [26]. AOM secreted during the growth of *C. vulgaris* (as stated in Section 3.2.1) counteracted the partial biodegradation of organics, resulting in poor COD removal. Similar negative effects of AOM on COD removal have been observed in a recent study that used microalgae to treat raw dairy wastewater [37].

The highest COD removal rates during the four cultivation cycles at 4 d, 6 d, and 8 d HRT were 24.00 ± 7.07 (the third cycle), 20.33 ± 0.47 (the second cycle), and 15.13 ± 3.71 (the first cycle) mg L^{-1} d^{-1}, respectively (Table S2). This indicates that shorter HRTs are more

favourable for achieving a faster COD removal, possibly because shorter HRTs benefit bacterial growth [38] and contain less microalgae (Fig. 1B). Moreover, the available organics were more sufficient at 4 d HRT compared to 6 d and 8 d HRT due to the poor biodegradability of DLD. However, the maximum COD removal at 4 d, 6 d, and 8 d HRT were 12.42 ± 3.15 (the third cycle), 15.82 ± 1.00 (the second cycle), and 16.43 ± 3.65 % (the third cycle), respectively, indicating that longer HRTs still benefit COD removal, although to a lesser extent. This might be because longer HRTs enriched bacteria that could biodegrade the refractory organics in the diluted DLD. Overall, MAS process is not an effective strategy for COD removal, with the highest COD removal of 16.43 ± 3.65 % obtained in the third cycle of 8 d HRT. Pre-treatment strategies to improve the biodegradability of DLD or post-treatment to remove the residual organics are necessary to achieve more effective COD removal.

3.3. Bacterial community

The bacterial diversity and structure in the effluent of the pre-culture and the third cycle of different HRTs were assessed using MiSeq high throughput sequencing, and the results are presented in Table 2. In all samples, a Good's coverage of greater than 0.99 was achieved, indicating adequate representation of bacterial diversity and structure in the sequence libraries. The Shannon index in all three systems was significantly lower than in the pre-culture ($p < 0.05$) (Table 2). The Simpson index at 4 d HRT also showed a significant decrease ($p < 0.05$), while at 6 d and 8 d HRT, it was similar to that of the pre-culture group ($p > 0.05$) (Table 2). These results indicate that the growth of *C. vulgaris* reduced the diversity of bacterial community, with the strongest effect observed at 4 d HRT. As shown in Table 2, the ACE index and Chao 1 index at 4 d and 6 d HRT were similar to the pre-culture ($p > 0.05$), while both indices decreased significantly ($p < 0.05$) at 8 d HRT, indicating that bacterial community richness was not affected until HRT exceeded 6 d.

As discussed above, HRTs have different effects on bacterial community. Compared to the pre-culture, bacteria in 4 d HRT maintained similar richness and decreased in diversity, indicating that microalgae selectively enriched specific bacteria without affecting their overall abundance. Similar selective effects of microalgae on bacteria was also reported previously [36]. 6 d HRT exhibited similar bacterial diversity and richness to the pre-culture, suggesting that bacteria and microalgae could coexist harmoniously at this HRT. Regarding 8 d HRT, the bacteria diversity was similar to the pre-culture, but the richness decreased, demonstrating the adverse effects of microalgae on bacteria. Microalgae and bacteria tend to have a compete relationship under nutrient deficiency conditions, and microalgae will consume oxygen and secrete inhibitory substances to supress bacteria [39]. In this research, the nutrient starvation was observed at 8 d HRT and the culture conditions favoured microalgae growth. The long HRT allowed the system to enrich more microalgae, consequently supressing the bacteria population. Therefore, 6 d HRT was conducive to the establishment of microalgal-bacterial symbiosis.

Table 2
Effective reads, numbers of OTUs, and calculated richness and diversity estimators were obtained from the effluent of pre-culture and the third cycle of different HRTs. It is based on a threshold of 97 % on the 16S rRNA gene level.

	Pre-culture	HRT = 4 d	HRT = 6 d	HRT = 8 d
Effective reads	62584 ± 12067	97970 ± 2815	107453 ± 2401	90369 ± 10420
OUT number	1348 ± 88	1021 ± 44	1197 ± 227	895 ± 129
Coverage	0.994 ± 0.001	0.997 ± 0.000	0.997 ± 0.000	0.997 ± 0.000
Shannon index	4.947 ± 0.086^{a}	3.101 ± 0.334^{b}	4.360 ± 0.029^{c}	3.871 ± 0.120^{c}
ACE index	1649.72 ± 64.39^{abc}	1406.04 ± 60.02^{abd}	1672.72 ± 34.89^{ac}	1180.25 ± 160.10^{bd}
Chao 1 index	1628.07 ± 35.99^{a}	1384.87 ± 42.50^{ab}	1532.44 ± 111.82^{a}	1165.69 ± 160.40^{b}
Simpson index	0.026 ± 0.005^{a}	0.218 ± 0.062^{b}	0.035 ± 0.009^{a}	0.087 ± 0.020^{a}

Note: The symbols a, b, c, and d represent the differences between groups, with a significance level (α) of 0.05.

The bacterial community compositions in the effluent of pre-culture and the third cycle of three different HRTs were monitored, and it was found that *Proteobacteria* and *Bacteroidetes* were the most dominant phyla in all samples (Fig. 5A). After three cycles of cultivation, the HRTs greatly influenced the bacterial community of MAS in all three systems. *Proteobacteria* was the most abundant phylum under 4 d HRT (85.0 %), while there was no significant difference in its abundance between pre-culture, 6 d, and 8 d HRT (about 67 %) ($p > 0.05$). This result indicated that 4 d HRT is optimal for the accumulation of *Proteobacteria*, while longer HRTs had a smaller effect on its abundance. Previous studies have shown that *Proteobacteria* can decompose COD and ammonia from piggery wastewater [40], and thus the large amount of *Proteobacteria* in our MAS system illustrates that the activated sludge contributed to the COD and nitrogen removal. The DLD used in our study has poor biodegradability, and the higher availability of organic matter under 4 d HRT likely explains the enrichment of *Proteobacteria*.

At the class level, the dominant classes of *Proteobacteria* in pre-culture were *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria*, accounting for 57.8 %, 7.1 %, and 2.9 %, respectively (Fig. 5B). However, after three cultivation cycles, a decrease in the abundance of *Alphaproteobacteria* was observed in all three systems. The abundance of *Alphaproteobacteria* decreased at 4 d, 6 d, and 8 d HRT, with the abundance being 32.8 %, 42.0 %, and 26.8 %, respectively (Fig. 5B). *Alphaproteobacteria* are known for their ability to degrade organic carbon, nitrogen fixation, and ammonia in aerobic environments [41], and have been reported to have a symbiotic relationship with microalgae [42]. However, in this study, a decline in the abundance of *Alphaproteobacteria* was observed (Fig. 5B). The decreased abundance of *Alphaproteobacteria* at 4 d HRT could be attributed to poor DLD biodegradability, competition from other bacteria, and a small amount of AOM due to the lowest *C. vulgaris* content. Although 8 d HRT resulted in the highest *C. vulgaris* content, the long cultivation time led to a nutrient shortage, resulting in lower *Alphaproteobacteria* abundance than 4 d HRT. However, 6 d HRT provided an appropriate cultivation time and *C. vulgaris* content, resulting in the highest abundance of *Alphaproteobacteria*.

In this study, the abundance of *Betaproteobacteria* in pre-culture, 4 d, 6 d, and 8 d HRT were 7.1 %, 2.4 %, 8.8 %, and 5.0 %, respectively (Fig. 5B). The highest abundance of *Betaproteobacteria* was observed at 6 d HRT, indicating that this time period is the most suitable for their accumulation. Shorter or longer HRT periods may suppress their growth. *Betaproteobacteria* are often referred to as PAOs or *Accumulibacter* [43], demonstrating that activated sludge also contributes to phosphorus removal. However, the low pH, which decreased from about 6.60 to below 6.00 from the second cycle (Fig. S2) in all three systems indicates the unsuitable working conditions for PAOs, as the optimal pH range for PAOs-based phosphorus removal is 6.4–7.2 in a short-term operation [44]. Furthermore, the low PAOs content also illustrates their limited contribution to phosphorus removal. Therefore, phosphorus removal was primarily owing to microalgae in this study. After three cycles of cultivation, the abundance of *Gammaproteobacteria* increased from 2.9 % to 49.5 %, 14.4 %, and 33.8 % at 4 d, 6 d, and 8 d HRT, respectively (Fig. 5B). This result suggests that 4 d is the most suitable HRT for the enrichment of *Gammaproteobacteria*, followed by 8 d and 6 d. *Gammaproteobacteria* are known as important denitrifying bacteria [45], and the increase in their abundance indicates that activated sludge could remove nitrogen in DLD through denitrification. Additionally, the genus *Nitrobacter* (a type of NOB) and *Nitrosomonas* (a type of AOB) [46] were detected in *Alphaproteobacteria* and *Betaproteobacteria*, respectively. This observation confirms the presence of AOB and NOB in the MAS system,

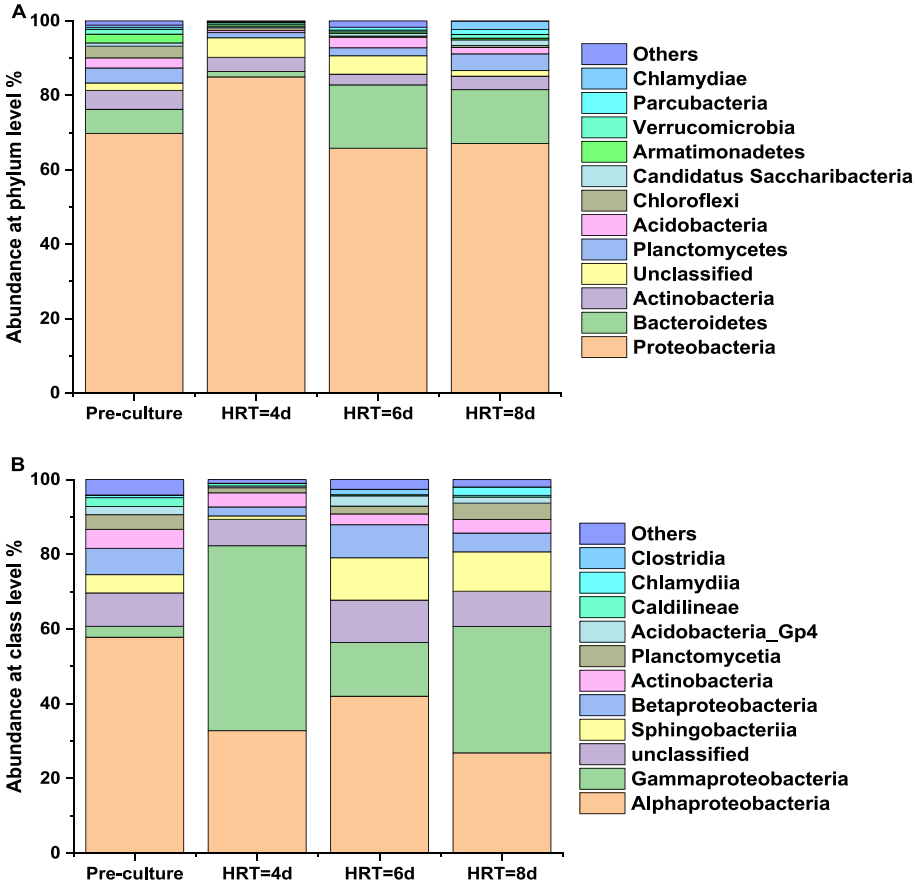

Fig. 5. Bacterial community classification of samples collected from the effluent of pre-culture and the third cycle of different HRTs at phylum (A) and class (B) levels.

suggesting the potential for TAN removal through nitrification. These findings indicate that HRT has significant influences on the abundance and components of *Proteobacteria*, and this phylum participated in COD and nutrient removal, confirming our analysis in Section 3.2.

Bacteroidetes was another dominant phylum in the pre-culture, accounting for 6.5 % of the total population (Fig. 5A). Although the abundance of *Bacteroidetes* decreased at 4 d HRT (1.5 %), it increased at both 6 d (17.0 %) and 8 d (14.5 %) HRT. The main class belonging to *Bacteroidetes*, *Sphingobacteriia*, followed the same variation trend (Fig. 5B). Previous studies have shown that *Bacteroidetes* are capable of biodegrading AOM, proteins, lignocellulose, and other complex organics [47,48]. These findings suggest that activated sludge played a role in removing AOM and organic carbon from DLD when HRT $\geq$ 6 d.

Activated sludge played a crucial role in removing COD and nutrient in the MAS system, and the HRT had a significant impact on the diversity and richness of the bacterial community. Although other phyla, such as *Planctomycetes*, *Chloroflexi*, and *Armatimonadetes*, were detected in small amounts, *Proteobacteria* and *Bacteroidetes* were the dominant phyla, illustrating the selective effects of microalgae on bacterial communities. A thorough analysis of the bacterial community structure is essential to establish a stable and efficient microalgal-bacterial consortium in a consistently-operated system. However, despite the enriched phylum's effectiveness, the effluent still contained a large amount of COD (over 300 mg/L). Therefore, further studies are necessary to identify more effective aerobic bacteria for constructing a microalgal-bacterial symbiosis system that can enhance COD removal from DLD.

3.4. Biomass composition

At the end of the experiments, biomass from the MAS process effluent was harvested to evaluate the effect of HRT on biomass composition and its potential as biofuels. Fig. 6 shows that the highest total lipid content was achieved at 8 d HRT (19.55 $\pm$ 0.44 %) while the impact of HRTs on protein content was not significant among different HRTs ($p > 0.05$). This lipid accumulation was attribute to nutrient starvation (Section 3.2), which triggered the accumulation of lipids in microalgae at longer HRTs [49,50]. The MAS biomass at 8 d HRT had a higher lipid content

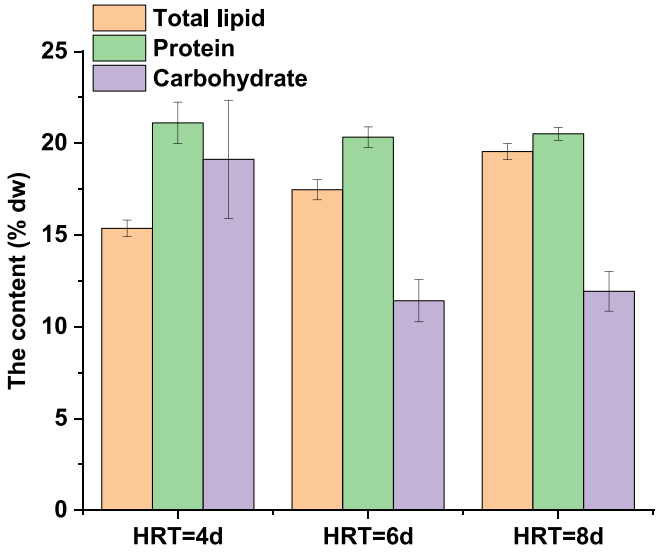

Fig. 6. Total lipid, protein, and carbohydrate contents in the dry MAS biomass harvested from the effluent of the third cycle at different HRTs.

than at 6 d HRT, as it experienced a longer nutrient starvation period. As for carbohydrate content, 4 d HRT had the highest carbohydrate content (19.12 ± 3.22 %), while 6 d and 8 d HRT had similar carbohydrate content ($p > 0.05$), but significantly lower than 4 d HRT ($p < 0.05$) (Fig. 6). Carbohydrates play important roles in microalgae development, as they are the main product of photosynthesis [51]. Therefore, the highest carbohydrate content at 4 d HRT was due to its rapid biomass growth rate and sufficient nutrient (Fig. S1, Fig. 2, and Fig. 4A). Nutrient starvation at 6 d and 8 d HRT (Section 3.2) suppressed the synthesis of carbohydrates while redirecting the reaction into fatty acid synthesis, resulting in the accumulation of lipids [50,52]. Interestingly, despite the differences in MAS biomass compositions harvested from different HRTs, they had similar HHV ($p > 0.05$), which is in the range of 20.63–21.72 MJ kg^{-1} (Table S3). These results suggest that although HRT had no significant impact on HHV of MAS biomass, it affected the biochemical composition. Longer HRT favored lipid accumulation due to nutrient starvation, while shorter HRT encouraged carbohydrate production.

3.5. Implication and significance

This study focuses on investigating the impact of HRT on the performance of MAS in wastewater treatment. Three distinct HRTs were employed individually to evaluate their effects on MAS performance in treating diluted DLD. The results indicate that HRTs significantly affected MAS biomass production, pollutant removal, chemical composition of biomass, and the relationships between microalgae and bacteria. Within the HRT range of 4 days to 8 days, a longer HRT benefits MAS biomass production and pollutants removal. The 8 d HRT achieved the highest biomass production (2.20 to 2.52 g/L) and demonstrated superior removal of TAN (100 %), TN (87.68 ± 4.57 %), and TP (100 %). The 6 d HRT fostered the development of microalgae-bacteria consortium. The MAS biomass exhibited the highest carbohydrate content of 19.12 ± 3.22 % at 4 d HRT due to the rapid MAS biomass growth rate, while 8 d HRT achieved the highest lipid content (19.55 ± 0.44 %) due to nutrient deficiency.

Currently, studies on the effects of HRT on MAS biomass performance in wastewater treatment are limited. Most existing studies using the MAS system for wastewater treatment have not explored the effects of HRTs on MAS biomass accumulation and energy potential (Table 3). Despite better nutrient removal in this study compared to others, there is a notable lower COD removal of 16.43 ± 3.65 % (Table 3), which cannot meet the discharge standard. Moreover, the HHV range of harvested MAS biomass (20.63–21.72 MJ kg^{-1}) was lower than other microalgae systems [12,22]. The relatively low energy content of MAS biomass poses challenges for its broader application.

The low COD removal in this study is primarily attributed to the low biodegradability of DLD resulting from anaerobic digestion. The challenge arises from the fact that microalgae can only utilize small molecular organics, making it difficult to decompose those macromolecular organic matters in DLD. Despite the effectiveness of activated sludge in degrading organic pollutants, the organic matters in DLD proved highly refractory, posing a challenge for their removal. Additionally, the increased COD during the last half of cycle 3 and cycle 4 at 4 d HRT (Fig. 4B) suggests that the accumulation of AOM is also a contributing factor to the low COD removal. Therefore, effective bacteria for biodegrading refractory pollutants should be employed, and DLD pretreatment strategies, such as ozonation [53], are recommended to increase the biodegradability of DLD.

The energy potential of the MAS biomass in this study is suboptimal for certain applications compared to pure microalgae due to the presence of activated sludge, which has proven to have low energy content [26]. Consequently, the introduction of activated sludge decreased the overall energy content of biomass harvested from MAS systems. To increase the energy potential of MAS biomass, it is crucial to improve the energy content of microalgae, the primary contributors to the energy content of MAS systems. While extending cultivation time to create nutrient-deficient surroundings is a feasible way to increase microalgae energy potential, it results in a longer treatment time and lower treatment volume [6]. Research efforts should explore methods such as genetic engineering, dosing with metal nanoparticles, and applying phytohormones [54] to enhance the energy content of microalgae without significantly extending treatment duration, considering both economic and environmental aspects for comprehensive system optimization.

MAS systems are promising in wastewater treatment, and HRT is significant to the performance of this process. In contrast to continuous bioreactor operation in previous research on HRTs [6,21], this research explored different HRTs in separate bioreactors at the same time, which proved to be time-saving when exploring the same amount of HRTs. However, this MAS system could be further improved to enhance COD removal and the energy potential of harvested biomass. Moreover, exploring a broader range of HRTs is needed to improve treatment efficiency and develop optimal treatment conditions for the MAS system. This is crucial from the perspectives of costs and real-world applications. Additionally, evaluations of capital costs and environmental impacts are necessary for the development of a circular economy.

4. Conclusion

This research explored the effects of three HRTs (4 d, 6 d, and 8 d) on the performances of MAS in diluted DLD treatment and nutrient recovery. In the range of 4 d to 8 d HRT, the results demonstrated that HRT had a significant effect on MAS-based biomass production, diluted DLD remediation, the relationships between microalgae and activated sludge, and the chemical composition of MAS biomass. Specifically, longer HRT favoured biomass accumulation, with the highest MAS biomass concentration (2.20–2.52 g/L) observed at 8 d HRT. However, MAS biomass productivity was decreased when HRT exceeded 6 days. Longer HRTs enhanced nutrient removal, the highest TN and TP removal (87.68 ± 4.57 % and 100 %) achieved at 8 d HRT, while both 6 d and 8 d HRTs could eliminate TAN. The MAS system exhibited unsatisfactory COD removal under the three HRTs due to the poor biodegradability of DLD and the adverse effects of AOM. HRTs significantly influenced bacterial communities, with 6 d HRT maintaining a high diversity and richness. Microalgae selectively enriched the phyla *Proteobacteria* and *Bacteroidetes* across all systems. HRT exhibited a minor impact on HHV but significantly altered MAS biomass composition. Extending the HRT increased MAS biomass lipid content due to nutrient starvation, while shorter HRT favoured carbohydrate accumulation. These findings provide valuable insights into the practical application of the MAS process for the resource utilization of liquid digestate and the construction of

Table 3
Comparation of biomass yield, pollutant removal, and MAS biomass lipid content with past studies.

HRTs	Wastewater type	Biomass yield	TN removal (%)	TAN removal (%)	TP removal (%)	COD removal (%)	Lipid content (%)	References
6 days	Municipal wastewater	_	81.5 ± 5.1	_	80 ± 12	79.5 ± 3.5	_	[6]
6 h	Domestic wastewater	5.2–15 gVS L^{-1}	47	_	94	_	_	[19]
3 days	Primary wastewater	_	63.9 ± 3	96 ± 2.1	99 ± 1	_	_	[21]
2 days	Municipal wastewater	_	88.3 ± 13.9	_	98.2 ± 1.6	83.5 ± 2.6	_	[23]
8 days	DLD	2.20–2.52 g/L	87.7 ± 4.6	100	100	16.43 ± 3.65	19.6 ± 0.4	This study

microalgal-bacterial symbiosis under a continuously operated MAS system at a larger scale.

CRediT authorship contribution statement

Siran Feng: Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Fen Liu:** Writing – review & editing. **Shunni Zhu:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Zhongbin Xu:** Writing – review & editing. **Lei Qin:** Writing – review & editing. **Pingzhong Feng:** Writing – review & editing. **Zhongming Wang:** Writing – review & editing. **Huan Chen:** Writing – review & editing. **Wenshan Guo:** Writing – review & editing, Supervision. **Huu Hao Ngo:** .

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

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cej.2024.149538.

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