Treating low carbon/nitrogen (C/N) wastewater in simultaneous nitrification-endogenous denitrification and phosphorous removal (SNDPR) systems by strengthening anaerobic intracellular carbon storage

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Abstract

A novel simultaneous nitrification denitrification and phosphorous removal-sequencing batch reactor (SNDPR-SBR) enriched with PAOs (phosphorus accumulating organisms), DPAOs (denitrifying PAOs), and GAOs (glycogen accumulating organisms) at the ratio of 2:1:1 was developed to achieve the simultaneous nutrient and carbon removal treating domestic wastewater with low carbon/nitrogen ratio (C/N ≤ 3.5). The SNDPR system was operated for 120 days at extended anaerobic stage (3 h) and short aerobic stage at low oxygen concentration (2.5 h) with short sludge retention time (SRT) of 10.9 d and hydraulic retention time (HRT) of 14.6 h. The results showed that at the stable operating stage, the average effluent chemical oxygen demand (COD) and PO4-P concentrations were 47.2 and 0.2 mg L−1, respectively, the total nitrogen (TN) removal efficiency was 77.7%, and the SND efficiency reached 49.3%. Extended anaerobic stage strengthened the intracellular carbon (mainly poly-β-hydroxybutyrate, PHB) storage, efficiently utilized the organic substances in wastewater, and provided sufficient carbon sources for denitrification and phosphorus uptake without external carbon addition. Short aerobic stage at low oxygen concentration (dissolved oxygen (DO): 1 ± 0.3 mg L−1) achieved a concurrence of nitrification, endogenous denitrification, denitrifying and aerobic phosphorus uptake, and saved about 65% energy consumption for aeration. Microbial community analysis demonstrated that P removal was mainly performed by aerobic PAOs while N removal was mainly carried out by denitrifying GAOs (DGAOs), even though DPAOs were also participated in both N and P removal.

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1. Introduction

Functional microorganisms in municipal wastewater treatment have various requirements of operational conditions, such as organic substance concentration, dissolved oxygen (DO) concentration, and sludge retention time (SRT) (Ma et al., 2009). This might cause inefficient nitrogen and phosphorus removal in traditional biological nutrient removal (BNR) process, especially treating the domestic wastewater with deficient carbon sources when denitrifying OHOs (ordinary heterotrophic organisms) would preferentially use organic carbon over PAOs (phosphorus accumulating organisms), and cause insufficient carbon sources for phosphorus removal. Therefore, highly efficient sewage treatment process and control strategies should be developed for dealing with wastewater with low carbon/nitrogen ratio (C/N, referred to chemical oxygen demand (COD)/total nitrogen (TN)) lower than 3.5.

Enhanced biological phosphorus removal (EBPR) can achieve a stable phosphorus removal by strengthening anaerobic phosphorus release and aerobic phosphorus uptake through the enriched PAOs, while simultaneous nitrification and denitrification (SND) can achieve an efficient nitrogen removal at low DO concentration (DO: 0.5 ~ 1.5 mg/L) (Helmer et al., 1998; Meyer et al., 2005; Yilmaz et al., 2008). Therefore, a novel process termed as SNDPR (simultaneous nitrification-denitrification and phosphorus removal) was developed by coupling EBPR with SND (Meyer et al., 2005; Zeng et al., 2003a). Compared with traditional A/A/O or A/O nutrient removal process, the SND in the SNDPR system could reduce the NO\(_x\) (NO\(_2^-\) - N and NO\(_3^-\) - N) concentration at low aeration intensity, and thus reducing the carbon demand for denitrifying OHOs and leading to more carbon sources available for EBPR.

Simultaneous removal of nutrient and carbon from wastewater with low C/N ratio has not been achieved in activated sludge SNDPR systems without external carbon addition. SNDPR has been studied in granular sludge systems (de Kreuk et al., 2005; Yilmaz et al., 2008; Wang et al., 2009a; Coma et al., 2012), hybrid sludge systems (Wang et al., 2009b), and biofilm reactors (Yang et al., 2010; Fu et al., 2009; Gieseke et al., 2002). Normally, a SNDPR system needs to be seeded with aerobic granular sludge or biofilms to achieve SND, but the TN removal efficiency was low (52%, Wang et al., 2009b). For activated sludge SNDPR systems without granular sludge and biofilm, the operational modes (e.g. anaerobic/aerobic duration, aerobic DO concentration) should be optimized. Moreover, most SNDPR studies have focused on synthetic wastewater (Zeng et al., 2003a; Lo et al., 2010; Coma et al., 2012), in which PAOs were easily enriched. For real domestic wastewater, especially for those with limited carbon sources (low C/N), the rapid enrichment of PAOs could not be achieved. Other microorganisms in real wastewater, such as GAOs (glycogen accumulating organisms) that have similar metabolisms to PAOs, but without contributing to phosphorus removal, Filipe et al., 2001; Saunders et al., 2003), might affect the performance of SNDPR systems. Previous studies found that the enrichment of PAOs failed due to the proliferation of GAOs (Mino et al., 1995; Saunders et al., 2003), but others reported that GAOs carried out denitrification in SNDPR systems (Zeng et al., 2003a).

This study aimed at developing a simple anaerobic/aerobic SNDPR system using a sequencing batch reactor (SBR) to achieve an efficient removal of nutrient and organic carbon treating domestic wastewater at low C/N ratio (≤3.5) without external carbon addition. Two unique features of the SBR cycles (e.g. extended anaerobic stage and short aerobic stage with low DO concentration) were developed to enhance the activities of different functional microorganisms (e.g. PAOs, GAOs, denitrifying PAOs (DPAOs), and denitrifying GAOs (DGAOs)) in the SNDPR system, and to achieve simultaneous N and P removal. The study also explored the nutrient removal pathway in the SNDPR system by determining the variations of intracellular and extracellular substrates, and elucidated the correlation of microbial population with the nutrient and carbon removal in the SNDPR system.

2. Materials and methods

2.1. Experimental device and operation process

A laboratory-scale open-mouthed SBR with a working volume of 8 L and made up of methyl methacrylate (PMMA) was used as the SNDPR-SBR system (Fig. 1), due to its simple operation protocols and flexible adjustment of each stage (e.g. anoxic mixing, aerobic stage). The system was operated for 120 days under extended anaerobic and short low aerobic conditions. Specifically, short low aerobic stage was used for the concurrence of SND and P uptake, and extended anaerobic stage was used for enhancing the utilization of carbon sources in wastewater and providing sufficient intracellular carbon for SND and P removal at the following short low aerobic stage. The cycle time of the SBR was 6 h, consisting of 180 min anaerobic reaction (including 36 min feeding period), 150 min aerobic reaction (including 2 min sludge wasting), 20 min settling, 5 min decanting phase, and 5 min idle phase. During the feeding period, 3 L domestic wastewater (composition described below) was added to the reactor with a hydraulic

![Fig. 1 – Experimental device of the SNDPR-SBR system.](image-url)
retention time (HRT) of 14.6 h. During the sludge wasting period, 200 mL of mixed liquor was discharged to achieve a SRT of 10.9 d and a mixed liquor suspended solid (MLSS) concentration of 3000 ± 300 mg L⁻¹. The SBR was operated at room temperature. DO concentration was maintained at 1.0 ± 0.3 mg L⁻¹ in the aerobic stage by using an online real-time control device (PLC). pH was controlled at 7.2 – 8.0 by addition of 0.2 mol L⁻¹ hydrochloric acid (HCl) to avoid chemical phosphorus precipitation.

2.2. Wastewater and seeding sludge

Domestic wastewater fed to the SNDPR-SBR system was taken from a septic tank in the residential area of Beijing University of Technology (Beijing, China). The main characteristics of the wastewater were: COD 142.4 – 268.3 mg L⁻¹, TOC (total organic carbon) 45.3 – 77.6 mg L⁻¹, TIC (total inorganic carbon) 38.4 – 75.8 mg L⁻¹, NH₄⁺ – N 50.2 – 69.4 mg L⁻¹, NO₃⁻ – N < 1 mg L⁻¹, NO₂⁻ – N < 1 mg L⁻¹, PO₄³⁻ – P 5.1 – 7.9 mg L⁻¹, TN 68.4 – 79.2 mg L⁻¹, and C/N ratio < 3.5. The activated sludge inoculated was taken from a pilot SBR system (volume: 8.8 m³), which had achieved a stable performance of biological nitrogen and phosphorus removal for 8 months.

2.3. Batch SBR tests

Besides the SNDPR-SBR system, a series of SBRs (working volume: 1.0 L each) were used for all batch tests. The first batch SBR test was carried out to determine the optimal influent COD and NO₃⁻ – N concentrations for the PAOs activities in the SNDPR-SBR system. The second batch SBR test was carried out to determine the proportion of DPAOs in PAOs, since previous studies showed that the ratio of anoxic P uptake rate and aerobic P uptake rate might reflect the proportion of DPAOs to PAOs (Wachtmeister et al., 1997). During all the batch tests, magnetic stirrers were used to keep the sludge in suspension and the rotation speed was controlled at around 100 r min⁻¹. pH value was measured online using a pH meter (pH/oxi340i, WTW Company, Germany) and controlled at 7.4 – 7.8 by adding 0.2 mol L⁻¹ HCl or 0.2 mol L⁻¹ sodium hydroxide (NaOH) solution.

In the first batch test (12 SBRs sealed), the sludge sample was the inoculated sludge after been washed for 3 times by centrifugation to remove any remaining N, P, and external carbon. The sample was then divided equally into each batch SBR, and mixed with domestic wastewater, sodium acetate (NaAc), and sodium nitrate (NaNO₃) solution to achieve an initial COD and NO₃⁻ – N concentration as described in Table 1 (test number: 1 – 12). Mixed liquor samples were taken every 10 min for water quality analysis.

In the second batch test (2 SBRs with 1 sealed and 1 open-mouthed, respectively), activated sludge was taken from the SNDPR-SBR system at the end of the anaerobic phase on the day 1, 40, 80, and 120. After been washed for 3 times, sludge sample was evenly distributed to 2 SBRs, and then mixed with distilled water and phosphorus solution to achieve an initial P concentration of 25 mg L⁻¹, the same as the SNDPR-SBR system. During experiments, the sealed SBR was added with NaNO₃ solution to achieve an initial NO₃⁻ – N concentration of 25 mg L⁻¹ and the denitrifying P uptake rate was measured, while the open-mouthed SBR was added nothing but aerated to measure the aerobic P uptake rate (Table 1, test number: 13 – 14).

2.4. Analysis methods

All samples were filtered through 0.45 μm filter paper before analysis. NH₄⁺ – N, NO₂⁻ – N, NO₃⁻ – N, and PO₄³⁻ – P were analyzed using an automatic flow injection analyzer (Lachat Quik-Chem8000, Lachat Instrument, USA). COD, MLSS, and MLVSS were analyzed according to the standard methods (APHA, 1998). TN, TOC, and TIC were analyzed using a TN/TOC analyzer (Multi N/C3000, Ananlitijena AG, Germany). Freeze-dried biomass was used to measure polyhydroxyalkanoates (PHAs) and glycogen (Gly). PHAs were determined by the sum of poly-β-hydroxybutyrate (PHB) and poly-β-hydroxyvalerate (PHV), which were analyzed as previously reported (Oehmen

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### Table 1 – Experimental conditions applied in batch SBR tests.

<table>
<thead>
<tr>
<th>Test name</th>
<th>Test number</th>
<th>Initial concentration (mg/L)</th>
<th>Batch SBR operation mode</th>
<th>Experimental purpose</th>
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<tr>
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<td>1</td>
<td>60</td>
<td>2 – 3</td>
<td>Anaerobic stir for 3.0 h</td>
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<td>80</td>
<td>2 – 3</td>
<td>–</td>
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<td>2 – 3</td>
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<tr>
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<td>240</td>
<td>2 – 3</td>
<td>–</td>
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<tr>
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<td>6</td>
<td>300</td>
<td>2 – 3</td>
<td>–</td>
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<td>180</td>
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</table>
et al., 2005). Gly was analyzed as previously reported (Zeng et al., 2003b).

Fluorescence in situ hybridization (FISH) was used to identify the dominant bacteria in the SNDPR-SBR system (Aman et al., 1990). FISH probes used were: EUB338 (comprising equal amounts of EUB338, EUB388-II, and EUB338-III) for most Eubacteria; PAOmix (comprising equal amounts of PAO462, PAO651, and PAO846) for Accumulibacter; GAOmix (comprising equal amounts of GAO431 and GAO989) for Competibacter (Yang et al., 2013).

2.5. SND efficiency

SND efficiency is defined as the loss of nitrogen at the aerobic stage (Lo et al., 2010) (Eq. (1)).

\[
\text{SND} = \left(1 - \frac{\text{NH}_4^+ + \text{NO}_2^- + \text{NO}_3^-}{\text{NH}_4^+ - \text{NH}_4^+}\right) \times 100\%
\]

Where, \(\text{NH}_4^+\) is the \(\text{NH}_4^+\)-N concentration at the end of anaerobic stage, \(\text{mg L}^{-1}\), \(\text{NH}_4^+\) is the \(\text{NH}_4^+\)-N concentration at the end of aerobic stage, \(\text{mg L}^{-1}\), \(\text{NO}_2^-\) is the \(\text{NO}_2^-\)-N concentration at the end of aerobic stage, \(\text{mg L}^{-1}\); \(\text{NO}_3^-\) is the \(\text{NO}_3^-\)-N concentration at the end of aerobic stage, \(\text{mg L}^{-1}\), \(\text{NO}_3^-\), \(\text{NO}_2^-\), \(\text{NH}_4^+\) are the concentrations of \(\text{NO}_3^-\)-N and \(\text{NO}_2^-\)-N at the beginning of anaerobic stage, \(\text{mg L}^{-1}\).

2.6. Anaerobic organic carbon (COD) consumption

Anaerobic organic carbon consumption (COD_{consum}) in the SNDPR-SBR system is defined as the consumption of influent COD at the anaerobic stage, which consists two parts. One is the COD consumed by exogenous denitrification of nitrite and nitrate, and the other is the COD stored as the intracellular carbon source (refers to the activities of PAOs and GAOs) (Eq. (2)–(3)).

\[
\text{COD}_{\text{consum}} = \text{COD}_{\text{dn}} + \text{COD}_{\text{intra}}
\]

\[
\text{COD}_{\text{dn}} = 2.86\text{NO}_3^-_{\text{ana}} + 1.71\text{NO}_2^-_{\text{ana}}
\]

Where, \(\text{COD}_{\text{dn}}\) is the COD consumed by denitrification at the anaerobic stage, \(\text{mg L}^{-1}\); \(\text{COD}_{\text{intra}}\) is the COD consumed by PAOs and GAOs for intracellular carbon storage, \(\text{mg L}^{-1}\); 2.86 is the theoretical value of COD consumption for denitrification of unit \(\text{NO}_3^-\)-N, \(\text{mg N} (\text{mgCOD})^{-1}\); 1.71 is the theoretical value of COD consumption for denitrification of unit \(\text{NO}_2^-\)-N, \(\text{mg N} (\text{mgCOD})^{-1}\); \(\text{NO}_3^-_{\text{ana}}\) and \(\text{NO}_2^-_{\text{ana}}\) are the concentrations of \(\text{NO}_3^-\)-N and \(\text{NO}_2^-\)-N at the beginning of anaerobic stage, \(\text{mg L}^{-1}\).

3. Results and discussion

3.1. Determining the optimal influent conditions for PAOs enrichment through the batch SBR tests

Influent COD and \(\text{NO}_3^-\)-N concentrations are the two major factors for PAOs activities in the SNDPR-SBR system. To determine the optimal influent conditions for PAOs enrichment, a series of batch SBR tests were conducted at different COD and \(\text{NO}_3^-\)-N concentrations. P release amount (termed as PRA) of the inoculated sludge increased with the anaerobic initial COD concentration (Fig. 2A). When the initial COD concentration increased from 60 to 300 mg L\(^{-1}\), the PRA increased from 4.6 to 13.5 mg L\(^{-1}\), and the P release rates (termed as PRRs) increased from 0.4 to 2.4 mgP (gVSS h\(^{-1}\)). PRRs were determined with the linear fittings at the first 60 min (30 min for COD at 60 mg L\(^{-1}\)) on the P release curves. PRA increased linearly with the COD concentrations of 60–180 mg L\(^{-1}\), and reached the plateau at the COD concentration higher than 180 mg L\(^{-1}\). Based on the biochemical model for phosphorus removal (Comeau et al., 1986), at the aerobic stage, PAOs take up organic carbon contained in wastewater and store them as intercellular carbon in the form of PHAs. The energy required for this process comes from the decomposition of intracellular poly-phosphate (poy-P). Hence, at low COD concentration (60–180 mg L\(^{-1}\) in this study), COD was the main factor for the phosphorus release of PAOs, while at high COD concentration (>180 mg L\(^{-1}\) in this study), poly-P decomposition became the limiting factor (Fig. 2A). Therefore, the optimal initial COD concentration for PAOs enrichment in the SNDPR-SBR was about 180 mg L\(^{-1}\).

P release performance of the inoculated sludge decreased with the anaerobic initial \(\text{NO}_3^-\)-N concentration at initial
COD concentration of 180 mg L\(^{-1}\) (Fig. 2B). With NO\(_3^-\)–N concentration increasing from 0.9 to 18.1 mg L\(^{-1}\), the corresponding PRA decreased from 12.0 to 0.7 mg L\(^{-1}\). PRR decreased from 3.3 to (–) 0.6 mgP (gVSS h\(^{-1}\)) with a linear fitting of \(y = 3.99 – 0.25x (R^2 = 0.972)\) (Fig. 2B), and denitrification rate (termed as DNR in the first 10 min) showed a linear relationship with NO\(_3^-\)–N concentration (\(y = 0.65 + 1.38x (R^2 = 0.996)\)) (Fig. 2B). PRR became negative when initial NO\(_3^-\)–N reached 13 mg L\(^{-1}\), and the decrease in PRAs became obvious at initial NO\(_3^-\)–N higher than 6 mg L\(^{-1}\), indicating that insufficient COD in the influent limited biological P release (Ekama and Wentzel, 1999). Hence, the anaerobic initial NO\(_3^-\)–N concentration should be controlled below 6 mg L\(^{-1}\) at initial COD concentration of 180 mg L\(^{-1}\) to enrich PAOs and make P release proceed well.

### 3.2. Simultaneous C, N, and P removal in the SNDPR-SBR system

The whole operation period of the SNDPR-SBR system was divided into 4 phases based on the influent COD concentration and nutrient removal performance. In Phase 1 (1 – 14 d, Fig. 3A), the average influent COD concentration was 233.0 mg L\(^{-1}\), with a low P release and uptake amount (2.5 and 1.7 mg L\(^{-1}\)) and a high PO\(_4^{3-}\)–P in effluent (3.8 mg L\(^{-1}\)). But aerobic nitrification and anaerobic exogenous denitrification proceeded well, with the average effluent NH\(_4^+\)–N, NO\(_3^-\)–N, and NO\(_2^-\)–N concentration of 0.9, 1.7, and 19.6 mg L\(^{-1}\), respectively, and an average TN removal efficiency of 65.2% (Fig. 3B). Good nitrification at the aerobic stage led to an average anaerobic initial NO\(_3^-\)–N concentration of 10.9 mg L\(^{-1}\), which consumed substantial amounts of influent organic carbon for exogenous denitrification. Thereby, insufficient carbon (COD\(_{intra}\) << COD\(_{dn}\), Fig. 3C) was left for P release and resulted in a poor P removal performance.

Based on the poor P removal performance in Phase 1, influent COD concentration was increased to 344.8 mg L\(^{-1}\) (to meet the optimal anaerobic initial COD concentration of about 180 mg L\(^{-1}\), Fig. 3A) in Phase 2 (15 – 45 d) to improve the P removal. Hereafter, COD\(_{intra}\) was increased (Fig. 3C), which increased the anaerobic PRA to 23.5 mg L\(^{-1}\) on the 26th day and decreased the effluent PO\(_4^{3-}\)–P concentration to lower than 0.5 mg L\(^{-1}\) on the 45th day. The average ΔPO\(_4^{3-}\)–P/ΔCOD\(_{intra}\) (P release amount per COD\(_{intra}\)) increased from 106.6 mgP/gCOD\(_{intra}\) in Phase 1 to 289.0 mgP/gCOD\(_{intra}\). The results confirmed that more organic carbon available could improve the P release at the anaerobic stage and the P uptake at the aerobic stage. However, effluent NH\(_4^+\)–N concentration slightly increased to 4.3 mg L\(^{-1}\) on the 45th d, and the average effluent NO\(_3^-\)–N and NO\(_2^-\)–N concentrations reduced to 0.1 and 15.3 mg L\(^{-1}\), respectively (Fig. 3B), indicating that ammonium oxidation was slightly affected at the low oxygen aerobic stage. Additionally, SND efficiency slightly increased from 12.7% in Phase 1 – 16.7% due to the increase of COD\(_{intra}\) (Fig. 3C).

In Phase 3 (46 – 85 d), influent COD concentration was maintained at 350.0 mg L\(^{-1}\) to further strengthen the P removal performance and SND efficiency. The effluent PO\(_4^{3-}\)–P concentration was consistently below 0.5 mg L\(^{-1}\), and the average effluent NH\(_4^+\)–N and NO\(_3^-\)–N concentrations were 2.4 and 11.1 mg L\(^{-1}\), respectively. The TN removal efficiency and the SND efficiency reached to 70.1% and 30.9%, respectively (Fig. 3B and C). The enhanced SND might be caused by the better utilization of extracellular carbon sources, which was used by endogenous denitrifying bacteria to reduce NO\(_x\). The average COD\(_{intra}\) reached 83.5 mg L\(^{-1}\) in Phase 3 (Fig. 3C), compared to 24.3 and 54.9 mg L\(^{-1}\) in Phases 1 and 2, respectively. Additionally, almost no extracellular carbon was left for exogenous denitrification at the low aerobic stage, and
COD at the end of anaerobic was almost the same with COD effluent (Fig. 3A). Hence, DPAOs and GAOs, which possibly proliferated with the enrichment of PAOs (Crocetti et al., 2002; Cech et al., 1993) and performed denitrification by using intracellular carbon (Zeng et al., 2003a; Chen et al., 2013), were considered to be responsible for the improvement of SND efficiency.

The nitrogen and phosphorus removal stability of the SNDPR-SBR system was maintained in Phase 4 (85 ~ 120 d) when influent COD concentration decreased to 254.7 mg L\(^{-1}\) (the same level as in Phase 1). The average TN removal efficiency stabilized at 77.7%, and the effluent PO\(_4^{3-}\)-P concentration was below 0.5 mg L\(^{-1}\). Compared with Phase 3, the SND efficiency was further enhanced to 49.3% probably due to the further enriched GAOs and DPAOs, but P release amount showed a slight drop (Fig. 3A) because of the decrease of COD\(_{\text{intra}}\) (Fig. 3C). Compared with Phase 1, the effluent of NO\(_3^-\)–N almost reduced by half due to the low COD\(_{\text{dn}}\) and high COD\(_{\text{intra}}\) at the anaerobic stage (COD\(_{\text{dn}} << \) COD\(_{\text{intra}}\), 19.4 and 55.1 mg L\(^{-1}\), respectively).

The results of the 4 phases indicated that EBPR coupling with SND in a SNDPR-SBR system achieved an efficient simultaneous removal of nutrient and organic carbon by strengthening the intracellular carbon storage at the extended anaerobic stage. This system has the unique advantage for treating wastewater with low C/N ratio and avoids the needs for external carbon addition.

### 3.3. Population dynamics and quantitative relations of PAOs and GAOs with SNDPR-SBR performance

The variation of microbial community over the operation period of the SNDPR-SBR system was quantified using FISH analysis and batch tests (Fig. 4). On day 1 in Phase 1 (poor P removal performance and low SND efficiency), PAOs (including 12.3% of DPAOs) and GAOs only accounted for 3% ± 1% and 2% ± 1% of the total biomass (Fig. 4A, B and E). This microbial community well corresponded with the SNDPR-SBR performance, which little P was released, only half of COD\(_{\text{consum}}\) was stored as COD\(_{\text{intra}}\), and little NO\(_3^-\) was removed by endogenous denitrification (Table 2).

On day 40 in Phase 2, PAOs (including 18.4% of DPAOs) reached 9% ± 2% of the total biomass with the increase of influent COD, and GAOs increased to 7% ± 1%. The minor enrichment of PAOs improved P removal performance, while the minor enrichment of GAOs and DPAOs improved SND efficiency and reduced nitrate concentration in effluent. Compared with Phase 1, the P removal efficiency almost doubled, the SND efficiency increased by 4%, and the nitrate concentration in effluent decreased by 21.9% (Table 2).

On day 80 in Phase 3, all of PAOs, DPAOs, and GAOs exhibited a huge increase with the sufficient COD in influent (350.0 mg L\(^{-1}\), Table 2). PAOs and GAOs enriched to 33% ± 2% and 15% ± 2% of total biomass, respectively, while DPAOs accounted for 42.1% of PAOs. Highly enriched PAOs substantially improved P removal efficiency to 95.2% (compared with 74.5% in Phase 2), and the average PO\(_4^{3-}\)–P in effluent lowered to 0.3 mg L\(^{-1}\) (compared with 1.6 mg L\(^{-1}\) in Phase 2). Further enriched GAOs and DPAOs improved TN removal efficiency to 70.1% (compared with 65.6% in Phase 2), with the average nitrate in effluent decreasing by 27.5% and SND efficiency increasing by 14.2%. Moreover, the enrichment of PAOs and GAOs increased COD\(_{\text{intra}}$/COD\(_{\text{consum}}\) to 70.6%. These results confirmed that the more PAOs and GAOs, the higher strengthened intracellular carbon storage, and the better nutrient removal efficiency in the SNDPR-SBR system. Previous studies found that GAOs were potentially detrimental to EBPR (Mino et al., 1995; Saunders et al., 2003), but this study found that GAOs coupling with PAOs could improve the nitrogen removal performance of EBPR.

On day 120 in Phase 4, both PAOs and GAOs maintained at a high population (38% ± 2% and 17% ± 3% of total biomass) and DPAOs accounted for half of PAOs, even though the influent COD was reduced to 254.7 mg L\(^{-1}\) (the same as Phase 1, Table 2). Compared with Phase 1, PRA increased by 10 times (Table 2) with PAOs increasing about 35%; NO\(_3^-\)–N in effluent decreased by 50%, SND efficiency increased 4 times, and TN removal efficiency increased by 16.1% accompanied by the increase of GAOs and DPAOs. Additionally, COD\(_{\text{intra}}$/COD\(_{\text{consum}}\) increased by nearly twice with the enrichment of PAOs and GAOs. The variations of the average PRA/ΔCOD\(_{\text{intra}}\) coincide with the variations of PAOs enrichment throughout the operating period.

![Fig. 4 Variations of microbial community structure in the SNDPR-SBR system. A and C: FISH images of total biomass on day 1 and day 120; B and D: FISH images of PAOs on day 1 and day 120; E: percentages of PAOs, DPAOs, and GAOs in total biomass during 120-day operational period.](image-url)
period (Figs. 3C and 4E, and Table 2), indicating that PAOs played an important part in intracellular carbon storage. Therefore, the population variations of PAOs and GAOs elucidated the changes of nutrient removal performance of SNDPR-SBR from Phase 1 to Phase 4 (Fig. 3).

### 3.4. Nutrient removal mechanism of the SNDPR-SBR system in a typical operation cycle

The variations of nitrogen, phosphorus, extracellular and intracellular carbon sources in a typical operation cycle (6 h) were analyzed on Day 120 (Phase 4, with enhanced SND performance) to investigate the nutrient removal mechanism of the SNDPR-SBR system. The anaerobic initial \(\text{NH}_4^+ - \text{N}\), \(\text{NO}_2^- - \text{N}\), \(\text{NO}_3^- - \text{N}\), \(\text{PO}_4^{3-} - \text{P}\), and COD concentrations were 24.0, 1.2, 6.4, 2.4, and 140.7 mg L\(^{-1}\), respectively (Fig. 5).

In the anaerobic stage (3 h), COD decreased to 48.2 mg L\(^{-1}\), followed by PHAs and \(\text{PO}_4^{3-} - \text{P}\) increasing to 11.6 mmolC L\(^{-1}\) and 25.6 mg L\(^{-1}\), respectively, and Gly decreasing to 8.3 mmolC L\(^{-1}\). The changes of COD, \(\text{PO}_4^{3-} - \text{P}\), PHAs, and Gly mainly happened in the first 80 min, while nitrogen almost unchanged throughout the anaerobic stage. After 80 min, COD and \(\text{PO}_4^{3-} - \text{P}\) changed slightly, but the formation of PHAs and the decomposition of Gly continued, indicating that both PAOs and GAOs participated in the composition of intracellular carbon, and the influent COD was first consumed (the first 80 min) and then absorbed and translated (the following 100 min) into internal carbon (mainly PHB, accounted for 85.7% of PHAs, Fig. 5). Thus, the extended anaerobic stage of 180 min was beneficial for the utilization of external carbon.

In the first hour of aerobic stage, \(\text{PO}_4^{3-} - \text{P}\) showed a rapid decline (decreased by 22.3 mg L\(^{-1}\)) accompanied by the degradation of PHAs and the consumption of Gly, indicating the occurrence of the aerobic phosphorus removal with/without denitrifying phosphorus removal. Simultaneously, \(\text{NH}_4^+ - \text{N}\) oxidation happened with little \(\text{NO}_2^- - \text{N}\) and \(\text{NO}_3^- - \text{N}\) being produced (Fig. 5) and led to a nitrogen loss of 4.1 mg L\(^{-1}\), indicating the occurrence of denitrifying phosphorus uptake or/endogenous denitrification driven by PHAs, since almost no COD was left for OHOs to conduct exogenous denitrification. In the following 90 min, SND happened, and \(\text{NH}_4^+ - \text{N}\) decreased by 12.8 mg L\(^{-1}\), \(\text{NO}_2^- - \text{N}\) and \(\text{NO}_3^- - \text{N}\) increased by 2.3 and 3.9 mg L\(^{-1}\), with a nitrogen loss of 6.4 mg L\(^{-1}\). \(\text{PO}_4^{3-} - \text{P}\) remained the same, but PHB decreased by 1.9 mmolC L\(^{-1}\) with Gly increasing by 1.5 mmolC L\(^{-1}\), revealing that the denitrification in SND was caused by denitrifying GAOs through utilizing PHB. SND contributed to a total aerobic nitrogen loss of 10.5 mg L\(^{-1}\) (the sum of 4.1 and 6.4) at the aerobic stage and reduced the effluent \(\text{NO}_3^- - \text{N}\) concentration to 10 mg L\(^{-1}\), thus ensuring the effective nitrogen removal performance of SNDPR-SBR system.

#### 3.5. Activities of PAOs, DPAOs, and GAOs in the SNDPR-SBR system

The intracellular carbon transformation stoichiometry at the anaerobic stage and the aerobic stage were calculated for the activities of PAOs, DPAOs, and GAOs in the SNDPR-SBR system (Table 3). At the anaerobic stage, \(\text{COD}_{\text{ana}}\) was 20.4 mg L\(^{-1}\) (12.2 mg L\(^{-1}\) + 1.71 x 6.4 mg L\(^{-1}\)) x 2.86, according to Eq. (3)), \(\text{COD}_{\text{intra}}\) was 72.1 mg L\(^{-1}\) (140.7 - 48.9 - 20.4 mg L\(^{-1}\), according to Eq. (2)); \(\text{PRA/} \text{COD}_{\text{intra}} (0.2 \text{ molP/molC})\) was lower than the reported PAO model value (0.5 molP/molC, Smolders et al., 1994a), indicating that a part of \(\text{COD}_{\text{intra}}\) (about 40%) was used for the PHAs storage of PAOs. \(\Delta \text{Gly/} \text{COD}_{\text{intra}} (0.85 \text{ molC/molC})\) was higher than the reported PAO model value (0.5 molC/molC, Smolders et al., 1994a) but lower than the reported GAO model value (1.12 molC/molC, Zeng et al., 2003b), indicating that both PAOs and GAOs contributed to the PHAs storage, and PAOs accounted for 43.5% (assuming that PAOs account for x, then GAOs were (1-x), and 0.5x + 1.12(1-x) = 0.85, so x was 0.435). The percentage of PAOs activity calculated from \(\text{PRA/} \text{COD}_{\text{intra}} (40%)\) coincided with the value calculated from \(\Delta \text{Gly/} \text{COD}_{\text{intra}} (43.5%)\),

<table>
<thead>
<tr>
<th>Phase</th>
<th>Influent (mg L(^{-1}))</th>
<th>Effluents (mg L(^{-1}))</th>
<th>Removal efficiency (%)</th>
<th>PRA (mgP L(^{-1}))</th>
<th>SND (%)</th>
<th>COD(<em>{\text{intra}})/COD(</em>{\text{consum}}) (mgP gCOD(_{\text{intra}}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>233.0</td>
<td>0.9</td>
<td>19.6</td>
<td>3.8</td>
<td>80.5</td>
<td>65.2 38.5</td>
</tr>
<tr>
<td>2</td>
<td>344.8</td>
<td>2.8</td>
<td>15.3</td>
<td>1.6</td>
<td>85.3</td>
<td>66.6 74.5</td>
</tr>
<tr>
<td>3</td>
<td>350.0</td>
<td>2.4</td>
<td>11.1</td>
<td>0.3</td>
<td>86.2</td>
<td>70.1 95.2</td>
</tr>
<tr>
<td>4</td>
<td>254.7</td>
<td>2.3</td>
<td>10.4</td>
<td>0.4</td>
<td>81.0</td>
<td>77.7 94.0</td>
</tr>
</tbody>
</table>

**Fig. 5** – Variations of nitrogen, phosphorous and organic carbon concentrations in a typical cycle of Phase 4 (Day 120).
which confirmed that the contribution of PAOs (40%) in the intercellular carbon storage was less than that of GAOs (60%).

In the first hour of aerobic stage, PUA/▵PHAs (0.35 molP/molC) was lower than the PAO model value (0.44 molP/molC, Coma et al., 2012; 0.41 molP/molC, Smolders et al., 1994b), but much higher than the DPAO model value (0.16 ~ 0.24 molP/molC, Guisasola et al., 2009), proving that phosphorus was mostly removed by aerobic PAOs instead of DPAOs. Additionally, △Gly/▵PHAs (0.70 molC/molC) was higher than the PAO model value (0.42 molC/molC, Smolders et al., 1994b) and the DPAOs model value (about 0.2 molC/molC, Guisasola et al., 2009), proving that both DPAOs and DGAOs participated in endogenous denitrification.

In the following 90 min of aerobic stage, PUA was almost zero (Fig. 5), thus PHAs were mostly consumed for endogenous denitrification driven by DGAOs. 0.24 molN/molC of △TIN/▵PHAs and 0.33 molN/molC of △ATIN/▵Gly were obtained for the stoichiometry of endogenous denitrification process in the SNDPR-SBR system, which elucidated the contributor of the nitrogen (4.1 mg L⁻¹) in the first hour of aerobic stage. Assuming that all 4.1 mg L⁻¹ of nitrogen loss (▵ATIN) was denitrified by DGAOs, the corresponding PHAs consumed should be about 1.22 mmolC L⁻¹ (4.1 mg L⁻¹ ÷ 14 mg mmol⁻¹ ÷ 0.24 molN/molC), and the PHAs consumed for phosphorus uptake should be 1.64 mmolC L⁻¹ (2.86 mmolC L⁻¹ ÷ 1.22 mmolC L⁻¹). Thus, the corresponding PUA should be 20.84 mg L⁻¹ (1.64 mmolC L⁻¹ ÷ 0.41 molP/molC ÷ 31 g mol⁻¹), which was lower than the actual value of 22.32 mg L⁻¹ (Fig. 5). Therefore, 4.1 mg L⁻¹ of nitrogen loss was removed by both DGAOs and DPAOs. However, the percentage of DPAOs activity needs further investigation, since the model of DGAOs should be explored and the electron acceptor (nitrite or nitrate) used for DPAOs and DGAOs should be determined.

The P removal in the SNDPR-SBR system was accomplished by P uptake of both aerobic PAOs and DPAOs, with aerobic PAOs playing the major role. The nitrogen removal was accomplished by SND, in which both DPAOs and DGAOs participated in denitrification, with DGAOs playing the major role. This finding was different from previous studies that DGAOs rather than DPAOs were responsible for denitrification activity (Zeng et al., 2003a), and that only certain types of GAOs were detected in denitrification (Coma et al., 2012). The difference of PAOs and GAOs activities may lie in the different microbial community structures (38% ± 2% of PAOs, 17% ± 1% of DPAOs, and 17% ± 3% of GAOs in this study; while 14.7% of PAOs and 8.6% of GAOs in Coma et al., 2012; not mentioned in Zeng et al., 2003a).

### 3.6 Feasibility and reliability of using an SNDPR-SBR to treat low C/N wastewater

The removal efficiency of nutrient and organic carbon in the SNDPR-SBR system was compared with other systems to demonstrate its superiority (Table 4). The P removal performance of the SNDPR-SBR was higher than SNDPRs inoculated with aerobic granule (Wang et al., 2009a; Bassin et al., 2012) or biofilms (Rahimi et al., 2011; Guadie et al., 2013), and flocculent SNDPR systems (Rahimi et al., 2011; Coma et al., 2012). The extended anaerobic stage and highly enriched PAOs might be the major reason for the high performance of SNDPR-SBR treating wastewater with low C/N ratio. Because the DO concentrations of other systems were all operated at a low level (Table 4) to achieve SND (von Münch et al., 1996; Bertanza et al., 1997; Pochana et al., 1999), but the extended anaerobic stage (3 h) used in this study was much longer than others (60 min, Zeng et al., 2003a; Lo et al., 2010; 90 min, Wang et al., 2009a, Table 4), which was helpful for the anaerobic P release and intercellular carbon storage of PAOs, and ensured the aerobic P uptake. Enriched PAOs could improve the P removal performance, and short SRT (10.9 d) in this study ensured the P removal by high amount of wasted sludge (Zeng et al., 2003b).

The SND efficiency of the SNDPR-SBR (49.3%) was lower than aerobic granule sludge SNDPRs (68%, Wang et al., 2009a) and hybrid sludge SNDPRs (75.9%, Lo et al., 2010), similar with biofilms SNDPRs (41.0%, Lo et al., 2010), but higher than fix-bed and flocculent sludge SNDPRs (31.2%, Rahimi et al., 2011; 16.7%, Lo et al., 2010; 2.5%, Rahimi et al., 2011). Thereby, adding granule sludge enhanced the performance of aerobic SND (Wang et al., 2009a; Lo et al., 2010) by providing anoxic zone and aerobic zone (de Kreuk et al., 2005), but the flocculent SNDPR in this study also achieved a high SND efficiency under extended anaerobic and low oxygen aerobic conditions. Additionally, the TN removal efficiency of the SNDPR-SBR (77.7%) was much higher than similar flocculent SNDPRs (26.7%, Lo et al., 2010; 75%, Coma et al., 2012), and granule sludge or biofilms (such as 52% in aerobic granule sludge SBR, Wang et al., 2009a; 49.5% in biofilm SBR, Lo et al., 2010; 71.5% in hybrid sludge SBR, Lo et al., 2010; 70% in fix-bed SBR, Rahimi et al., 2011). This comparison proved that the SNDPR-SBR system could achieve a high TN removal efficiency even the SND efficiency was lower than granular/biofilm SNDPRs. The possible reason might be the extended anaerobic stage and the highly enriched GAOs. Extended anaerobic stage provided sufficient carbon for DGAOs by strengthening influent carbon utilization, and thus alleviating the DO consumption for COD oxidation at the low oxygen stage, which resulted in efficient nitrification, denitrification and P absorption. Moreover, the aerobic duration and the DO concentration needed in the SNDPR-SBR system were about 50% ~ 70% lower than other systems (aerobic granule SBR, Wang et al., 2009a; biofilms and hybrid SBR, Lo et al., 2010; fix-bed SBR, Rahimi et al., 2011; aerobic granule SBR, Bassin et al., 2012) (Table 4). Therefore, substantial energy (about 65%) was saved during the short low aerobic stage in the SNDPR-SBR system.
EBPR coupling SND in an extended anaerobic and short low aerobic system is an efficient process for enhanced C, N, and P removal from low C/N wastewater. Extended anaerobic stage in the SNDPR-SBR system was confirmed to play an important role in nutrient removal. However, further studies are still needed to optimize the SNDPR-SBR system, such as the effect of DO concentration or aerobic duration on nutrient removal efficiency, the proportion of DPAOs and DGAOs, and the preferable electron acceptors for DPAOs and DGAOs.

4. Conclusions

A novel SNDPR-SBR system enriched with PAOs, DPAOs, and GAOs at the ratio of 2:1:1 was developed and successfully operated to treat wastewater with low C/N ratio (≤3.5). For the first time, extended anaerobic stage was used to enhance the utilization of influent carbon, and combined with short low oxygen aerobic stage to achieve a simultaneous C, N, and P removal without external carbon addition. About 65% energy was saved for aeration. Four major conclusions were drawn as follows:

- Extended anaerobic stage (3 h) achieved a sufficient storage of intracellular carbon (mainly PHB) in PAOs and GAOs (40% in PAOs and 60% in GAOs), and provided sufficient carbon sources for the SND and P uptake in the following short low aerobic stage.
- Nitrification, endogenous denitrification, aerobic and denitrifying phosphorus uptake were achieved at short aerobic stage (2.5 h). Endogenous denitrification driven by DGAOs and denitrifying phosphorus uptake driven by DPAOs improved the TN removal efficiency (77.7%) and SND efficiency (49.3%), and minimized the NOX impact on the subsequent anaerobic phosphorus release process.
- Short SRT (10.9 d) ensured the P removal by sludge wasting, and the effluent PO43−−P concentration was below 0.5 mg L−1.
- PAOs and GAOs accounted for 38% ± 2% and 17% ± 3% of total biomass, and DPAOs accounted for 45.9% of PAOs. High amounts of DPAOs and GAOs facilitated SND and P absorption at the short low aerobic stage.

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References


