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Optimum operational conditions for mixotrophic microalgae growth and nutrient recovery

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Abstract. Microalgae have been presented as microorganisms with great potential to recover nutrient from wastewater. Mixotrophic cultivation of microalgae in nutrient rich wastewater can help eliminating the deficiencies of both phototrophic and heterotrophic growth by allowing the independent optimisation of respiration and photosynthesis processes. Nutrient control and uptake by mixotrophic microalgae can be achieved either in a single or two-stage process using sequential reactors in a continuous flow system. Therefore, this work aims at studying mixotrophic microalgae growth in a two-stage biological process under continuous flow conditions with biomass recycle to recover nutrients from wastewater, considering the effects of different operational conditions (hydraulic retention time (HRT), cell retention time (CRT) and different nitrogen sources). The optimum operational conditions for algal nutrient uptake were identified to be 48 h HRT and 14 d CRT, using a mix of nitrogen sources (Ammonium-N to Nitrate-N ratio of 1:1) with 40.0% and 93.2% of phosphorus of nitrogen recovery in algal biomass, respectively.

1. Introduction

Microalgae are considered as a promising solution for nutrient control and recovery at wastewater treatment works due to their capacity to uptake nutrients and high algal biomass production with the additional benefit of enhanced biofuel production [1]. Microalgae can be separately cultivated under phototrophic, heterotrophic and mixotrophic conditions, as well as under a combination of them [2-3]. The most common microalgae cultivation mode is phototrophic. Microalgae utilise CO_2 and/or HCO_3^- and light as carbon and energy sources; molecular oxygen (O_2) and new algal cells are produced as a result of the photosynthesis process [4]. Organic carbon is consumed as an energy and carbon source by heterotrophic microalgae under fully dark conditions, using oxygen as a final electron acceptor. Despite the fact that heterotrophic microalgae growth overcomes the requirements for light to support phototrophic cultivation, it also has inherent disadvantages: (i) higher CO_2 emissions; (ii) the risk of bacterial and fungal contamination; and (iii) the need for an organic carbon source and a terminal electron acceptor (oxygen) [5]. Mixotrophic cultivation means that microalgae are able to grow either phototrophically or heterotrophically or both, in which mixotrophic metabolism can occur either simultaneously or separately. Both organic and inorganic carbon are assimilated as carbon sources.

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Microalgae release CO_2 by heterotrophic assimilation which is subsequently used during photosynthesis [6]. Light and organic materials are the energy sources for the fixation of inorganic carbon and aerobic respiration, respectively, and therefore microalgae growth does not solely depend on photosynthesis because organic carbon can support cell growth as well [7-8]. Different growth regimes were investigated to identify which of them resulted in higher algal biomass and biodiesel production rates. The results revealed that Chlamydomonas reinhardtii had the greatest growth rate and lipid accumulation under mixotrophic cultivation [9].

Mixotrophic cultivation of microalgae can occur either in a single reactor or in separated, sequential reactors. With separated reactors, microalgae take up nutrients under phototrophic conditions and further nutrient uptake and lipid accumulation can take place in subsequent heterotrophic reactors [2]. Batch cultures have been used for microalgae growth on the industrial scale; however, low biomass productivity, high harvesting costs and uncertain product quality can limit its applicability. In the last few decades, continuous flow systems have caught the attention of the industry due to their ability to maintain growth rates at close to maximum values, reduce harvesting costs and stabilise the characteristics of any final products [10].

In the current literature, there is a limited number of research works investigating the use of continuous flow systems for microalgae cultivation with the simultaneous benefit of nutrient control and recovery via microalgae uptake from wastewaters. In order to fill this gap, the work reported herein is aimed at identifying the optimum operational conditions for a two-stage biological process combining phototrophic and heterotrophic microalgae cultivation, and biomass recirculation under continuous flow conditions. In order to assess the performance of the proposed system, a series of experiments were performed under different operational conditions to test the effects of hydraulic retention time (HRT), cell retention time (CRT) and different nitrogen sources on microalgae growth, nutrient control and recovery.

2. Materials and methods

2.1. Microalgae species and Culture Conditions

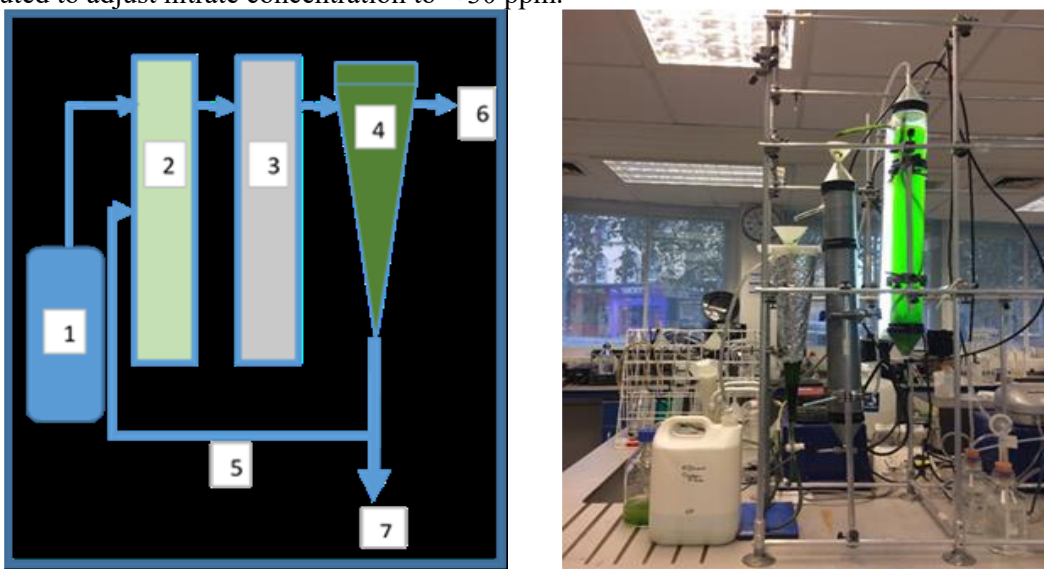
A pure culture of *Chlamydomonas reinhardtii* 11/32C was ordered from the Culture Collection of Algae and Protozoa, Scotland (CCAP), and propagated in 500 mL conical flasks containing 300 mL of Bold's Basal Media (BBM) which consists of 250 mg $NaNO_3$ L^{-1} , 75 mg K_2HPO_4 L^{-1}, 175 mg KH_2PO_4 L^{-1}, 25 mg $CaCl_2.2H_2O$ L^{-1}, 75 mg $MgSO_4.7H_2O$ L^{-1}, 25 mg NaCl L^{-1}), 50 mg EDTA L^{-1}, 31 mg KOH L^{-1}, 0.001 mL H_2SO_4, 11.42 mg H_3BO_3 L^{-1}, 8.82 mg $ZnSO_4.7H_2O$ L^{-1}, 1.44 mg $MnCl.4H_2O$ L^{-1}, 0.71 mg MoO_3 L^{-1}, 1.57 mg $CuSO_4.5H_2O$ L^{-1}, 4.98 mg $FeSO_4.7H_2O$ L^{-1} and 0.49 mg $Co(NO_3)_2.6H_2O$ L^{-1}, using a shaking incubator (Infors Multitron). Culture media was autoclaved at 121°C and 1 bar for 15 minutes. Controlled environmental conditions for temperature, photoperiod and light intensity during cultivation (axenic microalgae culture) were set at 25°C, 24 h of light and 40 μE/m2S, respectively.

2.2. Experimental setup for a two-stage biological process

The two-stage biological process setup includes seven main components as presented in the process flow diagram and the picture depicted in Figure 1.a. and b, respectively. The two-stage microalgal process was fed by synthetic wastewater (SWW) including 25 mg NH_4^+-N L^{-1}, 25 mg NO_3^--N L^{-1}, 15 mg PO_4^{3-}-P L^{-1}, 2800 mg $NaHCO_3$ L^{-1}, 567 mg $CH_3COONa.3H_2O$ L^{-1} which are typically found in the effluent of a conventional activated sludge process and the same concentrations of inorganic salts, trace elements and pH conditioners were used as those in the BBM media with the addition of 20 mg L^{-1} of cationic polymer (Zetag 50). The experimental setup for mixotrophic microalgae growth comprises a photobioreator (PBR) and a heterotrophic reactor (HTR) to which is connected in series, having an effective volume: 2.3 L (diameter: 7 cm; height: 61 cm). The effluent from the HTR was transferred by gravity to an Imhoff cone that allows algal biomass sedimentation and recycling to the PBR by pumping, while the supernatant is collected for characterisation. Settled microalgal biomass was recycled into the PBR at a specific rate according to the predefined VSS target concentration (mixed liquor volatile

suspended solids – MLVSS). A set amount of settled microalgal biomass (algal sludge) was discarded daily according to the predefined cell retention time.

Water samples were collected every other day from three sampling points including: PBR effluent (2); HTR effluent (3); and, Imhoff cone supernatant (final effluent) (6) (see Figure 1.a). Collected data was processed to assess process performance and monitor biomass growth and nutrient uptake. Microalgae growth was determined using gravimetric analysis for volatile suspended solids (VSS) (SM 2540E). Ammonium and phosphorus were analysed following the methods (SM 4500-NH_3.B and SM 4500-P.B, respectively). All analytical tests were conducted following standard methods reported by [11]. Nitrate was determined by Ion Chromatography. Ion Chromatographer (Metrohm 850 Professional IC) using a with Metrosep A supp 5 column (length: 150 mm, diameter: 4 mm) and an eluent comprising 1 mM NaHCO3 and 3.2 mM Na2CO3. Samples were passed through 0.45 µm Minisart syringe filters and diluted to adjust nitrate concentration to < 30 ppm.



(a) (b)

Figure 1. (a) Process diagram, (b) and picture of the experimental setup of the two-stage biological process.

2.3. Operational Conditions for a Two-Stage Biological Process

The two-stage biological process was operated under different operational conditions to optimise process performance based on operational parameters (hydraulic retention time (HRT) and cell retention time (CRT)) and nitrogen source availability in the SWW. The tested conditions are listed in Table 1.

Table 1. Experimental conditions.

Number of experiments	Variables			
	HRT (hours)	CRT (days)	NH4+-N concentration (mg L-1)	NO3--N concentration (mg L-1)
1	36	14	25	25
2	48	14	25	25
3	72	14	25	25
4	48	7	25	25
5	48	21	25	25
6	48	14	50	-
7	48	14	-	50

Each experiment was operated for approximately one month, which gave the possibility of producing plenty of data to test the stability and performance of the process under the set condition, i.e., an experiment with an HRT of 72 h (3 d) run for 30 days is the equivalent of running 10 replicates in a

batch reactor with a three-day residence time. Biomass concentrations in each reactor with different operational conditions at steady-state conditions are reported as average ± one standard deviation.

2.4. Calculations

2.4.1. Hydraulic Retention Time (HRT)

HRT is calculated dividing the volume of the reactor by the flowrate feeding the system (equation (1)). Flowrates for operating the two-stage biological process were calculated via equation (1). HRTs tested in the two-stage biological process were 36, 48 and 72 h, which corresponded to feeding flowrates of 2.5, 1.7 and 0.9 L d^{-1}, respectively.

$$HRT = \frac{V}{Q} \quad (1)$$

Where, HRT is hydraulic retention time (d), V is working volume of each reactor (L), and Q is flowrate of each reactor (L d^{-1}).

2.4.2. Cell retention time (crt)

The system was tested at 7, 14 and 21 d of CRT by carefully controlling the amount of algal biomass daily purged from the system (algal sludge); the corresponding volume of algal sludge purged daily was 660, 330 and 220 mL, respectively. CRTs and the volume of algal sludge purged were calculated via equation (2) and (3), respectively.

$$CRT = \frac{V\,(Xpbr+Xhtr)}{(Q-Qw)Xe+QwXr} \quad (2)$$

$$Q_w = \frac{Vt}{CRT} \quad (3)$$

Where; CRT is cell retention time (d), V is working volume of each reactor (L), V_T is total volume of system (L), X_{PBR} is average biomass concentration in PBR (mg L^{-1}), X_{HTR} is average biomass concentration in HTR (mg L^{-1}), Q is flowrate of each reactor (L d^{-1}), Q_w is flowrate of wasted algae (L d^{-1}), Xe is biomass concentration in final effluent (mg L^{-1}), and X_r is biomass concentration in the recycle line (mg L^{-1}). Operational parameters for the two-stage biological process are listed in Table 2.

Table 2. Operational parameters for the two-stage biological process.

Parameter	Value		
VT	4.6 L (2*2.3 L)		
HRT	36 h, 48 h, and 72 h (18 h, 24 h, and 36 h for each reactor)		Equation (1)
Q	2.5 L d-1	36 h	
	1.7 L d-1	48 h	
	0.9 L d-1	72h	
CRT	7 d, 14 d and 21 d		
Qw	660 mL d-1	7 d	Equation (3)
	330 mL d-1	14 d	
	220 mL d-1	21 d	
Xe	75 mg VSS L-1		MLVSS concentration in PBR and HTR were assumed 1 and 1.5 g L-1, respectively (Equation (2))
Xr	750 mg VSS L-1		

2.5. Statistical analysis

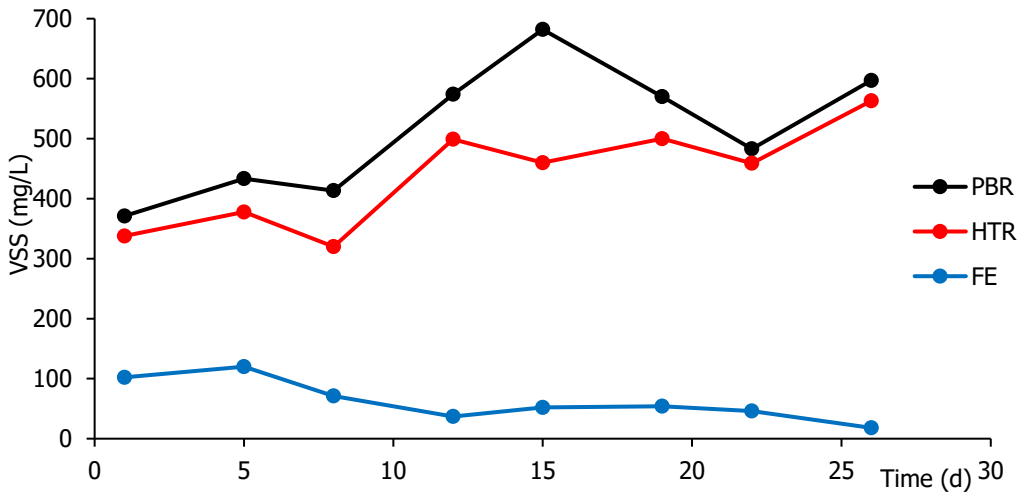
Data processing included statistical analyses for descriptive statistics and variable comparisons; all data were processed using IBM's SPSS Statistics 22 software suite. One-way analysis of variance (ANOVA) was used to evaluate the differences among the treatments. If ANOVA effects were significant, comparisons between the different means were made using *post hoc* least significant differences (LSD).

3. Results and discussion

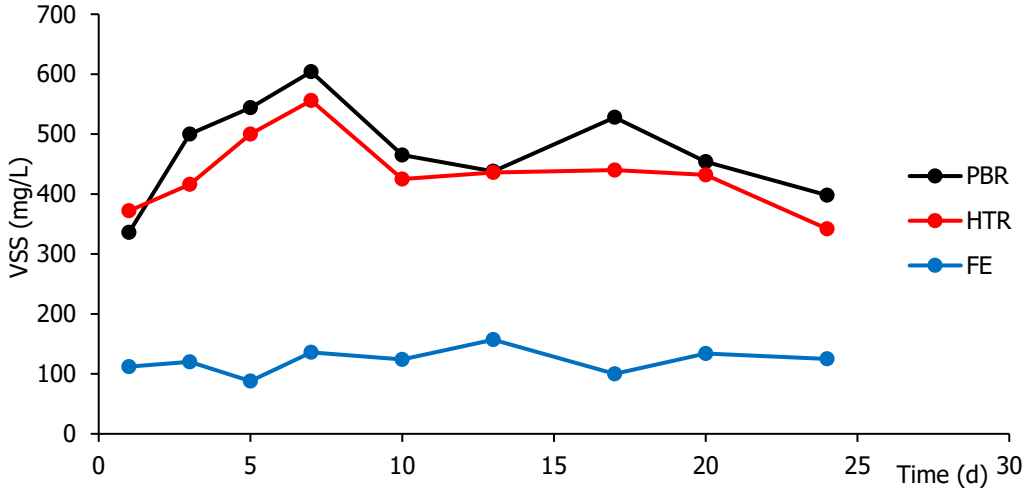
3.1. The Influence of HRT on Microalgae Growth and Nutrient Recovery

Algal biomass concentrations in the effluent of the PBR, HTR and sedimentation unit (final effluent - FE) as a response to different initial feeding flowrates in the two-stage biological process are presented in Figure 2. It can be clearly seen that a slightly higher biomass concentration was achieved in the PBR than in the HTR for all HRTs tested. Microalgae concentration in the PBR fluctuated between 370 and 680 mg VSS L^{-1}, 330 and 600 mg VSS L^{-1} and 380 and 570 mg VSS L^{-1} at 36 h, 48 h and 72 h HRT, respectively. Biomass concentrations in the HTR varied from 320 to 560 mg VSS L^{-1}, 340 to 560 mg VSS L^{-1} and 370 to 600 mg VSS L^{-1} at 36 h, 48 h and 72 h HRT, respectively. However, it was expected that a higher biomass concentration would be achieved in the heterotrophic reactor due to the fact that heterotrophic microalgae a have higher growth rate in comparison with the growth rate of phototrophic microalgae [12]. This can be attributed to the synthetic wastewater containing low organic carbon concentrations and bacterial contamination under heterotrophic conditions. There was a similar trend in the change of biomass concentration with HRTs under both phototrophic and heterotrophic conditions. The average dry weight concentration of microalgae declined with increasing HRT from 36 h to 48 h. This could be attributed to the reduced nutrient supply at higher HRT due to the decrease in flowrate [13]. A further increment in HRT to 72 h did not result in any significant difference in algal biomass concentration for both the PBR and HTR ($p > 0.05$).

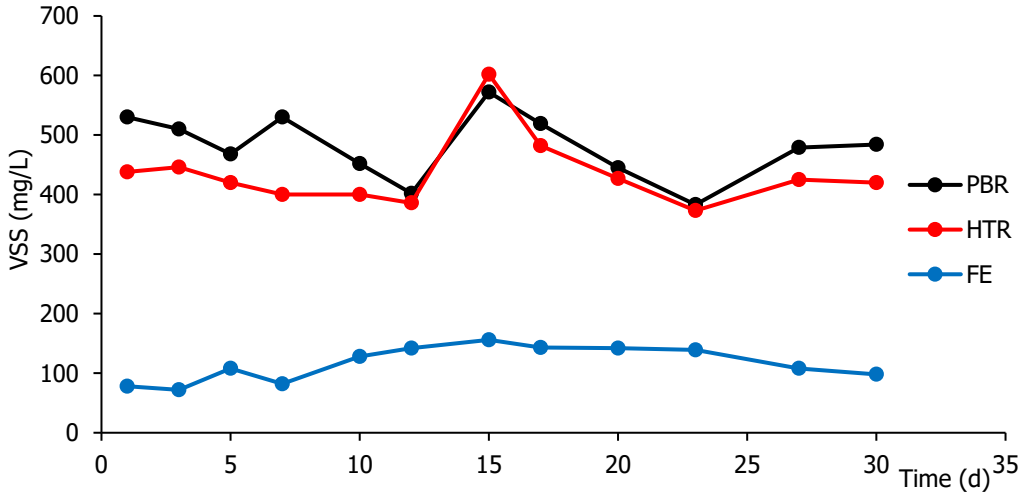
With regard to effluent quality, algal biomass in the final effluent showed different patterns with regard to the HRTs tested. At 48 h HRT, biomass concentration (dry weight) fluctuated between 90 and 160 mg VSS L^{-1}. While there was a constant decrease from 100 to 40 mg VSS L^{-1} at 36 h HRT, a continuous increase was observed from 80 to 150 mg VSS L^{-1} at 72 h HRT. The biomass concentration increased almost double with increasing HRT for 36 h to 48 h HRT; after that, dry weight concentrations of biomass in the FE remained stable at 72 h of HRT. Solid concentrations were quite high, even though a cationic polymer was added to the feeding media to increase the settling ability of the microalgae. Therefore, the additional process was required to improve the settlement quality of the algae and reduce solids concentration in the effluent for the implementation of a two-stage biological process in the wastewater treatment plant. Harvesting is considered the main limitation to the large-scale cultivation of microalgae due to its limited settlement capacity. Sedimentation, centrifugation and filtration are the most common methods to separate solid and liquid phases. All harvesting techniques have some deficiencies such as the land area requirement for sedimentation, and high energy consumption for the others [14].



(a)



(b)



(c)

Figure 2. Microalgae concentration under different hydraulic retention times: (a) 36 h HRT, (b) 48 h HRT, and (c) 72 h HRT.

The uptake of nutrients from the two-stage biological process was determined by analysing phosphate, ammonium and nitrate in the PBR, HTR and FE samples. Phosphorus concentrations in each reactor at 36 h, 48 h and 72 h HRT are presented in Figure 3 and similar trends were observed. At 36 h HRT, phosphorus concentrations in FE increased from 9.8 to 11.3 mg P L^{-1} at Day 12, thereafter it remained stable while there was a constant decline in phosphorus concentration in FE from 15.0 to 9.0 mg P L^{-1} at 48 h HRT. At 72 h HRT, the phosphorus concentration in FE fluctuated around 9.0 mg P L^{-1}. P in the effluent at 36 h was significantly different than the ones at 48 h and 72 h ($p < 0.05$), whereas P in the effluent did not change significantly between 48 h and 72 h ($p = 0.348$). Phosphorus uptake efficiency incremented from 24.6% by 40% with the increase of HRT from 36 h to 48 h due to the increased time, providing more time to assimilate phosphorus [13][15]; it then remained invariant (40%) at 72 h HRT. Therefore, 48 h HRT was chosen as the optimum because prolonged HRT requires a larger reactor volume.

Ammonium recovery rates in the PBR were found to be 90.1, 88.4 and 89.4% at 36 h, 48 h and 72 h HRT, respectively, while nitrate was fully consumed under phototrophic conditions. There were not any changes in ammonium concentration under heterotrophic conditions.

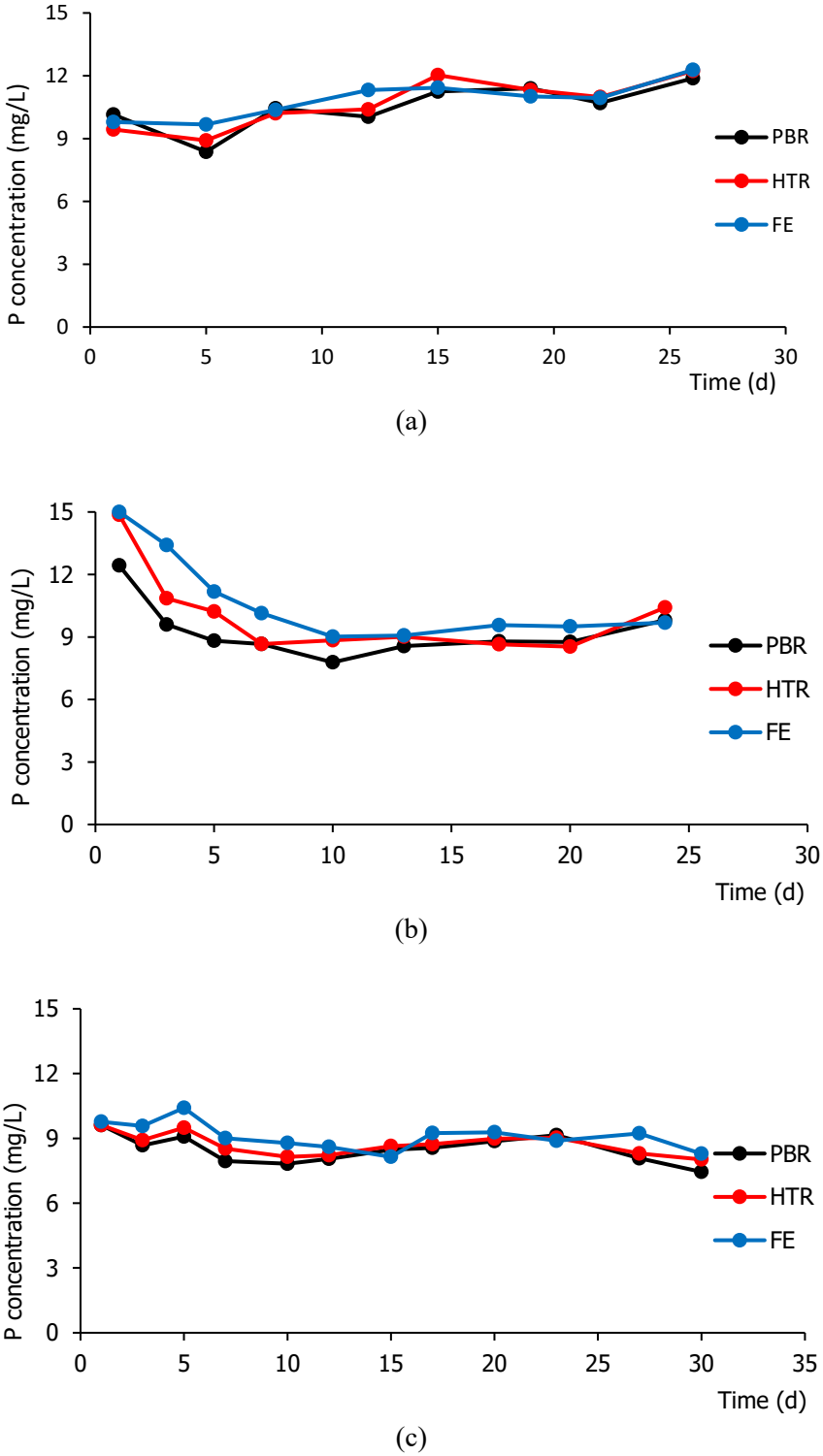
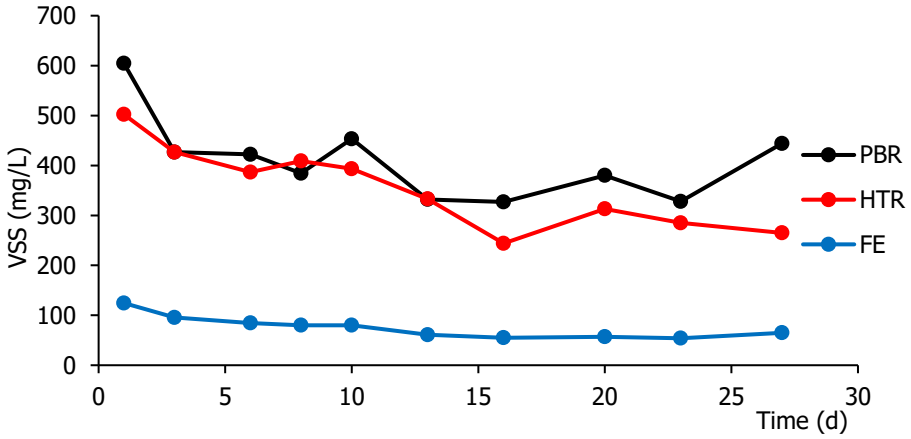


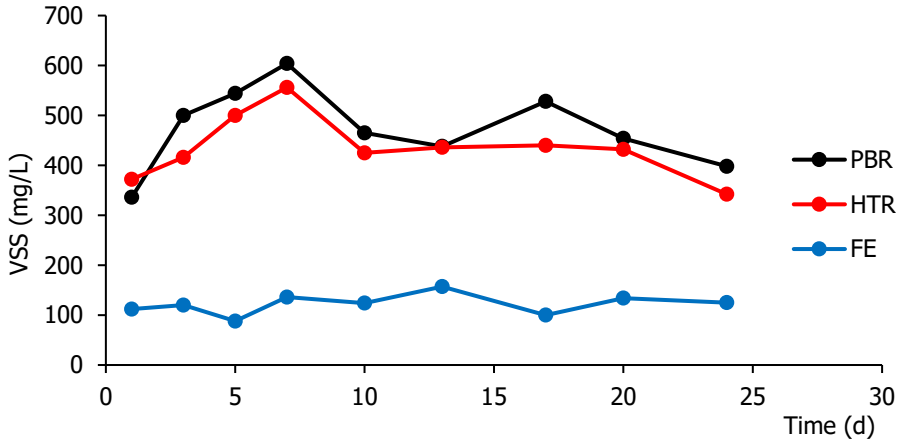
Figure 3. Changes in P concentration during the cultivation period for different HRTs: (a) 36h HRT, (b) 48h HRT and (c) 72h HRT.

3.2. The Impact of CRT on Microalgae Growth and Nutrient Recovery

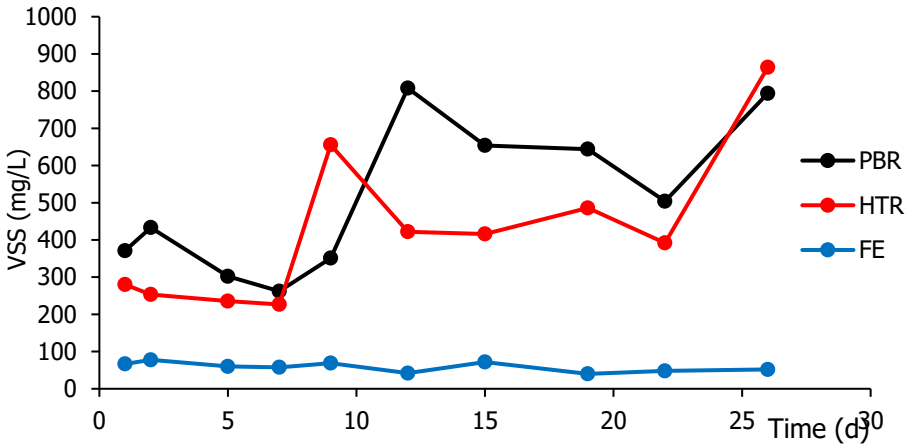
The two-stage biological process was operated at different CRTs of 7 d, 14 d and 21 d at 48 h HRT using a mix of different nitrogen sources (Figure 4). The same growth trend was observed in the PBR and HTR throughout the experiments for cell retention times ($p = 0.103$). A slightly higher microalgae concentration was obtained in the PBR than in the HTR. Dry weight concentrations fluctuated from 330 to 600 mg VSS L^{-1}, from 330 to 600 mg VSS L^{-1} and from 260 to 810 mg VSS L^{-1} for the PBR and from 250 to 500 mg VSS L^{-1}, from 340 to 560 mg VSS L^{-1} and from 230 to 860 mg VSS L^{-1} for the HTR at 7 d, 14 d and 21 d CRT. Between 7 d and 14 d CRT, average algal biomass concentrations in both the PBR and the HTR increased. Algal biomass concentrations in the PBR significantly increased at 21 d CRT ($p < 0.05$) whereas a slight increase was observed in HTR ($p = 0.397$). It can be clearly anticipated that biomass concentration increased with incrementing cell retention times at fixed hydraulic retention times under phototrophic and heterotrophic conditions There is a good agreement with [16], reporting that lower biomass concentrations were obtained at lower CRTs with *Chlamydomonas reinhardtii.* Biomass concentration in the final effluent differed from 50 to 120 mg VSS L^{-1}, from 90 to 160 mg VSS L^{-1} and from 40 to 80 mg VSS L^{-1} at 7 d, 14 d and 21d CRT along with the cultivation period. FE included an approximate two-fold dry weight concentration at 14 d HRT (128 ± 20 mg VSS L^{-1}) which was significantly different from figures obtained at 7 d and 21 d CRT (76 ± 22 and 59 ± 13 mg VSS L^{-1}, respectively) ($p < 0.05$).



(a)



(b)

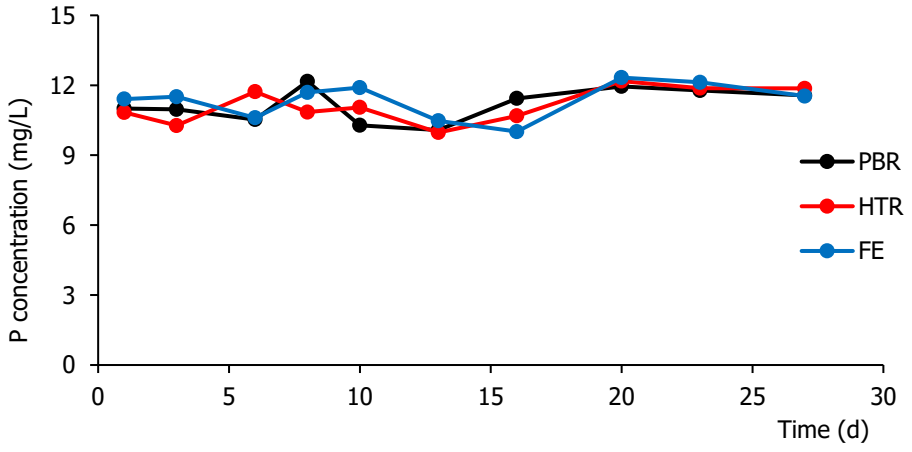


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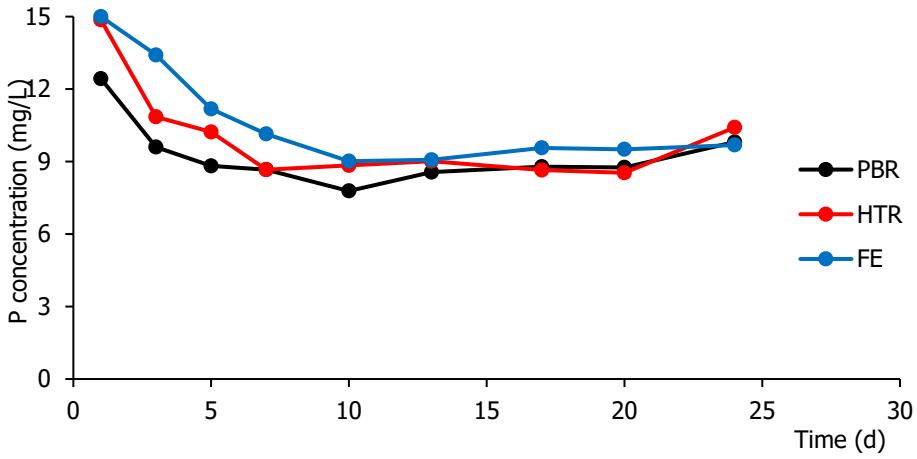
Figure 4. Algal biomass concentration variation with different CRTs: (a) 7 d CRT, (b) 14 d CRT, and (c) 21 d CRT.

Phosphorus concentrations over the two-stage biological process at the tested CRTs at 7 d, 14 d and 21 d are demonstrated in Figure 5. Overall, any significant differences in P concentration throughout the continuous flow system was not reported with varying CRT, meaning that the P in each reactor showed a similar trend ($p > 0.05$). Average P concentrations under steady-state conditions at 7 d and 21d CRT were 11.3 and 11.9 mg P L^{-1}, consistent with lower P recovery efficiencies via *Chlamydomonas reinhardtii* which were obtained as 24.7% and 20.7%, respectively ($p = 0.408$). There was a significantly difference in P concentration in the final effluent and recovery efficiency of P uptake at 14 d CRT (9.0 mg L^{-1} P and 40%, sequentially) ($p < 0.05$). Greater phosphorus uptake at 14 d CRT could be attributed to higher biomass concentration at this time compared to the one reported at 7 d CRT. Although biomass concentration continued to increase when further increasing CRT to 21 d, there was a decrease in P uptake which could be the result of biomass decay and excretion of phosphorus from algae cells into the media [16]. Hence, 14 d CRT was considered the optimum producing the lowest content in the final effluent.

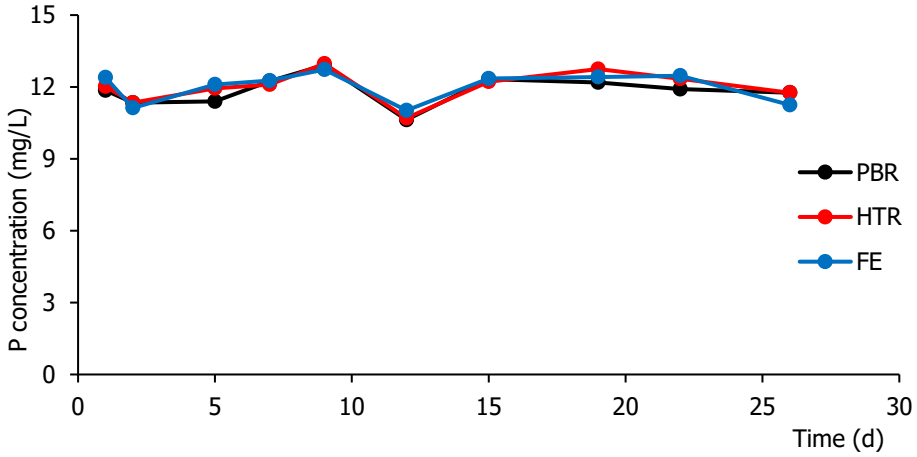
The ammonium recovery rates achieved in the PBR were 100.0%, 88.4% and 87.7% at 7 d, 14 d and 21 d CRT, respectively, and nitrate was fully consumed by microalgae under phototrophic conditions. Heterotrophic conditions offered only limited contribution to nitrogen recovery.



(a)



(b)

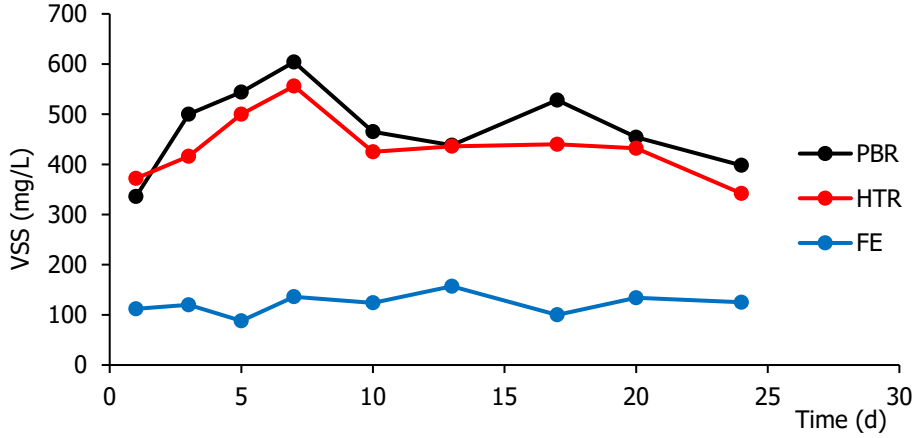


(c)

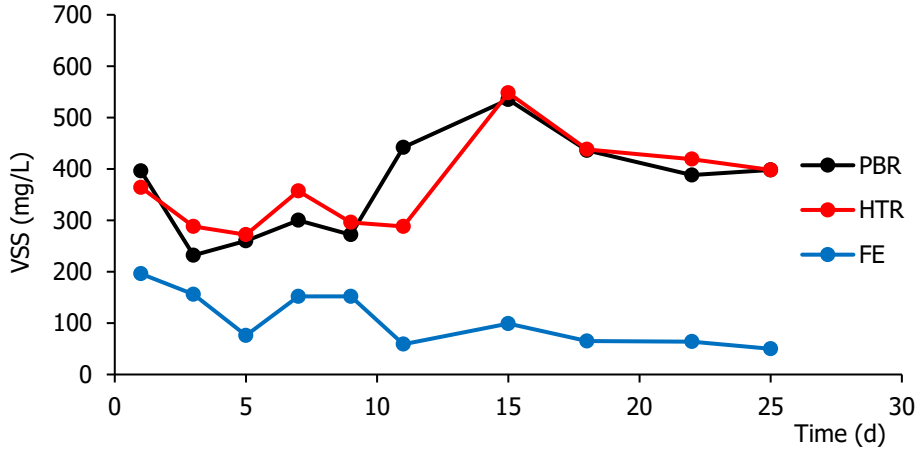
Figure 5. The effect of CRT on phosphate uptake and recovery: (a) 7 d CRT, (b) 14 d CRT, and (c) 21 d CRT.

3.3. The Effect of Different Nitrogen Sources on Microalgae Growth and Nutrient Recovery

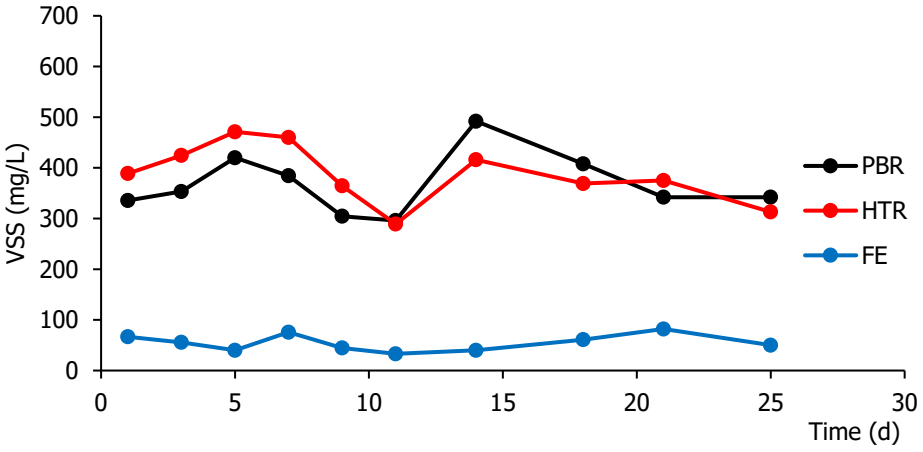
Chlamydomonas reinhardtii was cultivated in a feeding medium including different nitrogen sources as a mix of ammonium and nitrate, ammonium alone, and nitrate alone (Figure 6). Similar microalgae concentrations were achieved in the PBR and HTR. Nitrogen sources did not show any significant effect on algal biomass concentration ($p = 0.605$ for PBR and $p = 0.243$ for HTR). Biomass growth fluctuated between 330 and 600 mg VSS L^{-1}, between 230 and 540 mg VSS L^{-1}, and between 300 and 490 mg VSS L^{-1} for the PBR, and from 340 to 560 mg VSS L^{-1}, from 270 to 550 mg VSS L^{-1}, and from 290 to 470 mg VSS L^{-1} for the HTR when a mix of ammonium and nitrate, solely ammonium and nitrate were used as the nitrogen sources, respectively. The dry weight of biomass in the final effluent differed from 90 to 160 mg VSS L^{-1}, from 50 to 200 mg VSS L^{-1} and from 30 to 80 mg VSS L^{-1} with different nitrogen sources as a mix of ammonium and nitrate, solely ammonium and nitrate, respectively. There was a similar effluent quality, as based on average solid concentration, between a mix of NH_4^+-N and NO_3^--N, (128 ± 20 mg VSS L^{-1}) and solely NH_4^+-N (107 ± 19 mg VSS L^{-1}) ($p < 0.05$). With the use of NO_3^--N in the synthetic wastewater, average microalgae concentration was decreased by half to 55 ± 16 mg VSS L^{-1} VSS, respectively ($p = 0.502$).



(a)



(b)

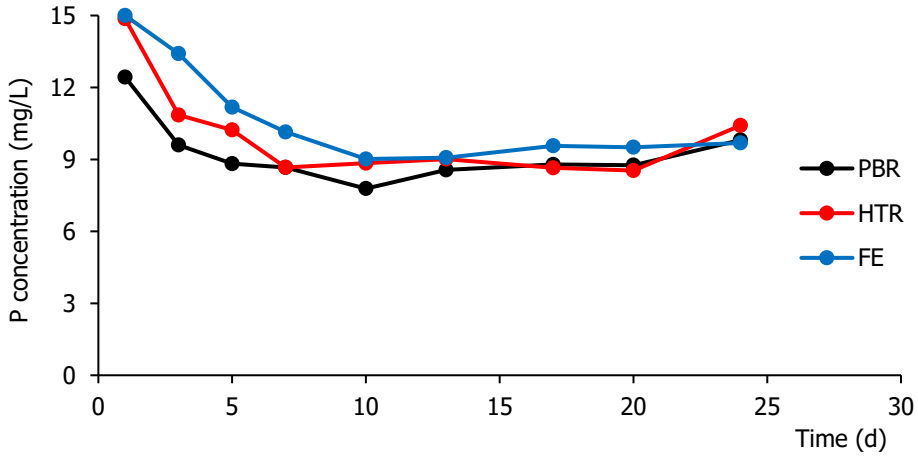


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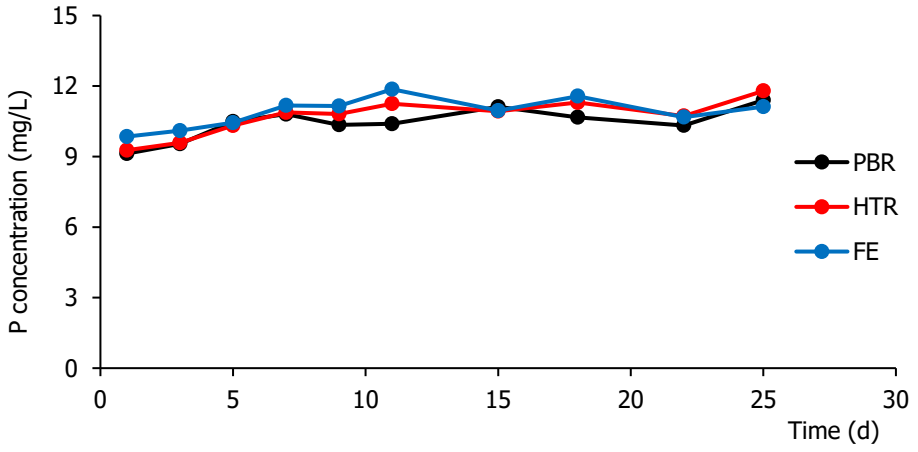
Figure 6. Biomass growth during cultivation with different nitrogen sources: (a) a mix of NH_4^+-N and NO_3^--N (25:25), (b) NH_4^+-N, and (c) NO_3^--N.

Figure 7 exhibits the influences of different nitrogen sources on P concentration in the continuous flow system for mixotrophic microalgae growth. It can be clearly seen that there was not any significant differences in phosphorus uptake by *Chlamydomonas reinhardtii* in each reactor when cultivating with different nitrogen sources ($p = 0.764$). The lowest P concentration in the effluent achieved was 9.0 mg P L^{-1} using a mixture of ammonium and nitrate, correspond to 40% P uptake efficiency. This was significantly different from when NH_4^+-N and NO_3^--N were used as the nitrogen source ($p = 0.001$). Similar P concentrations in the FE were obtained as 11.3 mg P L^{-1} when *Chlamydomonas reinhardtii* was cultivated in ammonia culture and nitrate culture, consistent with the P uptake efficiencies attained being 24.7% ($p = 0.924$). Thus, a mix of ammonium and nitrate was the favoured nitrogen source for the two-stage biological process. The mixture of nitrogen sources was preferred for *Chlamydomonas reinhardtii* rather than ammonia and nitrate in the view of biomass growth and phosphorus uptake [17].

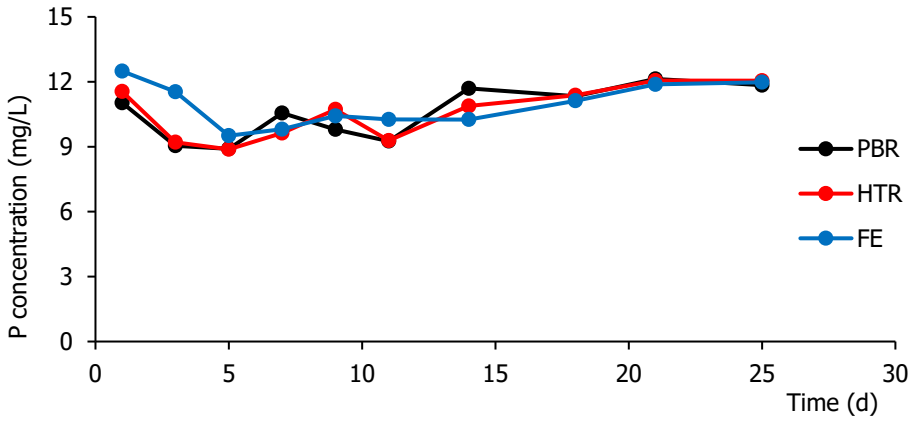
An ammonium recovery rate of 88.4% was achieved and nitrate was taken up via *Chlamydomonas reinhardtii* at phototrophic cultivation when a mixture of ammonium and nitrate were used as the nitrogen source. Ammonium uptake efficiency was reduced to 66.4% when ammonium alone was used. Nitrate was fully consumed via microalgae uptake under phototrophic conditions in nitrate alone culture. There was a small contribution to nitrogen recovery in the HTR.



(a)



(b)



(c)

Figure 7. P concentration throughout the cultivation period with different nitrogen sources: (a) a mix of NH_4^+-N and NO_3^--N (25:25), (b) NH_4^+-N, and (c) NO_3^-- N.

4. Conclusion

This research aimed to identify the optimum operational conditions for the two-stage biological process combining phototrophic and heterotrophic microalgae cultivation with biomass recirculation under continuous flow conditions with regard to nutrient control and recovery via biological uptake. Although the contribution from the HTR to nutrient recovery was limited under all experimental conditions, *Chlamydomonas reinhardtii* recovered phosphorus and nitrogen at 40% and 93.2%, respectively, under optimum operational conditions of 48 h HRT, 14 d CRT and a mix of nitrogen sources.

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