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The competition among heterotrophs under anoxic conditions for carbon source

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ABSTRACT

Bacterial utilization of molecules is recognized as an important step in the cycling of organic matters. In this study, a bench-scale continued-flow intermittent-aeration biological reactor (CIBR) was applied and the competition for acetate among heterotrophs with different influent C/N ratios under anoxic conditions was investigated. The results revealed that phosphorus release occurred after the depletion of nitrate when influent C/N ratio was lower than 400:30. With the influent C/N ratios of 800:30 and 1200:30, the denitrification and phosphorus release occurred simultaneously, and the activity of polyphosphate accumulating organisms (PAOs) was inhibited by the intermediates (NO_3-N, NO_2-N, NO, NH_2OH, etc) produced in denitrification. Based on the COD mass balance calculation, the theoretical C/P ratio for phosphorus release was 1.99, and the theoretical C/N ratios for the denitrification, denitratation and denitritation were 3.89, 2.33 and 3.56, respectively. The competitiveness for carbon source among heterotrophs follows the order: denitrifiers > PAOs > other heterotrophic organisms. The mechanisms include the competition for carbon source and the inhibition of intermediates (NO_3-N, NO_2-N, NO, NH_2OH, etc) in denitrification. The low influent C/N ratio and nitrite accumulation during denitrification were both beneficial to improve the efficiency of carbon utilization.

Keywords: CIBR; Heterotrophs; Carbon source competition; Phosphorus release; COD mass balance calculation

1. Introduction

The biological nutrient removal (BNR) processes can achieve simultaneous removal of carbon, nitrogen and phosphorus from wastewater by forming aerobic/anoxic/anaerobic environments. Denitrifiers and phosphate accumulating organisms (PAOs) play the main roles in nitrogen and phosphorus removal, which consume carbon source. However, COD is commonly deficient for complete denitrification and phosphorus release from municipal or domestic wastewater. Therefore, some measures were applied to improve the utilization efficiency of available carbon source in BNR, such as optimizing the design and protocol or adding external carbon sources (e.g., ethanol, and glycerol) [1,2]. Besides, volatile fatty acids (VFAs) from sludge fermentation was also often employed [3]. Additionally, combing biological process with sand filter has been recently proposed to improve BNR efficiency in the full scale [4].

Previous researchers have found that nitrate could affect phosphate and phosphorus release occurred after the depletion of nitrate [5]. However, Chuang et al. observed simultaneous denitrification and phosphorus release under

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anoxic condition when carbon source was enough [6]. The hypothesis of the competition between denitrifiers and PAOs for carbon source has been put forward and widely accepted [7]. Although the competition for carbon source between denitrifiers and PAOs with different influent C/N ratios has been studied [8], few quantitative data exist regarding the competition and carbon source distribution between denitrification and phosphorus release. Whether the utilization efficiency of carbon source can be improved by simultaneous denitrification and phosphorus release under anoxic conditions is still lacking of enough research.

It is known that the majority of microorganisms involved in the metabolic reactions under anoxic and anaerobic conditions include denitrifiers, PAOs and other heterotrophic organisms (e.g. glycogen accumulating organisms (GAOs), anaerobic fermentative bacteria and ordinary heterotrophic organisms (OHO) [9,10]. These heterotrophs can compete for available carbon source. Many studies report that when acetate is used as carbon source, PAOs can be more effective in consuming acetate than other heterotrophic organisms under strict anaerobic conditions, while the denitrifiers are more prone to acetate under the anoxic condition. Only when the activity of PAOs is inhibited or there is surplus COD after denitrification and phosphorus release, carbon source can be obtained by other heterotrophic organisms [11,12]. The utilization of carbon source by other heterotrophic organisms is considered as COD "loss". Previous studies have focused on the competition for carbon source between PAOs and GAOs or only among the denitrifiers [13–15], but the competition for carbon source among denitrifiers, PAOs and other heterotrophic organisms is not clear, especially under the anoxic condition. Therefore, a study regarding the competition for carbon source among heterotrophs under anoxic condition is desirable to investigate the pathways of carbon source utilization by denitrifiers, PAOs and other heterotrophs.

Copp and Dold suggested that if the C/N ratio for denitrification was higher than 2.86, the COD "loss" was resulted from the non-denitrifiers [16]. Therefore, the C/N ratio leading to a maximum conversion of all nitrogen compounds to nitrogen gas with minimum organic carbon is defined as "the theoretical C/N ratio". Similarly, the theoretical C/P ratio presents the relationships between P-release and O_2 equivalents, indicating no COD "loss" caused by other heterotrophic organisms under anaerobic condition. There also exists the theoretical C/N ratio for denitrification under anoxic condition. The alternating aerobic/anoxic/anaerobic environments offer different nutrients and energy gradients to the microbial population. Denitrifiers have priority in competition for carbon source under anoxic condition when nitrate exists, which results in less COD available for PAOs and other heterotrophic organisms. The difference between anoxic and anaerobic conditions is determined by the existence of nitrate rather than heterotrophic populations. Therefore, the acetate metabolism of PAOs and the theoretical C/P ratio under anoxic condition are the same as those under anaerobic condition. The quantitative correlation among heterotrophs for carbon source should be delineated based on the COD mass balance calculation. The amounts of carbon source consumed by denitrifiers and PAOs are calculated on the theoretical C/N and C/P ratios, and the remaining part of total influent COD was considered as the carbon source consumed by the other heterotrophic organisms.

Therefore, anoxic and anaerobic batch tests were performed in a bench-scale continued-flow intermittent-aeration biological reactor (CIBR) [17,18]. The theoretical C/P for phosphorus release was calculated by altering the anaerobic feeding time. The theoretical C/N ratio for the denitrification could be obtained by varying the influent C/N ratios, and based on the COD mass balance calculation. Then carbon source distribution among heterotrophs was investigated, and the pathways of improving the utilization efficiency of carbon source by denitrifiers and PAOs were evaluated.

2. Materials and Methods

2.1. Experimental equipment

A schematic diagram of bench-scale CIBR is depicted in Fig. 1. The reactor was comprised of Plexiglas, with a total volume of 325.72 L (250 L for the reaction zone with 0.5 m × 0.5 m × 1.15 m, 15.44 L for the three-phase separator and 60.28 L for the settling zone with 0.5 m × 0.2 m × 1 m). The inflow rate of wastewater was 0.6 m^3/d with the hydraulic retention time (HRT) of 10 h, and the aeration rate of 1.2 m^3/h was controlled by a gas flow meter with the gas-water ratio of 48:1. Water temperature was maintained at 26 ± 1°C. Sludge retention time (SRT) was set at 15 d, leading to MLVSS between 1800 and 2400 mg/L throughout the experimental periods, and organic load was about 0.133–0.178 kg BOD/(kg MLVSS·d). Activated sludge mixed liquor was stirred by an agitator at a speed of 200 rpm. The surface load of the settling zone was 0.28 $m^3/(m^2 \cdot d)$, and the settling time was 2.4 h. Online instruments for dissolved oxygen (DO), oxidation reduction potential (ORP) and pH analysis, together with an electric control system which was a PC equipped with an online control software were used to achieve a real-time self-control. A strict aerobic-anoxic-anaerobic environment was formed in CIBR through alternative operation of aeration/stirring/stirring. The aerobic and anaerobic durations were controlled at 3.5 h and 1.5 h, respectively. The anoxic stirring duration was determined by the online control system to identify characteristic points on the DO, ORP and pH curves. Samples were collected at intervals, according to pH and ORP variations.

2.2. Seed sludge and synthetic wastewater

The activated sludge used as inoculum in the experiment was taken from an aeration tank at Long wang zui Sewage Treatment Plant (A^2/O process, 150000 m^3/d) in Wuhan, China. The composition of the synthetic wastewater fed into the reactor was (mg/L): sodium acetate, 250; NH_4Cl, 30; KH_2PO_4, 5; $NaHCO_3$, 200–250. The micro element solution (1.0 mL/L of the wastewater) contained (mg/L): $MgSO_4 \cdot 7H_2O$, 10; $CaCl_2$, 10; $FeSO_4$, 0.1; $FeCl_3 \cdot 7H_2O$, 1.5; $CuSO_4 \cdot 5H_2O$, 0.03; $CoCl_2 \cdot 7H_2O$, 0.15; KI, 0.03; H_3BO_3, 0.15; $MnCl_2 \cdot 7H_2O$, 0.12; $ZnSO_4 \cdot 7H_2O$, 0.12; EDTA, 3; $(NH_4)_6Mo_7O_{24} \cdot 7H_2O$, 0.06; $NiSO_4 \cdot H_2O$, 0.20.

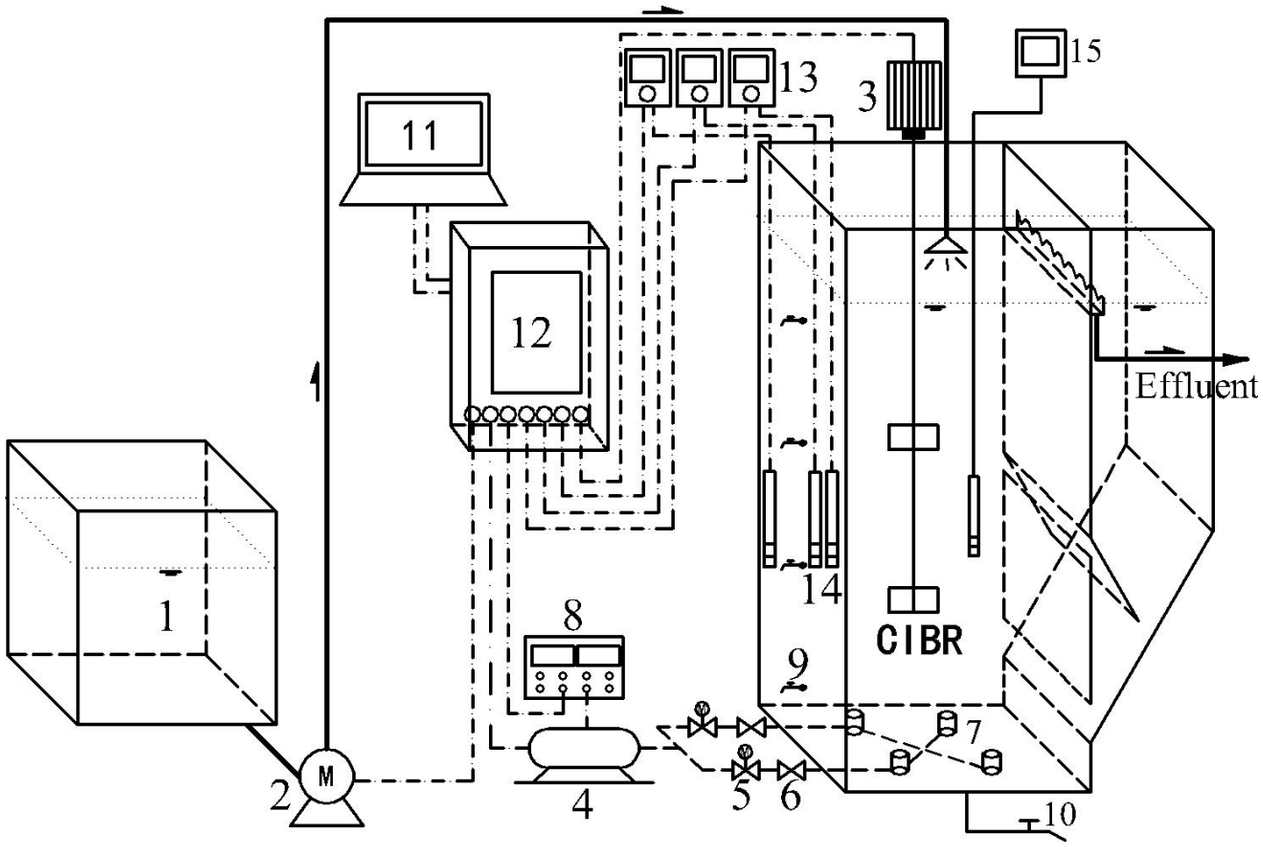

Fig. 1. Schematic diagram of CIBR. (1) water tank; (2) peristaltic pump; (3) agitator; (4) air pump; (5) electric valve; (6) hand valve; (7) air diffuser; (8) frequency converter; (9) sampling tube; (10) mud valve; (11) computer; (12) electric control cabinet; (13) DO/ORP/pH monitors; (14) electrode tubes; (15) temperature controller.

2.3. Batch experiments

2.3.1. Anaerobic batch tests

The seed sludge in five 5-L reactors for anaerobic batch tests was taken from the bench-scale CIBR at the end of the anoxic stage. The mixed liquor volatile suspended solid (MLVSS) concentrations were 1951, 2079, 2051, 1955 and 2122 mg/L, respectively. The influent of the five reactors and bench-scale CIBR was all the synthetic wastewater with the same compositions, and the HRT was set at 10 h. But the influent nutrient concentrations in the reactors were twice as those in the bench-scale CIBR, and the influent flow rates in the reactors were reduced by half via the control of five peristalsis pumps. Five reactors were continuously stirred to make sure that PAOs had the chance to obtain carbon source. The feeding time of which was controlled at 0, 0.25, 0.5, 1.5 and 2.5 h, respectively. Samples were collected at intervals and analyzed for the PO_4^{3-}-P concentration.

2.3.2. Anoxic batch tests

The seed sludge for anoxic batch tests was taken from the bench-scale CIBR at the end of the aerobic stage. MLVSS concentrations were 2079, 2102, 1984, 2138 and 2159 mg/L, respectively. Five reactors were continuously stirred so that the carbon source in the influent could be utilized quickly by activated sludge. The composition of the synthetic wastewater for anoxic batch tests were the same as anaerobic batch tests, except the COD concentrations. A highly concentrated acetate solution was added to five reactors, and the COD concentrations reached 120, 250, 400, 800 and 1200 mg/L, respectively, with the relevant C/N ratios of 120:30, 250:30, 400:30, 800:30 and 1200:30, respectively, The influent pumped into the anoxic reactors was controlled in the same way as the anaerobic reactors. The pH and ORP sensors were installed for monitoring pH and ORP in the anoxic reactors, and the anoxic duration was determined by identifying the "nitrite knee" on the ORP profiles and the "nitrite apex" on the pH profiles, which indicated the end of denitrification. Samples were collected at intervals, according to pH and ORP variations.

2.4. Analyses

Mixed liquids were collected and immediately filtered through 0.45 μm cellulose microfiber filters. The filtrate was analyzed for COD, NH_4^+-N, NO_2^--N, NO_3^--N, PO_4^{3-}-P and MLVSS, according to the standard methods [19]. The values of DO, ORP and pH were measured with American JENCO online instruments (6311 and 6308DT, JENCO, America).

3. Results and discussions

3.1. Competition for carbon source between PAOs and other heterotrophic organisms under anaerobic condition

Different amounts of carbon source for phosphorus release were supplied by adjusting the anaerobic feeding time. Fig. 2 shows the anaerobic phosphorus release under different feeding time. The carbon source could be transformed into the intracellular polymers and stored in activated sludge under anaerobic condition. Thus, the small amount of phosphorus (4.01 mg/L) was released by PAOs, even though the feeding time is zero after activated sludge was switched from anoxic to anaerobic conditions. The amounts of phosphorus release increased with the increasing of feeding time. Besides, COD in the influent was immediately consumed because of the competition for carbon source between PAOs and other heterotrophic organisms, leading to the COD concentrations under different feeding time all below the detective limit.

The necessary carbon source for phosphorus release could be obtained during the continuous feeding time of 0.5 h (Fig. 2). The C/P ratios for the carbon uptake and phosphorus release under different feeding time were calculated, and the smaller values (2.01 and 1.98) were obtained at feeding time of 0.25 and 0.5 h. When feeding time was longer than 0.5 h, surplus acetate in influent was utilized by other heterotrophic organisms, leading to a significant disappearance of COD. Fermentation in this system should be minimal, because the acetate was used as the input COD. The COD "loss" was possibly associated with poly-β-hydroxybutyrate (PHB) formation by GAOs [20]. It could be inferred that with feeding time of 0.5 h, the influent carbon source could be used by PAOs without the competition from other heterotrophic organisms (GAOs). It also proved that PAOs could be more effective than other heterotrophic organisms in consuming volatile fatty acid (VFA) in this study. Thus, the arithmetic mean of these two values was considered as the theoretical minimal C/P ratio of 1.99 in this study. Similar result was also obtained by the other researchers [21], who reported the C/P ratio was 2 when acetate was used as the sole carbon source.

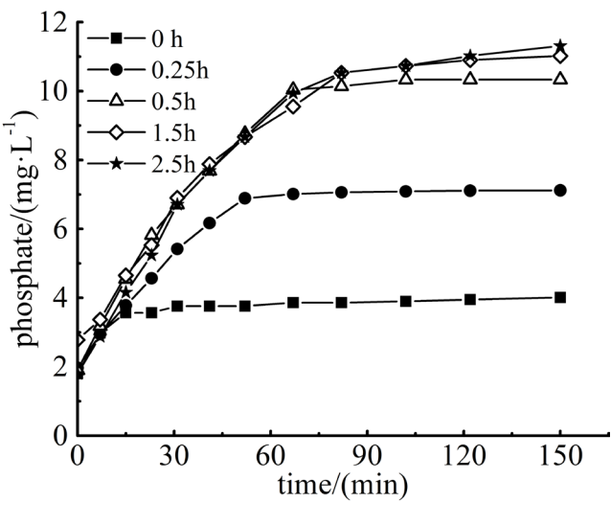

Fig. 2. Effect of the feeding time on phosphorus release at anaerobic phase.

3.2. Competition for carbon source between PAOs and other heterotrophic organisms under anoxic condition

3.2.1. Effect of influent C/N ratios on denitrification and phosphorus release

Nitrite accumulation during denitrification under different influent C/N ratios was observed in this study (Fig. 3). This was due to the interaction of carbon source, low C/N ratio and high temperature, which resulted in the higher specific growth rate of ammonia oxidizing bacteria (AOB) than the nitrite oxidizing bacteria (NOB) [22]. Maximum nitrite accumulation was achieved when the concentration of NO_3^--N was lower than 0.5 mg/L. The rates of nitrate and nitrite reduction were mainly limited by DO levels and insufficient carbon source in the influent when influent C/N ratios were lower than 400:30, while the denitrifiers showed highest activity with surplus COD supplied at the influent C/N ratios of 800:30 and 1200:30 [23]. Hence, the rates of nitrate and nitrite reduction, and nitrite accumulation were all calculated based on the zero-order reaction. These results are similar with previous research [9]. The accumulated nitrite began to reduce only after the nitrate was depleted, because the nitrate reductases no longer competed with nitrite reductases for the electron donors. Therefore, denitrification process could be divided into two major biological processes, namely, denitratation (i.e., nitrate reduction to nitrite) and denitritation (i.e., nitrite reduction to N_2). Phosphorus release under anoxic conditions could also be divided into three phases: no release of phosphorus, slow release of phosphorus and fast release of phosphorus.

COD in the continuous influent was consumed completely, and there was no available carbon source in the reactor, due to the competition among heterotrophs for limited carbon source. The COD concentrations under different influent C/N ratios were below the detection limit, except that the influent C/N ratio was 1200:30, the remaining COD at the end of anoxic phase was about 38.86 mg/L (Fig. 3d). This portion of COD was not accumulated until the end of denitritation, and the accumulated COD amount was 21.12 mg/L.

3.2.2. Correlation between denitrification and phosphorus release

To study the correlation between denitrification and phosphorus release quantitatively, the time durations and rates of denitrification and phosphorus release under different influent C/N ratios were calculated (Table 1).

Influent C/N ratios made a remarkable effect on denitrification and phosphorus release. The rates of nitrate reduction, and accumulated nitrite reduction, together with slow and fast phosphorus release rates, increased with the rise of influent C/N ratios, and the maximal rates of nitrate reduction and accumulated nitrite reduction were obtained at higher influent C/N ratios of 800:30 and 1200:30 (Table 1). Fig. 4 shows the variations of reaction

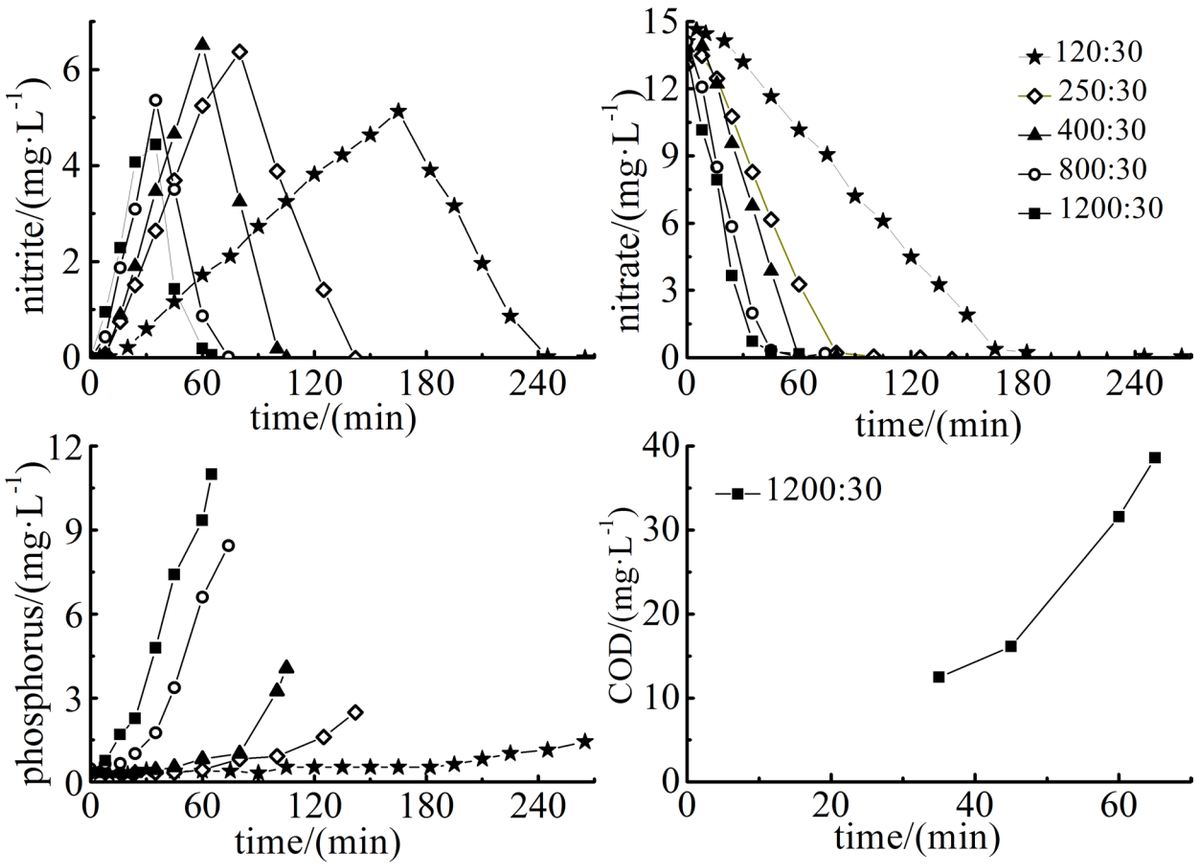

Fig. 3. Variations of nitrite, nitrate, phosphate and COD concentrations under different influent C/N ratios.

Table 1
Durations and rates of denitrification and phosphorus release under different influent C/N ratios

C/N	120:30	250:30	400:30	800:30	1200:30
Duration of denitrification (min)	265	142	95	74	65
Duration of denitratation (min)	0–165	0–80	0–50	0–45	0–35
Nitrate reduction rate (mg·L^{-1}·(gMLVSS)$^{-1}$)	1.72	3.56	5.35	6.46	6.72
Duration of denitritation (min)	165–265	80–142	50–95	45–74	35–65
Maximal concentration of nitrite (mg·L^{-1})	5.13	6.37	6.51	5.36	4.44
Nitrite reduction rate(mg·L^{-1}·(gMLVSS)$^{-1}$)	1.23	1.90	2.84	3.00	3.04
Duration of slow P release (min)	182–245	45–100	24–50	16–35	0–24
Slow P release rate (mg·L^{-1}·(gMLVSS)$^{-1}$)	0.20	0.22	0.27	1.07	1.52
Duration of fast P release (min)	245–265	100–142	50–95	35–74	24–65
Fast P release rate (mg·L^{-1}·(gMLVSS)$^{-1}$)	0.47	0.68	1.42	3.22	3.67

time with different influent C/N ratios. The order relationship between denitrification and phosphorus release was determined by nitrate reduction and fast phosphorus release. When influent C/N ratio was lower than 400:30, fast phosphorus release started after the nitrate was reduced to 0.5 mg/L, meanwhile, fast phosphorus release occurred prior to the completion of nitrate reduction. In other words, simultaneous denitrification and P-release occurred. It was because that the denitrifiers could compete for available carbon source with PAOs. As VFA was a key factor to trigger phosphorus release, the different rates of phosphorus release might be resulted from by a gradient decrease in the carbon source requirement for de nitrification.

3.2.3. COD mass balance calculation

Different influent C/N ratios would lead to the variations of carbon source distribution between denitrification and phosphorus release. The COD mass balance calculations afforded some useful new information. The existence of steady-state controlled process conditions needed to be considered. The influent COD included: the COD incorporated into the sludge mass through cell synthesis and adsorption, the COD in the effluent and the COD utilized by the heterotrophs, The COD in the effluent was negligible because there was no available carbon source in the effluent. COD for cell synthesis and adsorption was difficult to be measured accurately, and then it was considered according to the calculations of acetate uptake associated with denitrification and P-release. The actual and theoretical C/N and C/P ratios during denitrification, denitratation and denitritation were calculated individually. Some assumptions were made in the COD mass balance calculation: (1) Total influent COD was the total quantity of organic matter during the feeding duration; (2) Influent carbon source was utilized only by denitrifiers and PAOs, and denitrifiers were more sensitive towards acetate than PAOs; (3) The actual C/P ratio and theoretical P-release were calculated based on the theoretical C/N ratios for denitrification, denitratation and denitritation, while the actual C/N ratios were calculated based on the theoretical C/P ratio obtained under anoxic conditions. Based on these assumptions, the actual and theoretical C/P ratios were calculated (Table 2).

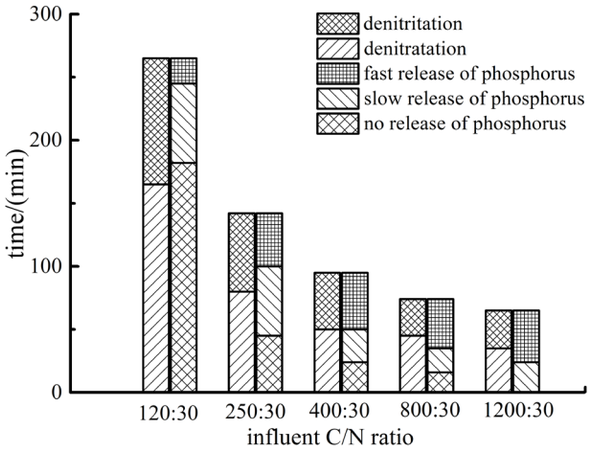

Fig. 4. Variations of denitrification and phosphorus release with different influent C/N ratios.

3.2.3.1. Theoretical C/N ratio for denitrification

When influent C/N ratios were 120:30, 250:30 and 400:30, the actual C/N ratios for denitrification were 3.89, 3.95 and 4.15, respectively (Table 2). These values are very close, indicating that other heterotrophic organisms had no

Table 2
COD mass balance calculation under anoxic condition

Influent C/N ratio		120:30	250:30	400:30	800:30	1200:30
Denitrification	Duration (min)	265	142	97	74	65
	Total influent COD (mg)	265	295	315	495	650
	NO_3^--N removal (mg)	64.4	68.65	68.26	68.92	64.14
	Actual P release (mg)	7.21	12.45	20.31	42.19	54.95
	Theoretical P release (mg) (COD /NO_3^--N = 3.89, C/P = 1.99)	7.25	14.45	29.13	113.0	147.91
	Actual C/P ratio (COD/NO_3^--N = 3.89)	2.01	2.31	2.86	5.34	5.37
	Actual C/N ratio (C/P = 1.99)	3.89	3.95	4.15	5.94	6.78
Denitratation	Duration (min)	165	80	52	45	35
	COD for Nitrate reduction (mg)	165	166.64	175	180	350
	Actual P release (mg)	2.67	4.08	4.11	16.87	23.93
	Theoretical P release (mg) (COD /NO_3^--N = 2.33, C/P = 1.99)	2.52	3.36	7.29	69.96	100.65
	Actual C/P ratio (COD /NO_3^--N = 2.33)	1.90	2.58	3.52	8.28	8.38
	Actual C/N ratio (C/P = 1.99)	2.33	2.46	2.42	3.87	4.72
Denitritation	Duration (min)	100	62	45	29	30
	COD for Nitrite reduction (mg)	100	129.15	150	193.35	300
	Maximal NO_2^--N (mg)	25.55	31.85	32.55	26.8	22.21
	Observed P release (mg)	4.55	8.35	16.21	25.35	31.02
	Theoretical P release (mg) (COD/NO_2^--N = 3.56, C/P = 1.99)	4.55	7.95	17.15	49.19	57.95
	Actual C/P ratio (COD/NO_2^--N = 3.56)	1.99	1.89	2.11	3.86	3.72
	Actual C/N ratio(C/P = 1.99)	3.56	3.53	3.62	5.33	5.97

chance to utilize carbon source. In other words, denitrifiers had priority in competition with PAOs for the carbon source during denitrification. Thus, the actual C/N ratio of 3.89 for the denitrification with influent C/N ratio of 120:30 was considered as the theoretical minimal value. Using acetic acid as the carbon source, the chemical equation including cell synthesis has been proposed by Mateju et. al as follows [24]:

$$0.819CH_3COOH + NO_3^- \rightarrow 0.068C_5H_7NO_2 + HCO_3^- + 0.301CO_2 + 0.902H_2O + 0.466N_2 \quad (1)$$

Based on the Eq. (1), the reduction of 1 g NO_3^--N theoretically consumes 3.51 g acetate (or 3.74 g COD) and produces 0.55 g new cells. The theoretical optimal C/N ratio is calculated to be 3.74, under the situation without competition from other heterotrophs. This value is a little lower than the experimentally actual value in this study. It may be because that even though the similar biological systems were used to treat various wastewaters, the optimal C/N ratios might be different under the distinct environmental conditions. Therefore, the optimal C/N ratio for denitrification under a special condition needs to be determined experimentally.

3.2.3.2. Competition for carbon source among denitrifiers and PAOs with influent C/N ratio lower than 400:30

When influent C/N ratio was lower than 400:30, all the actual and theoretical amounts of phosphorus release were very low. This was because insufficient carbon source was preferentially utilized by the denitrifiers and little residual carbon source was used by PAOs to release phosphate. The actual C/P ratios during denitrification were close to or slightly higher than the theoretical value of 1.99 for anaerobic phosphorus release. It suggested that other heterotrophic organisms had no opportunity to obtain the limited carbon source, and the theoretical C/P ratio under anoxic condition was similar with that under anaerobic condition.

3.2.3.3. Competition for carbon source among heterotrophs at influent C/N ratios of 800:30 and 1200:30

With influent C/N ratios of 800:30 and 1200:30, the actual amounts of phosphorus release were significantly higher than those when C/N ratios were less than 400:30. These results confirmed that denitrifiers had obvious competitive advantages over PAOs for influent carbon source. The actual C/P ratios and C/N ratios were all higher than the theoretical values. It indicated that influent carbon source was utilized not only by the denitrifiers and PAOs, but also by the other heterotrophic organisms. However, the theoretical amount of phosphorus release was much larger than the actual one, which indicated that the activity of PAOs was inhibited and the other heterotrophic organisms had a chance to obtain carbon source. It is generally accepted that nitrate can affect phosphorus release [25,26]. However, the inhibition on PAOs was observed during denitritation when nitrate was absent. Some studies have proposed that some denitrification intermediates (NO_3-N, NO_2-N, NO, NH_2OH, etc) could make an inhibitory effect on PAOs [27–29]. These intermediates were produced during denitrification with low influent C/N ratios [30]. Nitrite could make adverse effect on phosphorus release, due to the inhibition of free nitric acid (FNA). But in this study, the concentration of FNA was very low (<0.005 mg/L), leading to no significant inhibition on PAOs. Van Niel et al., proposed that the phosphate release was completely inhibited by 0.3 mM nitric oxide (NO) in the liquid phase and the adenylate kinase activity was completely inhibited by 0.15 mM NO in cell extracts of the sludge [27]. Hence, the inhibition on phosphate release might be caused by some undetected denitrification intermediates (NO_3-N, NO_2-N, NO, NH_2OH, etc) [31–33].

3.2.3.4. Carbon source competition during denitrification, denitratation and denitritation

The actual C/N ratios during denitratation were also very close when influent C/N ratios were lower than the 400:30. The similar phenomenon was observed during denitritation. It indicated that denitrifiers were more sensitive towards acetate than PAOs and other heterotrophic organisms during denitratation and denitritation. The actual C/P ratios during denitratation and denitritation were close to or slightly higher than the theoretical value of 1.99 for anaerobic phosphorus release. The actual and theoretical amounts of phosphorus release were very low. It suggested that other heterotrophic organisms did not obtain carbon source, due to their weaker competitiveness. The actual C/P ratios during denitratation, denitritation and denitrification were close to 1.99 with influent C/N ratios of 120:30, which proved that the theoretical C/P ratio under anoxic condition was close to that under anaerobic condition. The theoretical C/N ratios for denitratation and denitritation were 2.33 and 3.56, respectively. It is reasonable that the actual C/N ratio during denitrification was higher than that during denitratation and denitritation, but the actual C/N ratio during denitritation was higher than denitratation, which contradicted the traditional theory. According to the semi-reaction of nitrate and nitrite reduction, 40% COD is saved in denitrification process when NO_2^--N is used as the electron acceptor instead of NO_3^--N. The C/N ratio for the NO_3^--N reduction was 3.89 in this study, and the C/N ratio for NO_2^--N reduction was 1.67, the theoretical C/N ratio for denitritation would be 2.33. However, the actual C/N ratio for denitrification was much higher than 2.33. The fractions of COD used for the reduction of the accumulated nitrite to total influent COD during denitritation were 36.85, 38.29 and 37.63 %, respectively.

This portion of "COD loss" might be caused by other denitrification intermediates, because one or more undetected intermediates could accumulate during the denitrification under the inhibitory conditions (temperature, C/N ratio, carbon source, and so on) [34]. Carbon source was also consumed by those undetected intermediates. If this portion of COD utilized by undetected intermediates was not excluded from the total influent COD during denitritation, the theoretical denitritation C/N ratio would be overestimated.

3.2.4. Competitiveness of heterotrophs

The results mentioned above confirmed that when the acetate was use as carbon source in the synthetic wastewater with low C/P ratio, the competitive abilities of the heterotrophs for the carbon source were in the following order: denitrifiers >PAOs > other heterotrophic organisms. This result indicated that the energy produced by anoxic microbial respiration during denitrification was far more than the anaerobic respiration by PAOs, GAOs and other heterotrophic organisms. Besides, the electrode potential of anoxic nitrate respiration ($NO_3^- \rightarrow NO_2^-$, N_2O, N_2) is +0.4, which is higher than anaerobic carbonate respiration (–0.25 for $CO_2 \rightarrow CH_3COO^-$, –0.30 for $CO_2 \rightarrow CH_4$). Therefore, denitrifiers have the strongest ability to combine electron donors (carbon source). Furthermore, GAOs use glycogen as their sole energy source for VFA uptake, whereas PAOs possess both poly-P and glycogen reserves. Thus, GAOs have a higher Gly/VFA ratio than PAOs. But compared to the metabolism of PAOs on poly-P, the metabolism of GAOs on glycogen is more complicated and less effective in the energy generation, which is unfavorable for the GAO growth [35]. Moreover, the other heterotrophic organisms, including fermentative bacteria and OHO, do not have the ability of storing the carbon source like PAOs and GAOs.

3.2.5. Competition mechanisms of heterotrophs for carbon source

Competition mechanism was mainly induced by the heterotrophs for the carbon source, and denitrifiers had an obvious competitive advantage over PAOs for the insufficient carbon source [36–37]. With influent C/N ratios of 800:30 and 1200:30, when denitrification was finished, the residual COD could be used by the PAOs prior to the other heterotrophic organisms. However, the presence of intermediates (NO_3-N, NO_2-N, NO, NH_2OH, etc) in denitrification inhibited the activity of PAOs, which led to a small proportion of COD was used by other heterotrophic organisms. Competition mechanism was involved with the competition among heterotrophs for carbon source and the inhibition of PAOs by the intermediates (NO_3-N, NO_2-N, NO, NH_2OH, etc) in denitri fication [38,39].

3.3. Carbon source distribution under anoxic condition

Since the theoretical C/P and C/N ratios were obtained by the COD mass balance calculation, the carbon source distribution among denitrifiers, PAOs and other heterotrophic organisms during denitrification, denitratation and denitritation with different influent C/N ratios were determined (Table 3).

The fractions of COD utilized by the denitrifiers decreased with the increasing of influent C/N ratio, while the opposite trend was shown in PAOs and other heterotrophic organisms (Table 3). The increasing of influent C/N ratio had different effects on the efficiency of carbon source utilization by the different functional bacteria. The denitrifiers were not significantly affected by the increasing of influent C/N ratio while the other heterotrophic organisms were remarkably influenced. When influent C/N ratio was lower than 400:30, the fractions of COD utilized by PAOs and other heterotrophic organisms were very low during denitratation. During the denitritation, the other heterotrophic organisms could not obtain carbon source, while the fractions of COD utilized by PAOs increased. The fraction of COD utilized by PAOs (Table 3) during denitratation was significantly lower than those during denitritation, which

Table 3
Carbon source distribution among heterotrophs under anoxic condition

	C/N	Total COD (mg)	Denitrifiers		PAOs		Other heterotrophic organisms	
			COD (mg)	Fractions (%)	COD (mg)	Fractions (%)	COD (mg)	Fractions (%)
Denitrification	120:30	265	250.65	94.58	14.35	5.42	0.00	0.00
	250:30	295.89	267.18	90.32	24.85	8.39	3.81	1.29
	400:30	323.34	265.44	82.09	40.5	12.52	17.44	5.39
	800:30	493.35	268.15	54.36	84.15	17.06	141.03	28.59
	1200:30	544.42	249.51	45.83	109.55	20.13	185.39	34.05
Denitratation	120:30	165	149.88	90.82	5.3	3.20	9.86	5.97
	250:30	166.63	159.76	95.09	8.2	4.91	0.00	0.00
	400:30	173.36	158.71	91.56	8.2	4.72	6.44	3.72
	800:30	300	160.34	53.44	33.6	11.20	106.08	35.36
	1200:30	350	149.17	42.62	47.75	13.64	153.12	43.74
Denitritation	120:30	100	90.94	90.93	9.05	9.07	0.00	0.00
	250:30	129.15	112.52	87.11	16.65	12.89	0.00	0.00
	400:30	150	115.93	77.25	32.3	21.54	1.82	1.21
	800:30	193.37	95.41	49.35	50.55	26.15	47.43	24.51
	1200:30	194.42	79.05	40.65	61.8	31.80	53.58	27.55

indicated that phosphorus release during denitratation was strongly inhibited. Besides, the fraction of COD utilized by the other heterotrophic organisms was also found to be smaller than that during denitrification and denitratation. In general, low influent C/N ratio and the presence of nitrite accumulation during denitritation were beneficial to improve the efficiency of carbon source utilized by the denitrifiers and PAOs.

4. Conclusion

The competition for the acetate (under anoxic condition) among different heterotrophs in a CIBR was investigated in this study. The results revealed that the influent C/N ratios had significant influence on the competition for acetate among different heterotrophs. Phosphorus release occurred after the depletion of nitrate when influent C/N ratio was lower than 400:30. With the influent C/N ratios of 800:30 and 1200:30, the denitrification and phosphorus release occurred simultaneously, and the activity of polyphosphate accumulating organisms (PAOs) was inhibited by the intermediates (NO_3-N, NO_2-N, NO, NH_2OH, etc) produced in denitrification. Based on the COD mass balance calculation, the theoretical C/P ratio for phosphorus release was 1.99, and the theoretical C/N ratios for the denitrification, denitratation and denitritation were 3.89, 2.33 and 3.56, respectively. The competitiveness for carbon source among heterotrophs follows the order: denitrifiers > PAOs > other heterotrophic organisms. The mechanisms include the competition for carbon source and the inhibition of intermediates in denitrification. The low influent C/N ratio and nitrite accumulation during denitrification were both beneficial to improve the efficiency of carbon utilization.

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