# Feasibility of Partial Nitrification Combined with Nitrite-Denitrification Phosphorus Removal and Simultaneous Nitrification−Endogenous Denitrification for Synchronous Chemical Oxygen Demand, Nitrogen, and Phosphorus Removal

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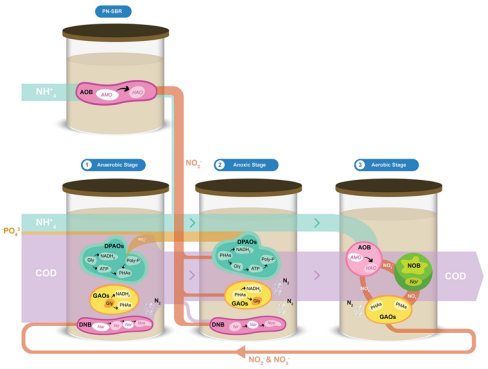


**ABSTRACT:** A combination of partial nitrification and nitrite-denitrifying phosphorus removal and simultaneous nitrification-endogenous denitrification (nDNPR-SNED) in two sequencing batch reactors was developed for synchronous chemical oxygen demand (COD), nitrogen (N), and phosphorus (P) removal by regulating dissolved oxygen (DO) and influent nitrite concentrations. COD, total nitrogen, and P removal efficiencies of 87.4 ± 0.5, 91.6 ± 1.1, and 97.8 ± 0.6%were obtained after 112 days of anaerobic/anoxic/aerobic operation. Mass balance analysis confirmed that 91.9% of the COD was stored as intracellular carbon at the anaerobic stage, and 99.6% of $PO_4^{3-}$−P and 99.8% of $NO_2^-$−N were eliminated via the nDNPR process at the anoxic stage, and at the aerobic stage, the SNED process contributed to 68.7% nitrogen removal. Genera of *Candidatus Competibacter*, *Dechloromonas*, *Ellin6067*, and *Nitrospirae* were the dominant consortia with a relative abundance of 26.5, 16.5, 1.0, and 1.1%, respectively. In the metabolic pathway model, *β*-hydroxybutyrate was the main endogenous drivingorce for nitrogen and phosphorus removal. Compared with conventional biological nitrogen and phosphorus removal processes, the combined process could achieve 6.7% saving in the total cost. The proposed approach provides an economic and technical alternative for C-, N-, and P-laden wastewater treatment, reducing both carbon demand and aeration consumption.

KEYWORDS: C/N ratio, nitrite denitrifying phosphorus removal, partial nitrification, phosphorus-accumulating organisms, simultaneous nitrification-endogenous denitrification

## 1. INTRODUCTION

Efficient nitrogen (N) and phosphorus (P) removal has been regarded as a crucial issue with the severe increase in the eutrophication phenomenon.[1] The partial nitrification (PN) process of converting ammonium ($NH_4^+$−N) to nitrite ($NO_2^-$−N) was considered a promising way in biotechnological nitrogen removal.[2] Particularly, PN-based systems, including partial nitrification−denitrification and partial nitrification−anaerobic ammonium oxidation, showed high potential in treating ammonia-containing wastewater such as landfill leachate,[3] sludge digestion liquid, and abattoir.[4] Notably, due to the limited carbon source in these wastewaters, N removal frequently takes precedence, resulting in an inadequate P removal performance.

The denitrifying phosphorus removal (DNPR) process has been considered an attractive alternative method for simultaneous P and N removal under anoxic conditions using $NO_x^-$−N ($NO_3^-$−N and $NO_2^-$−N) as electron acceptors.[5] It has been confirmed that certain species of phosphate-accumulating organisms (PAOs) hold complete denitrification genes.[6] In the DNPR process, polyhydroxyalkanoates (PHAs) have been shown to provide a carbon source for both N removal and P uptake via denitrifying PAOs (DPAOs). Compared to conventional nitrogen and phosphorus removal processes, DPAOs can reduce carbon consumption by 50% and aeration consumption by 30%.[7] Recently, in P removal systems, glycogen-accumulating organisms (GAOs) have been found to coexist with DPAOs, which reduce the demand for the carbon source needed for simultaneous nitrogen and phosphorus removal.[8] Studies found that GAOs had the ability of denitrification since they could reduce $NO_3^-$−N or $NO_2^-$−N in wastewater.[9] Under

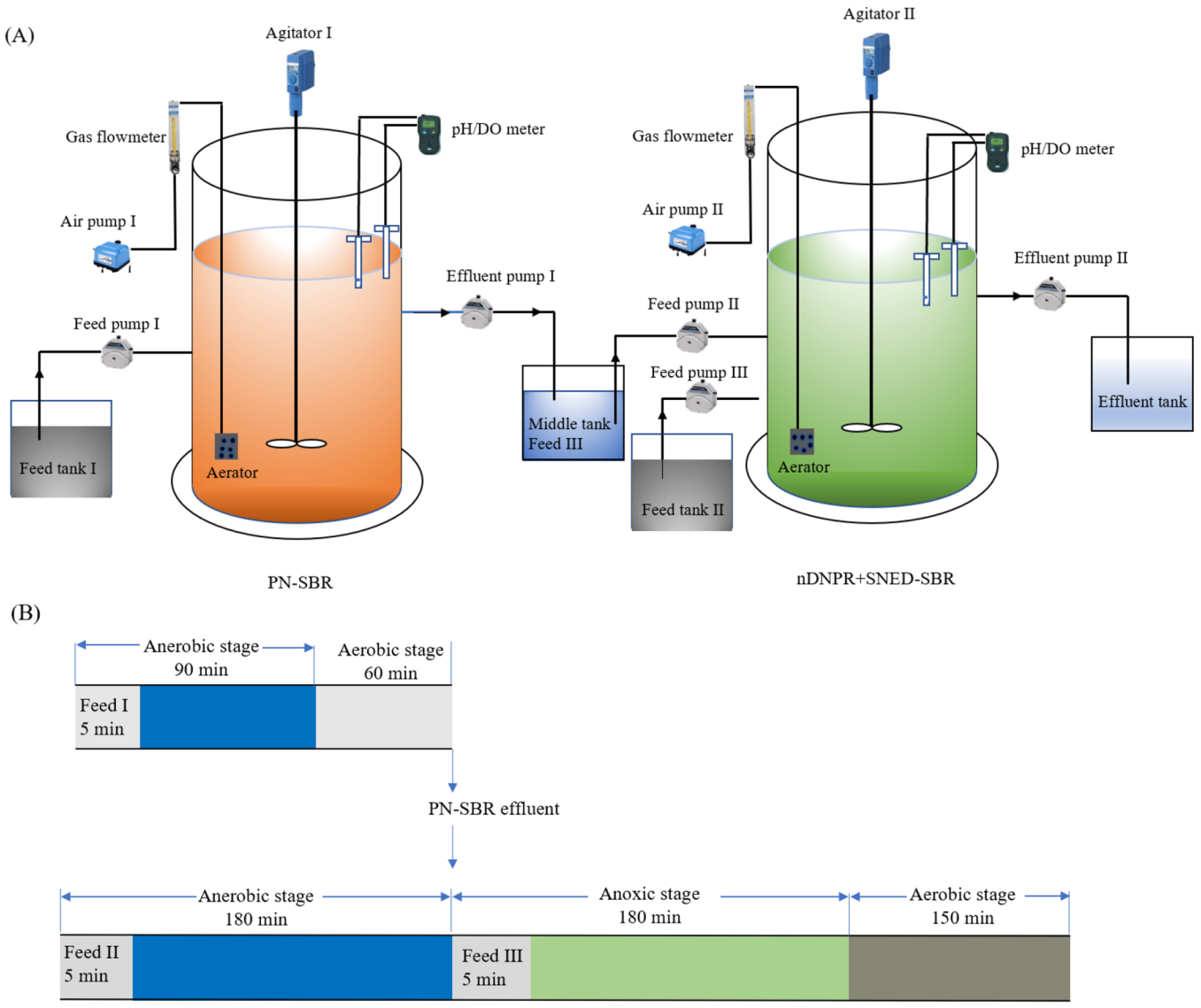


**Figure 1.** Schematic of the PN-SBR and nDNPR-SNED reactor (A) and the operation procedure of the combined system (B).

anaerobic conditions, GAOs could store biodegradable organics as PHAs, while under aerobic conditions, PHAs were used as energy sources for the simultaneous nitrification and endogenous (partial) denitrification (SNED) process.[10] In the SNED process, ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) can oxidize ammonia to $NO_2^-$–N or $NO_3^-$–N through a (partial) nitrification process, and at the same time, in the presence of GAOs, $NO_2^-$–N or $NO_3^-$–N could be reduced to nitrogen gas ($N_2$). Unlike the traditional simultaneous nitrification and denitrification (SND) process, the existence of GAOs instead of denitrifying bacteria (DNB) may avoid the addition of an external carbon source. Ji et al. revealed that GAOs were the functional bacteria in the endogenous partial denitrification (EPD) process of municipal sewage (C/N = 1.7) treatment, leading to a total nitrogen (TN) removal efficiency of 91.2%.[11] Thus, combining DNPR and SNED processes could be a step in the right direction toward maximizing the simultaneous removal of N, P, and chemical oxygen demand (COD).

In this work, nitrite is proposed as an electron acceptor in the DNPR-coupled SNED system, and the resulting system is dubbed nitrite-denitrifying phosphorus removal (nDNPR) combined with SNED. In the proposed nDNPR-SNED system, DPAOs can conduct $PO_4^{3-}$–P uptake using $NO_2^-$–N as an electron acceptor through the nDNPR process. Compared to the nitrate denitrifying phosphorus removal process, nDNPR could save 50% carbon consumption and reduce sludge production.[12] Moreover, advanced TN removal could be achieved in the following SNED process as the endogenous denitrification and (partial) nitrification proceeded concurrently at low dissolved oxygen (DO) concentrations (0.3–0.5 mg/L). Therefore, the nDNPR-SNED process would be more attractive than traditional biological wastewater treatment in saving carbon and reducing aeration. The nitrite needed for the nDNPR process could be provided through the PN of ammonia-rich wastewater.[13]

Therefore, the novelty of this study is underlying the detailed mechanism of the integrated PN and nDNPR-SNED system for synchronous COD, N, and P removal from municipal wastewater. The metabolic pathways and nutrient mass balance were investigated. Furthermore, the key functional bacteria involved in the biodegradation of municipal wastewater in the integrated system were identified. Finally, an economic analysis was performed to assess the feasibility of

Table 1. Operation Conditions of the PN System over the Course of the Experiment

| items | phase 1 | phase 2 | phase 3 | phase 4 |
|---|---|---|---|---|
| operational mode | A/O | A/O | A/O | A/O |
| $NH4^-$−N influent (mg/L) | 106.5 ± 2.4 | 106.8 ± 2.4 | 205.0 ± 3.6 | 200.7 ± 0.7 |
| aerobic duration (min) | 90 | 90 | 900 | 60 |
| influent volume (L) | 3.0 | 3.0 | 3.0 | 3.0 |
| SRT (d) | 10.0 | 10.0 | 10.0 | 10.0 |
| DO (mg/L) | 2.5−4.0 | 0.5−1.0 | 0.5−1.0 | 0.5−1.0 |
| HRT (h) | 8.0 | 8.0 | 8.0 | 6.6 |

Table 2. Operation Conditions of nDNPR-SNED in Different Phases

| items | acclimation phase 1 (1−28 d) | enhancement of nDNPR phase 2 (29−66 d) | achievement of nDNPR-SNED phase 3 (67−81d) | combination of PN and nDNPR-SNED phase 4 (82−112 d) |
|---|---|---|---|---|
| operational mode | A/A/O | A/A/O | A/A/O | A/A/O |
| anoxic $NO_2^-$−N influent (mg/L) | 51.3 ± 0.7 | 49.7 ± 0.4 | 51.3 ± 0.4 | 73.9 ± 0.7 |
| drainage ratio (L/L) | 3/8 | 5/10 | 5/10 | 5/10 |
| DO (mg/L) | 1.0−1.5 | 0.3−0.5 | 0.3−0.5 | 0.3−0.5 |
| aeration rate (L/min) | 1.0 | 0.4 | 0.4 | 0.4 |

nDNPR-SNED combined with PN for COD, N, and P removal.

## 2. MATERIALS AND METHODS

**2.1. Operation of the Reactors.** *2.1.1. PN Reactor.* The PN system was conducted in a lab-scale sequencing batch reactor (PN-SBR) with a working volume of 8 L (Figure 1A) with an operation period divided into four phases. The operation mode of the PN-SBR was anaerobic/aerobic (A/O) (Figure 1B). The PN-SBR was optimized under different nitrogen loading rates (NLRs) by increasing the influent ammonia concentration and reducing the hydraulic retention time (HRT) (Table 1). The anaerobic duration of 90 min was maintained constant, while the aeration time was reduced from 120 min in phases 1, 2, and 3 to 60 min in phase 4. The PN reactor was used with a constant-temperature water bath to control its operation conditions at room temperature (23 °C ± 2 °C), and the DO concentration was controlled using a volumetric flowmeter. The sludge retention time (SRT) was maintained at 10 d during the whole operational period.

The inoculum of the PN-SBR was harvested from a secondary sedimentation tank of a wastewater treatment plant (WWTP) in Shandong University, Qingdao, China. After inoculation, the mixed liquor suspended solids (MLSSs) of the system were kept at 3.5 ± 0.3 g/L.

*2.1.2. nDNPR-SNED Reactor.* The nDNPR-SNED system was performed in a lab-scale SBR with a working volume of 11 L (Figure 1A). The reactor was operated under room temperature (23 ± 2 °C) regulated by a constant-temperature water bath. The experimental operation lasted for 112 days and was divided into four phases according to the operational mode (Table 2). The reactor mode in one cycle was anaerobic/anoxic/aerobic (A/A/O) (Figure 1B): 3 L of municipal wastewater was added during the first 5 min of the anaerobic stage (180 min). Then, in the anoxic stage (180 min), 2 L of the partially nitrified effluent was pumped into the SBR within 5 min. The following aerobic stage (150 min) was performed at low DO concentrations, and the settling/decanting time was 10 min.

Inoculation sludge in the nDNPR-SNED system was collected from a simultaneous nitrification, denitrification, and phosphorus removal (SNDPR) system in a lab-scale reactor with a stable phosphorus and nitrogen removal performance. The concentration of MLSSs in the nDNPR-SNED reactor was kept at 2.8 ± 2.0 g/L by discharging 50 mL of the sludge at the end of each cycle.

**2.2. Wastewater.** Anaerobic digestion liquor was fed into the PN system converting ammonium to nitrite, which was required for nDNPR. The anaerobic digestion liquor was diluted to adjust the influent $NH_4^+$−N concentration to the values mentioned in Table 1. The main characteristics of the anaerobic digestion liquor before diluted were as follows: SS, 2000.0 mg/L; $NH_4^+$−N, 500.0−800.0 mg/L; and $PO_4^{3-}$−P, 55.2−80.9 mg/L.

The municipal wastewater fed in the nDNPR-SNED system was collected from the primary sedimentation tank effluent of a WWTP in Shandong University, Qingdao, China. The main characteristics of the influent substrate were as follows: COD, 195.3−307.2 mg/L; $NH_4^+$−N, 38.6−79.2 mg/L; $NO_2^-$−N <1.0 mg/L; $NO_3^-$−N, <1.0 mg/L; $PO_4^{3-}$−P, 3.3−8.4 mg/L; and TN, 39.2−79.4 mg/L.

**2.3. Analytical Approaches.** The liquid samples harvested from both systems were filtered through 0.45 μm filters before analysis. $NH_4^+$−N, $NO_2^-$−N, $NO_3^-$−N, $PO_4^{3-}$−P, glycogen (Gly), and MLSS were analyzed according to the standard methods.[14] The influent and effluent COD was measured by a COD speedy testing instrument (Massinno, MI-80K, China). β-Hydroxybutyrate (PHB) and poly (β-hydroxyvalerate) (PHV) concentrations were determined using a gas chromatograph (GC 2014, Shimadzu, Japan), and PHAs were the total amount of PHV and PHB. Detail analysis methods for PHV, PHB, and Gly are given in Text S1 and S2. The pH and DO concentration were measured using a portable pH-DO detector (Hach, HQ30d, USA). All samples were analyzed in triplicate.

**2.4. Microbial Analysis.** The sludge samples were collected from the two reactors: the PN-SBR (on days 1 and 70) and nDNPR-SNED system (on days 1, 81, and 112) for detecting the microbial community structures. Bacterial primers 338F and 806R were used to amplify the 16S rRNA gene in the V3−V4 region. The abundance of functional organisms was measured using an Illumina MiSeq sequencing PE250 platform at Shanghai Personal Biotechnology Co., Ltd (Shanghai, China). The extraction and amplification methods

were carried out in accordance with the manufacturer's instructions before submitting the extracted genetic materials to be sequenced. The UCLUST software program (v5.2.236) was employed to classify the optimized sequence, and the 97% identity threshold was used to divide the optimized sequence into operational taxonomic units. LEfSe analysis was used to detect biomarkers in each sludge sample,[15] and the threshold of the linear discriminant analysis (LDA) score was 4.5.

**2.5. Calculations.** *2.5.1. nDNPR Efficiency.* The nDNPR efficiency is the percentage of the nitrite denitrifying phosphorus uptake amount at the anoxic stage to the total phosphorus absorption, calculated according to eq 1.

$$\text{nDNPR efficiency} = \frac{{PO_{4An,e}}^{3-} \times (V_W - V_{An}) - {PO_{4A,e}}^{3-} \times V_W}{{PO_{4An,e}}^{3-} \times (V_W - V_{An}) - {PO_{4O,e}}^{3-} \times V_W} \times 100\% \quad (1)$$

where $PO_{4An,e}^{3-}$, $PO_{4A,e}^{3-}$, and $PO_{4O,e}^{3-}$ are the ${PO_4}^{3-}$−P concentrations at the end of the anaerobic stage, the anoxic stage, and the aerobic stage, respectively, mg/L; $V_W$ is the working volume of the reactor, L; and $V_{An}$ is the anoxic influent volume, L.

*2.5.2. $COD_{ins}$ Efficiency.* In this study, anaerobic organic carbon consumption ($COD_{AC}$) is the COD consumed during the anaerobic stage. $COD_{dn}$ is the COD utilized by the traditional denitrification process reducing ${NO_2}^-$−N or ${NO_3}^-$−N to $N_2$, and $COD_{ins}$ is the COD that was consumed by DPAOs and GAOs.

$$COD_{dn} = 2.86\Delta{NO_3}^- - N + 1.71\Delta{NO_2}^- - N \quad (2)$$

$$COD_{ins}\% = \frac{COD_{AC} - COD_{dn}}{COD_{AC}} \times 100\% \quad (3)$$

where 1.71 and 2.86 are the theoretical values of COD consumed in the denitrification process per unit ${NO_2}^-$−N and ${NO_3}^-$−N, respectively, mg COD/mg N[16] and $\Delta{NO_2}^-$−N and $\Delta{NO_3}^-$−N are the variation in the concentrations of ${NO_2}^-$−N and ${NO_3}^-$−N during the anaerobic operation, respectively, mg/L.

*2.5.3. Economic Analysis.* The net present value (NPV) and total cost (TC) were explored to determine the potential application of the combined system. The calculation methods are as follows

$$TC = CAPEX + \sum_n \frac{PC}{(1+i)^n} \quad (4)$$

$$NPV = -CAPEX + \sum_n \frac{BE - PC}{(1+i)^n} \quad (5)$$

where PC represents the periodic cost, BE refers to the benefits value, and $i$ and $n$ are the discount rate (8%) and life span of the reactor in years, respectively.[17]

## 3. RESULTS AND DISCUSSION

**3.1. Nitrogen Removal Performance and Functional Microbial Structure Variation in the PN-SBR.** The PN-SBR provided nitrite to verify the achievement of nitrite consumed by PAOs and GAOs in the nDNPR-SNED system. The inoculated sludge was pretreated by aeration for 24 h to shorten the acclimation time. The nitrogen removal performance of PN is shown in Figure S1. In phase 1 (days 1−8), the influent ${NH_4}^+$−N concentration was 106.5 ± 2.4 mg/L, and the DO concentration at the aerobic stage was maintained at 2.5−4.0 mg/L. The ${NH_4}^+$−N removal efficiency increased to 81.1%, while the nitrite accumulation rate (NAR) was lower than 5.5%, indicating that most ${NH_4}^+$−N was converted to ${NO_3}^-$−N. In this phase, the inoculated sludge mainly performed the nitrification function, and the existence of NOB led to the presence of ${NO_3}^-$−N in the effluent. In order to suppress NOB activity, the DO concentration at the aerobic stage was reduced to 0.5−1.0 mg/L (phase 2; days 9−23) since it was reported that AOB had a higher affinity with low DO than NOB.[18] The ${NH_4}^+$−N removal efficiency decreased to 71.1%, while the NAR was augmented to 84.2%, corresponding to an increase in ${NO_2}^-$−N concentration from 9.7 to 28.5 mg/L. The increase in concentration of ${NO_2}^-$−N in the effluent was mainly due to PN. In other words, AOB played the dominant role in this phase because the activity of NOB was more depressed than that of AOB under a low DO concentration. Similar findings were reported by Ruiz et al. at DO concentrations ranging from 0.7 to 1.4 mg/L.[19] In phase 3 (days 24−48), the influent ${NH_4}^+$−N concentration was increased to 205.0 ± 3.6 mg/L. The results revealed an increase in the ${NH_4}^+$−N removal efficiency to 96.5%; however, the NAR significantly decreased to 55.2%, accompanied by an increase in effluent ${NO_3}^-$−N concentration to 34.3 mg/L. Accordingly, pH and DO concentration for a typical operation cycle on day 48 (phase 3) were monitored every 5 min (Figure S2). In the first 60 min of the aerobic stage, the DO concentration was still low (less than 0.03 mg/L), which was mainly led by the nitrification of ${NH_4}^+$−N. Meanwhile, the decrease in pH also explained the possible occurrence of ${NH_4}^+$−N oxidation. However, in the last 30 min of the aerobic stage, pH and DO concentration increased up to 7.49 and 1.1 mg/L, respectively, which reflected that nitrification mainly happened in the first 60 min of aeration. Moreover, with continuous aeration, the increase in DO concentration could promote NOB survival which converted nitrite to nitrate.[20] To avoid nitrite oxidation, the aerobic operation time was optimized from 90 to 60 min in phase 4 (days 49−70). The ${NH_4}^+$−N removal efficiency and NAR in phase 4 significantly reached 93.9 and 99.1%, respectively, which indicated the successful operation of the PN process. Besides, concentrations of free ammonia (FA) under different NLRs were evaluated (eq 6) to determine the effect of FA on the PN process.[21] Temperature ($T$) and pH were measured during the operation, and the FA concentration was 0.9 and 1.2 mg/L on days 23 and 40, respectively. It has been proven that FA can inhibit the activity of AOB at the concentration of 10−15 mg/L and that of NOB at the concentration of 0.1−1.0 mg/L.[21] In this study, NOB could be more sensitive to FA which could explain the high accumulation of ${NO_2}^-$−N in phase 4.

$$C_{FA} = \frac{C_{NH_4^+} \times 10^{pH}}{\exp^{(6344/273+T)} + 10^{pH}} \quad (6)$$

In addition, the comparison of the microbial community structure at the phylum and genus levels on days 1 and 70 is shown in Figure 2A,B. The phylum *Proteobacteria* had the highest abundance among all bacteria in both samples, which was reported as the dominant bacteria in PN systems.[22] The relative abundance of *Proteobacteria* increased from 56.2% on day 1 to 66.8% on day 70, consistent with the increase in the

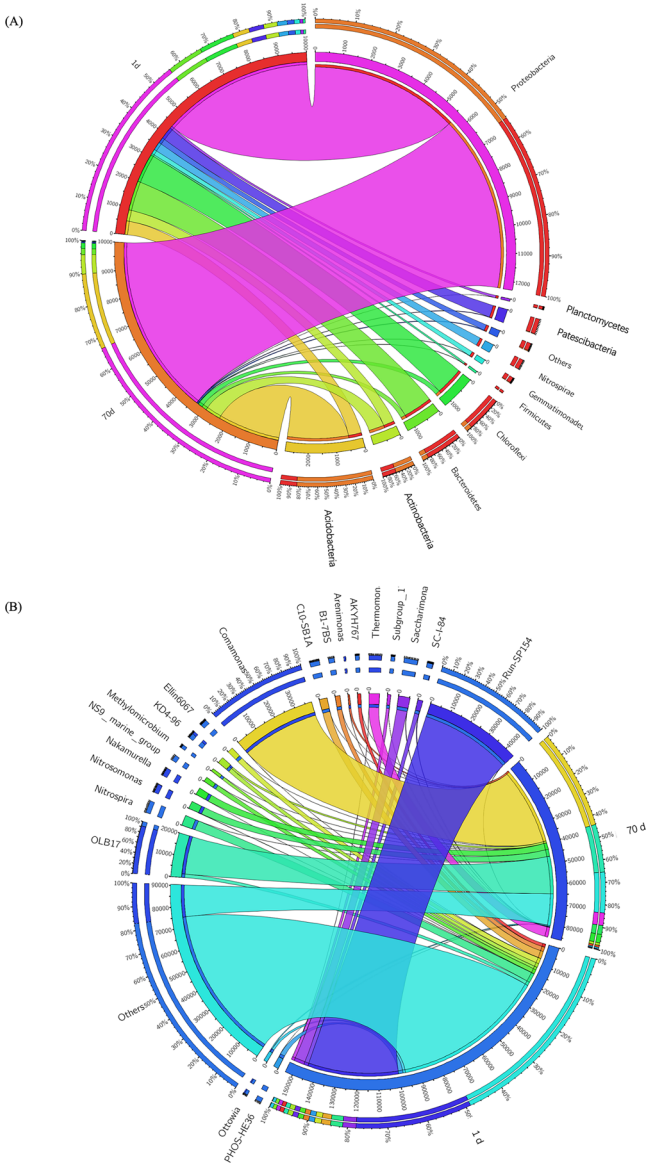


**Figure 2.** Microbial community structure at the phylum level of the top 10 (A) and at the genus level of the top 20 (B) before and after startup.

NAR on day 70. However, *Nitrospirae* (NOB) decreased from 3.0 to 0.0%, which explained the absence of $NO_3^-$–N in the effluent (Figure S1). In addition, at the genus level, the abundance of *Nitrosomonas* (AOB) increased from 0.07 to 3.95%, which further confirmed that the PN process was completed.

**3.2. Nutrient Removal in the nDNPR-SNED System.** Variations in COD, N, and P during different phases in the nDNPR-SNED system are shown in Figures 3 and S3. In phase 1 (days 1–28), the influent COD, TN, and $PO_4^{3-}$–P concentrations of 269 ± 6.1, 64.4 ± 2.7, and 6.4 ± 2.3 mg/L, respectively, were fed to the system in the anaerobic stage. COD, TN, and $PO_4^{3-}$–P removal efficiencies in this stage amounted to 85.4 ± 7.1, 76.7 ± 6.9, and 94.4 ± 2.3%, respectively. $COD_{ins}$ efficiency in the anaerobic stage was 65.4 ± 7.2% and that of $COD_{dn}$ was 22.72 ± 5.9 mg/L, higher than that of other phases, indicating that heterotrophic DNB are also consuming COD. The results were mainly caused by the considerable $NO_3^-$N that remained from the last cycle effluent. At the anoxic stage, the initial anoxic influent of $NO_2^-$–N was 51.3 ± 0.7 mg/L with a $PO_4^{3-}$–P–P to $NO_2^-$–N ratio ($PO_4^{3-}$–P/$NO_2^-$–N) of 1.68, which was reported to be the desirable ratio to enhance DPAOs.[23] As a result, the phosphorus uptake amount (PUA) at the anoxic stage driven by DPAOs gradually increased from 0.3 ± 0.1 mg/L on day 1 to 13.4 ± 0.3 mg/L on day 28. Most phosphorus was removed during the anoxic stage, with a maximum nDNPR efficiency of 76.4%, indicating the achievement of the nDNPR process in phase 1. In the following aerobic stage, the oxygen flow remained constant, and oxygen was mainly used to conduct the nitrification process, leading to increased DO concentrations (Table 2). This might explain the fluctuation of SNED efficiency in the aerobic stage between 13.2 and 46.6% (Figure 3D). Likewise, a previous study found that the DO concentration higher than 1.5 mg/L led to an unfavorable performance of SNED.[24]

In phase 2 (days 29–66), the DO concentration was reduced to 0.3–0.5 mg/L during the aerobic stage to enhance the SNED process, while the influent $NO_2^-$–N concentration during the anoxic stage was maintained at 49.7 ± 0.4 mg/L. The performance of P removal was improved, with nDNPR efficiency reaching 99.3 ± 0.4% (Figure 3B), confirming that DPAOs achieved complete P removal at the anoxic stage. However, $NH_4^+$–N was detected (~3.7 mg/L) in the effluent because of the low DO content. After a few days of operation, the concentration of $NH_4^+$–N in the effluent returned to below 0.1 mg/L, which manifested that nitrifying bacteria had adapted to the DO variation (Figure 3C). Meanwhile, the TN removal efficiency and the SNED efficiency were enhanced to 87.5% and 57.3%, respectively. The effluent $NO_2^-$–N and $NO_3^-$–N decreased to 1.4 and 6.2 mg/L, respectively, indicating the enhancement of the SNED process.[25] It is noteworthy that the residual $NO_x^-$–N might enhance the activity of DNB in the following cycle. Due to the coexistence of DPAOs and DNB, the COD consumption in the anaerobic stage remained at 76.7% (Figure 3A). During phase 3 (days 67–81), the nDNPR-SNED system was operated steadily with average $COD_{ins}$, nDNPR, and SNED efficiencies of 82.8, 99.9, and 67.8%, respectively. The system presented stable nitrogen and phosphorus removal when the influent $NO_2^-$–N concentration was 51.3 ± 0.4 mg/L. However, the existence of nitrite in wastewater is unstable; when it comes to treat real wastewater, the supplementation of nitrite must be considered.

In phase 4 (days 82–112), the PN-SBR was optimized to produce higher nitrite concentrations (Table 2). The effluent of the PN reactor was introduced to the nDNPR-SNED system as an anoxic influent. At the anoxic stage, the $NO_2^-$–N concentration was 70.9 ± 0.7 mg/L, which is higher than the theoretical P uptake process demand, and the residual $NO_2^-$–N could be used by GAOs to conduct partial denitrification. The $COD_{ins}$, TN, and P removal efficiencies were 86.2 ± 1.4, 87.4 ± 0.5, 91.6 ± 1.1, and 97.8 ± 0.6%, respectively, which proved the potential of the integrated system for synchronous COD, N, and P removal with a stable and efficient process performance. In the proposed process, wastewater containing $NH_4^+$–N was converted to nitrite in the PN system, which could provide electron acceptors to the nDNPR-SNED system. Compared with traditional biological wastewater treatment, both aeration energy and carbon consumption were reduced.

**3.3. Composition and Dynamics of the Functional Microbial Community Involved in the nDNPR-SNED System.** The diversity of overall microbial communities on

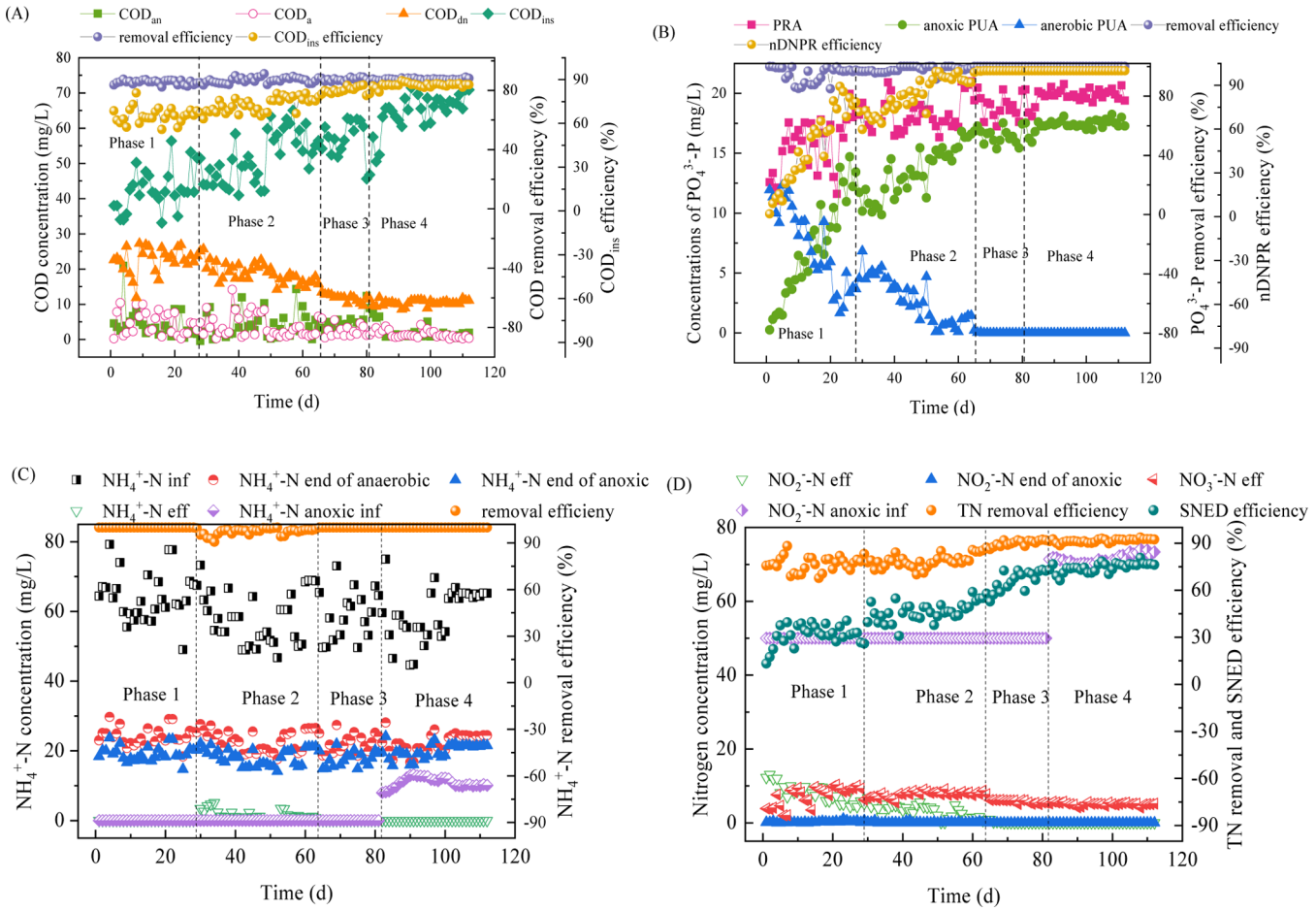


**Figure 3.** COD and P removal performance of the nDNPR-SNED system over the 112 day operational period. (A) Consumption of COD at different stages and COD removal efficiency; (B) P removal efficiency, phosphorous removal amount (PRA) at the anaerobic stage, PUA at the anoxic and aerobic stages, and nDNPR efficiency at the anoxic stage; (C) variations in $NH_4^+$−N concentration and removal efficiency; and (D) variations in $NO_2^-$−N and $NO_3^-$−N concentrations, TN removal efficiency, and SNED efficiency.

days 1, 81, and 112 for samples collected from the nDNPR-SNED system was analyzed at the phylum level (Figure 4A). The dominant phyla, including *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Parcubacteria*, *Nitrospirae*, *Planctomycetes*, and *Saccharibacteria*, were detected in each sample. It was reported that PAOs were often found in the phyla *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes*.[26] In this study, the abundance of these three phyla accounted for 76.6% (day 1), 79.7% (day 81), and 86.4% (day 112), corresponding to higher phosphorus removal efficiency in phase 3 and phase 4. The abundance of *Chloroflexi*, which played a vital role in the process of nitrification−denitrification,[27] reduced from 7.7% (day 1) to 3.5% (day 81) and then increased to 4.8% (day 112). This finding was commensurate with the results of TN removal shown in Figure 3D. Notably, the abundance of the phylum *Nitrospirae* (NOB) varied from 4.1% on day 1 to 0.8% on day 81 and then 1.5% on day 112, which was consistent with previous studies that demonstrated the suppression of NOB activity at low DO concentrations.[28]

Furthermore, the microbial dynamics of the nDNPR-SNED system at the genus level is presented in Figure 4B. The high abundance of *Dechloromonas*, *Candidatus Microthrix*, *Pseudomonas*, *Rhodocyclaceae*, and *Candidatus Accumulibacter* (the total count of 4.7% on day 1, 13.6% on day 81, and 19.6% on day 112) explained the stable performance of phosphorus removal of the system. These aforementioned genera were widely reported as organisms belonging to DPAOs and PAOs.[29,30] Moreover, on day 112, the relative abundance of *Dechloromonas* (16.5%) was higher than that of *Candidatus Microthrix* (0.05%) and *Candidatus Accumulibacter* (0.6%). However, in traditional enhanced biological phosphorus removal system, *Candidatus Accumulibacter* not *Dechloromonas* was reported as the dominant PAOs.[31] The reason behind this diversification of PAOs could be the revolution of electronic acceptors from oxygen to nitrite. In addition, the total abundance of genera *Candidatus Competibacter* and *Defluviicoccus* reported as GAOs[32] accounted for 27.4% on day 112, and *Candidatus Competibacter* (26.5%) was found to facilitate the EPD process via reduction of nitrite.[33] The increased abundance in GAOs may be due to the increased nitrite concentration in the anoxic influent at this phase. The existence of *Ellin6067*, deemed as AOB,[34] increased to 1.0% on day 112. Meanwhile, *Nitrospirae* (4.1% on day 1, 0.8% on day 81, and 1.1% on day 112) ensured the stable nitrification performance of the system. The reduction of *Thauera* from 20.6% (day 1) to 1.7% (day 112) demonstrated that most of the COD at the anaerobic stage was converted to $COD_{ins}$, which was consistent with the results in Section 3.2. In addition, on day 112, the coexistence of DNB and partial denitrifying bacteria, including *Denitratisoma* (1.5%), *Terrimonas* (0.9%), and *Thauera* (1.7%), which are related to the

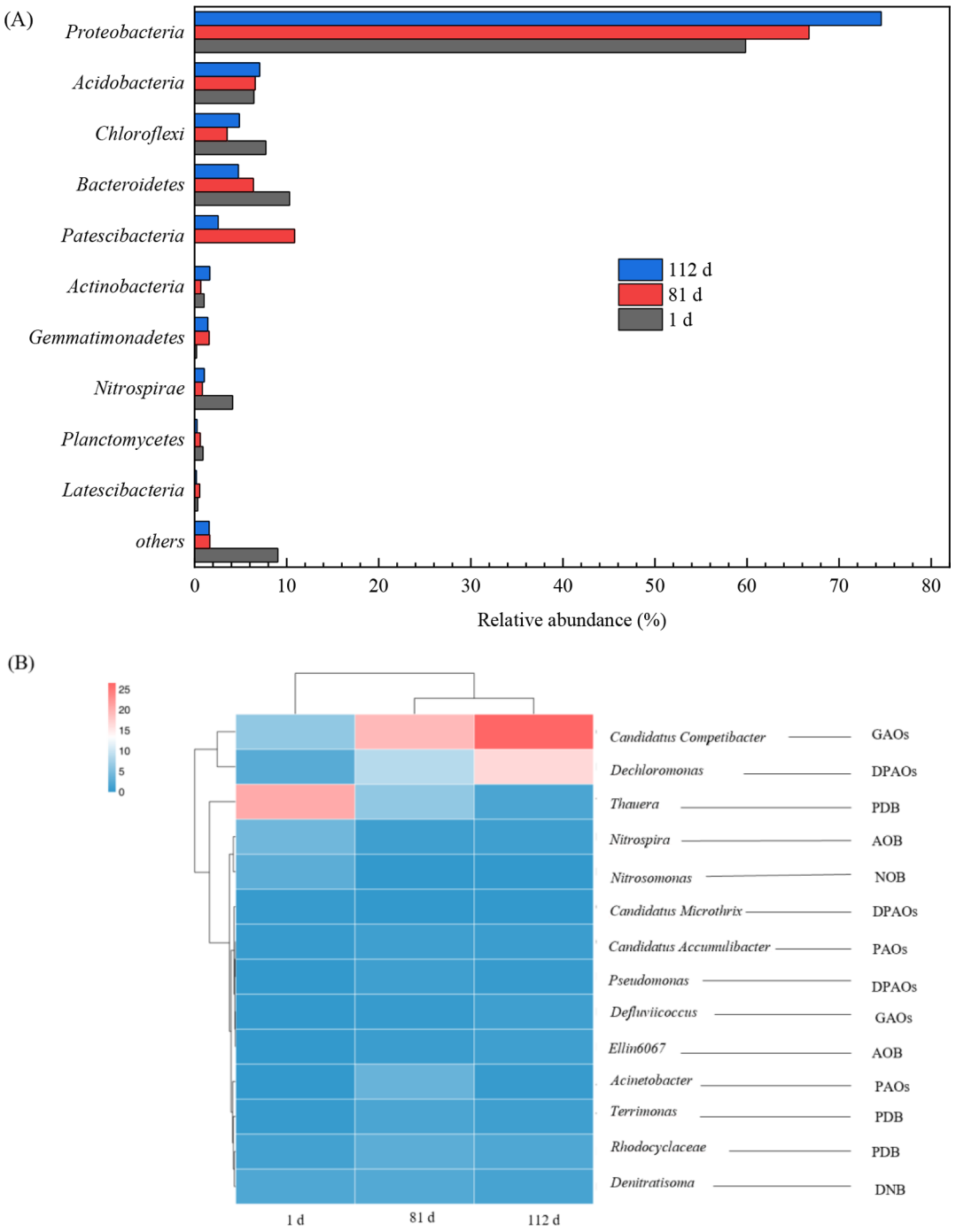


**Figure 4.** Classification and relative abundance of key functional bacteria in different phases: (A) phylum level and (B) genus level.

denitrifying community,[35] guaranteed further $NO_x^-$–N removal at the anoxic stage.

LEfSe analysis was performed to investigate the differences in microbial communities under different operating conditions (Figure 5). The biomarkers showed high LDA scores (LDA > 4.5, alpha value according to the factorial Kruskal–Wallis test<0.01) (Figure S4), which reflected different structures of bacterial communities with biological significance. Due to the subordination between biomarkers of different classification levels, the results of LEfSe analysis are given in Table S2. Differences among these classes were statistically significant with a *P*-value<0.05. With the decrease in DO concentration, genus *Nitrospirae* and phyla *Chloroflexi*, *Acidobacteria,* and *Gemmatimonadetes* were detected in phase 2 and phase 3. Genera *Thauera* and *Denitratisoma,* deemed as denitrifying organisms, were responsible for $NO_x^-$–N removal. The biomarker genera with variations from phase 3 to phase 4 included *Candidatus Competibacter*, *Dechloromonas*, and *Rhodobacter*. Among them, *Dechloromonas* and *Rhodobacter,* reported as DPAOs,[32] were related to the increase in nitrite concentration. Moreover, GAOs (*Candidatus Competibacter*) were related to nitrite accumulation explaining that GAOs demonstrated more competition in COD consumption under anaerobic conditions.

### 3.4. Mechanism Study of the nDNPR-SNED System.

Figure 6 shows the fluctuations in C, N, and P contents, as well

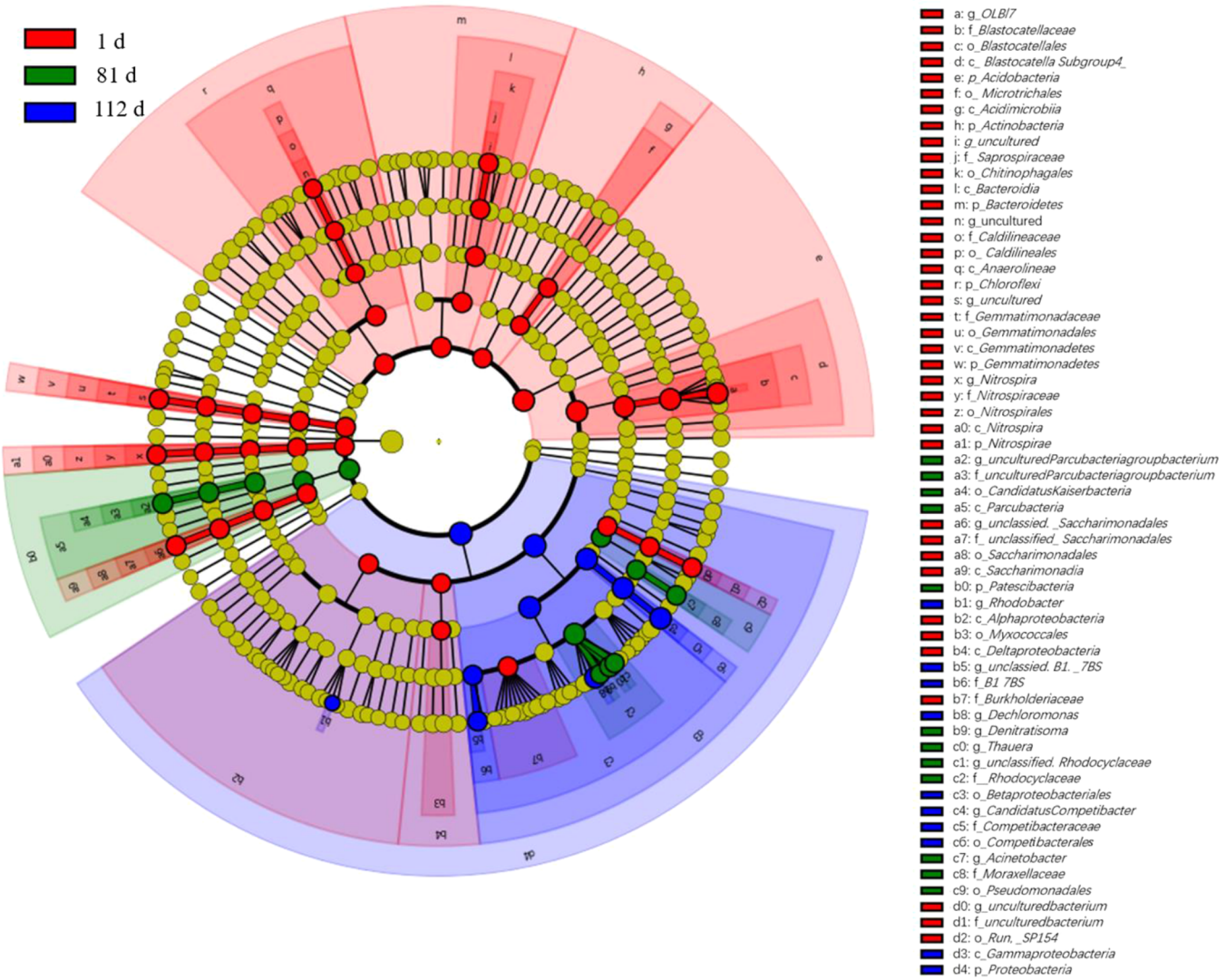


**Figure 5.** Comparative LEfSe analysis of microbial abundance among the bacteria in the three phases.

as intracellular metabolites, in a typical cycle of the nDNPR-SNED system on days 100−112 at a steady performance of the reactor; the average data of three separate cycles are provided for better clarity. At the anaerobic stage (0−180 min), the COD concentration witnessed a rapid decline from 99.2 ± 0.3 to 46.9 ± 0.1 mg/L, while PHB and PHV increased from 4.3 and 1.9 mmol/L to 12.7 and 2.3 mmol/L, respectively. PHA at this stage was mainly composed of PHB. PHB was conducive to the synthesis under low carbon source conditions.[36] In the first 90 min of the anaerobic stage, the $PO_4^{3-}$−P concentration reached 22.2 mg/L, indicating that volatile fatty acids (VFAs) were stored by DPAOs for P release (Figure 6A). The PRA/ΔPHA ratio in the anaerobic stage of the system amounted to 0.16 mol P/mol C, which was lower than the reported PAO model value of 0.5 mol P/mol C.[37] ΔPHA/ΔCOD was 1.79 mol C/mol C, which was close to the reported GAO metabolic model of 1.85 mol C/mol C,[38] suggesting that GAOs also participated in intracellular carbon storage in the anaerobic stage. Besides, the decrease in $NO_x^-$−N content was mainly due to DNB via the denitrification process. Hence, the reduction in COD in the anaerobic stage was caused by the utilization of DNB, PAOs, and GAOs (Figure 3A).

In the following anoxic stage (181−360 min), the effluent of the PN-SBR was fed into the nDNPR-SNED system to initiate the nDNPR process. The $PO_4^{3-}$−P and $NO_2^-$−N concentrations significantly decreased, resulting in a high nDNPR efficiency of 99.7% (Figure 3B). From 180th to 210th min, COD consumption was 4.9 mg/L, which might be utilized by DNB for nitrite reduction. More specifically, from 180th to 340th min, nitrite and $PO_4^{3-}$−P concentrations decreased from 15.2 and 17.9 mg/L to 1.8 and 0.04 mg/L, respectively (Figure 6A), accompanied by PHB and PHV declining from 12.7 and 2.3 mmol/L to 7.9 and 1.8 mmol/L, respectively (Figure 6B), confirming the occurrence of nDNPR. Moreover, the ratio of ΔGly to ΔPHA was 0.56 mol C/mol C, which is higher than the PAO model value of 0.42 mol C/mol C and lower than the GAO model value of 0.75 mol C/molC,[38] indicating that both PAOs and GAOs are involved in the endogenous denitrification process. The mass balance presented in Figure 6C shows that 29 and 16 mg of $NO_2^-$−N was consumed by PD and EPD, respectively, with a reduction of 49.5 mg of COD. PUA/Δ$NO_2^-$−N was 1.18 mg P/mg N, which was lower than the reported anoxic metabolism model values of DPAOs (1.7 and 2.1 mg P/mg N). Therefore, at the anoxic stage, a combination of nDNPR,

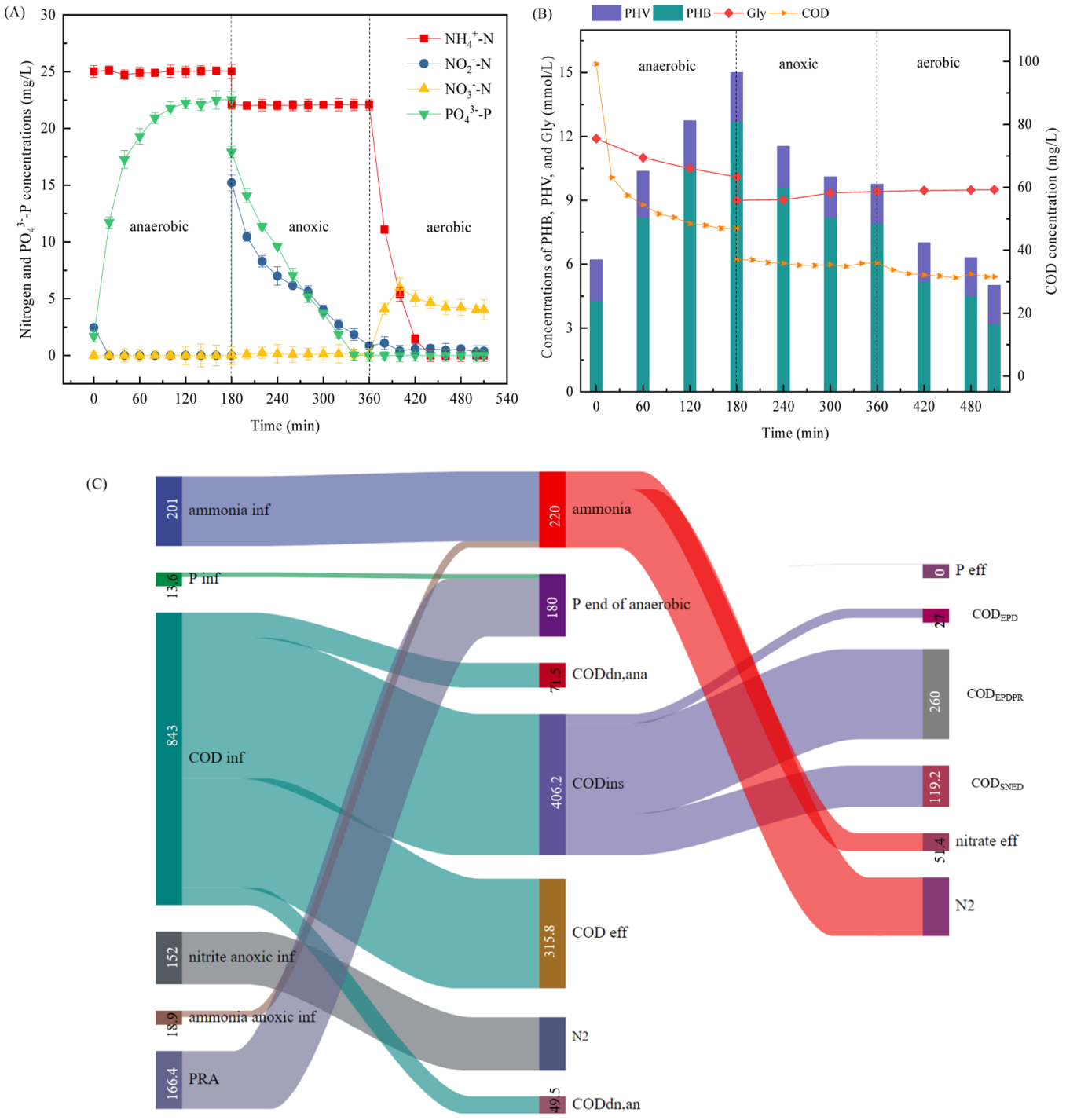


**Figure 6.** Variations in nutrient, PHV, PHB, and Gly concentrations in a typical cycle on day 112: (A) nitrogen and $PO_4^{3-}$−P concentrations; (B) COD, PHB, PHV, and Gly concentrations; (C) model-based mass balance evaluation. Notes: PRA, phosphorus removal amount; $COD_{ins}$, COD consumed for anaerobic intracellular carbon storage at the anaerobic stage; $COD_{dn,ana}$, COD consumed by exogenous denitrification of nitrite and nitrate at the anaerobic stage; $COD_{dn,an}$, COD consumed by exogenous denitrification of nitrite and nitrate at the anoxic stage; $COD_{EPD}$, COD consumed by the EPD process at the anoxic stage; $COD_{EPDPR}$, COD consumed by the EPDPR process at the anoxic stage; $COD_{SNED}$, COD consumed by the SNED process at the aerobic stage.

PD, and EPD processes occurred, which were responsible for the removal of $PO_4^{3-}$−P and $NO_2^-$−N. At the anoxic stage, Gly synthesis and PHA decomposition returned to the initial anaerobic level, realizing the circulation of endogenous metabolites.

At the aerobic stage (361−510 min), $NH_4^+$−N was oxidized to $NO_2^-$−N and $NO_3^-$−N under low DO concentration, as shown in Figure 6A. $NH_4^+$−N decreased from 20 to 1.4 mg/L in the first 60 min of the aerobic stage; the peak concentration of the produced $NO_3^-$−N was 6.1 mg/L. The total amount of $NO_x^-$−N production was lower than that of the $NH_4^+$−N reduction, and PHB decreased to 5.2 mmol C/L, implying that $NO_x^-$−N was reduced by GAOs via the SNED process during this period. Furthermore, the continuous decrease in $NO_3^-$−N

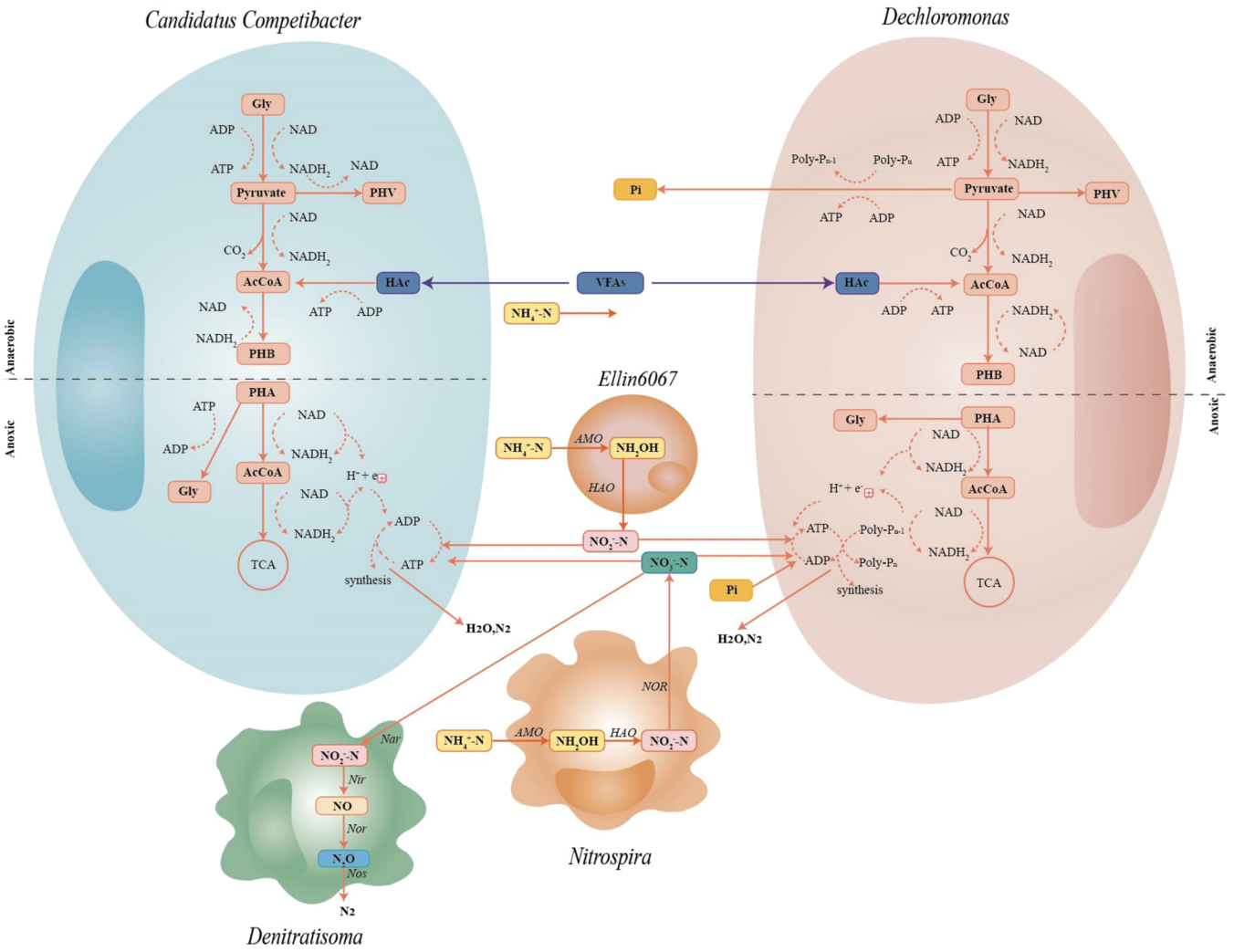


**Figure 7.** Metabolism of functional bacteria in the combined system.

(from 420th to 480th min), even after $NH_4^+$−N was completely consumed while no $NO_2^-$−N was detected in the effluent, was attributed to the SNED process. After 2.5 h aeration, the effluent COD, $NO_2^-$−N, and $NO_3^-$−N were 31.5, 0.3, and 5.1 mg/L, respectively, and the SNED process contributed to 68.7% of N removal.

In addition, a metabolic pathway model was developed for the combined system based on the physico-chemical analysis and the microbial dynamics of the present study, as shown in Figure 7. In the anaerobic stage of nDNPR-SNED, COD was mainly utilized by DPAOs, GAOs, and DNB. The genus *Dechloromonas* (DPAOs) decomposed polyphosphate (Poly-P) in the cell and released $P_i$ into the medium in the form of soluble phosphate. In this process, *Dechloromonas* absorb and convert the external carbon source, that is, VFAs, into PHA under anaerobic conditions. Gly degradation provides a small part of energy [adenosine triphosphate (ATP)], while Poly-P degradation provides most ATP, accompanied by a large amount of phosphate release. At the same time, the biochemical model of *Candidatus Competibacter* (GAOs) was similar to that of PAOs; however, the energy for PHA storage mainly came from Gly decomposition.[38] DNB consumed COD in the denitrification process, converting the residual $NO_x^-$−N to $N_2$ using enzymes, including *Nar*, *Nir*, *Nor*, and *Nos*. At the anaerobic stage, 91.9% of COD was stored as PHV and PHB by DPAOs and GAOs, respectively.

At the anoxic stage, $NO_2^-$−N and $P_i$ could be reduced through the nDNPR process by *Dechloromonas* during the uptake of $P_i$ into the cell for Poly-P synthesis using PHA as carbon sources. *Dechloromonas* used intracellular-stored PHA as an electron donor and nitrite as the electron acceptor in an anoxic environment to absorb excessive and sufficient extracellular phosphate. A part of PHA was used for the growth of new cells, while the rest was used for the excess uptake of Poly-P. Simultaneously, Gly was supplemented by PHA degradation, including that of PHV and PHB, and the energy was provided from ATP. At the aerobic stage, GAOs played a dominant role in the decomposition of PHA via the SNED process. Oxygen was mainly utilized for nitrification by *Ellin6067* (AOB) and *Nitrospirae* (NOB), and GAOs promoted N removal using PHB stored during the anaerobic stage.

**3.5. Feasibility of nDNPR-SNED Combined with the PN System for Synchronous COD, N, and P Removal.** Herein, the combination of PN and nDNPR-SNED was proven to be an efficient and cost-saving process for advanced synchronous nutrient removal (C, N, and P) without any external carbon addition. To validate the feasibility of the combined system, an economic evaluation was performed in

this study in comparison with the conventional biological nutrient removal (BNR) process[39] (Table 3). In the economic

**Table 3. Economic Analysis of the Combined Processes with the Conventional BNR Process**

| parameters | conventional BNR[39] | this study |
|---|---|---|
| flow quantity ($m^3$/day) | 10000.0 | 10000.0 |
| total volume ($m^3$) | 10416.7 | 10416.7 |
| construction cost ($) | 2083333.3 | 2083333.3 |
| aeration energy (kWh/year) | 29613600.0 | 21060000.0 |
| pumping energy (kWh/year) | 116785.7 | 116785.7 |
| operational cost ($/year) | 3000038.6 | 2144678.6 |
| maintenance cost ($/year) | 412500.0 | 412500.0 |
| TC ($) | 2815487.4 | 2631971.4 |
| NPV ($) | | 23040.1 |
| savings per TC (%) | | 6.7 |

study, the flow rate of influent wastewater was proposed to be 10,000 $m^3$/day. Meanwhile, the TC and NPV mentioned in eqs 4 and 5 were used as evaluating parameters, respectively.[17] Methods of calculating the operation cost and maintenance cost were estimated according to a previous study with an electricity price of 0.1 $/kWh.[17] As shown in Table S1, the operation cost mainly consisted of three parts including construction cost, aeration energy, and pumping energy. Besides, aeration energy was determined according to the oxygen transmission efficiency of 3.6 kg $O_2$/kWh.[40] The construction cost was calculated to be 200 $/$m^3$, and the volumes of PN and nDNPR-SNED were considered based on the HRT mentioned in Section 2.1. The NPV in this work was $23040.1, meaning that the combined process became significantly cost-effective after the enhancement of nDNPR. Moreover, compared with the traditional BNR process in the WWTP, the proposed approach could save 6.7% in TCs, mainly due to aeration energy and carbon source saving. The present study concluded that applying the nDNPR-SNED system in the WWTP is economically beneficial.

In municipal wastewater treatment, sludge digester liquor contains a considerable amount of $NH_4^+$−N, which can be oxidized to $NO_2^-$−N via PN. Therefore, the effluent of PN combined with municipal wastewater could be supplied to the nDNPR-SNED system. Consequently, the proposed system could be applied in sewage treatment plants for C, N, and P removal. Compared to traditional nitrogen and phosphorus removal biotechnologies, the nDNPR-SNED system could achieve higher N and P elimination in a cost-effective way.

## 4. CONCLUSIONS

PN combined with the nDNPR-SNED process was successfully applied for mainstream wastewater treatment without external carbon addition with removal efficiencies of 87.4 ± 0.5, 91.6 ± 1.1, and 97.8 ± 0.6% for COD, TN, and P, respectively. PN converted $NH_4^+$−N to $NO_2^-$−N and stably supplied nitrite to the nDNPR-SNED system. Variations in microbial community and performance of nutrient removal were studied in the nDNPR-SNED system at three stages. At the anaerobic stage, 91.9% of COD was stored as PHV and PHB by DPAOs and GAOs, respectively. The coexistence of *Candidatus Competibacter*, *Dechloromonas*, and *Thauera* ensured the high efficiency of N and P removal at the anoxic stage, while *Candidatus Competibacter* was more competitive during higher nitrite concentration with DO concentration below 1.0 mg/L. *Candidatus Competibacter*, *Ellin6067*, and *Nitrospirae* were responsible for the SNED process at the aerobic stage, contributing to 68.7% of nitrogen removal. Compared with traditional biological nitrogen and phosphorus removal processes in the WWTP, the proposed approach could save 6.7% in TCs. This combination provided a more effective and economical way for simultaneous COD, N, and P removal.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsestwater.2c00126.

Analysis methods of PHV and PHB, analysis methods of Gly, nomenclature list of abbreviations and characters with suffix, cost calculating parameters of the combination system, biomarkers from different phases of the nDNPR-SNED system, nitrogen removal performance of the PN system, DO and pH in one typical operation cycle of the PN system, COD and $PO_4^-$−P removal performance of the nDNPR-SNED system, and LDA analysis of the nDNPR-SNED system (PDF)

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### Notes

The authors declare no competing financial interest.

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