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Variation in the quantity and composition of phosphorus accumulating organisms in activated sludge driven by nitrate-nitrogen

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Abstract−Anaerobic/anoxia sequencing batch reactor (A/ASBR) system was used to analyze the quantity and composition of each branch of phosphorus accumulating organisms (PAOs) in activated sludge under different nitrate-nitrogen (NO_3^--N) concentrations by using real-time quantitative polymerase chain reaction (PCR) technology. The study determined whether NO_3^--N and its concentration change were the main driving factors for the variation of the quantity and composition of each branch of PAOs. The results show that with the increase of NO_3^--N concentration from 10 mg/L to 40 mg/L, the number of bacterial 16S rRNA genes in the A/ASBR reactor changed slightly at 6.81×10^{11}-7.53×10^{11} copies/g dry sludge. The number of PAO genes (Acc 16S rRNA) increased from 1.98×10^{11} to 3.53×10^{11} copies/g dry sludge, and the total number of ppk1 genes increased from 1.25×10^{11} to 3.59×10^{11} copies/g dry sludge. Additionally, the number of polyphosphate kinase (ppk) genes in Accumulibacter branch IA, IIC and IID was high, and the changes were positively related to the concentration of NO_3^--N, while the number of branches in IIA, IIB and IIF was very low. The dosing concentration of NO_3^--N was the main driving factor for the change of PAOs and their branch number and composition in the A/ASBR reactor.

Keywords: A/ASBR, Real-time Quantitative PCR, PAOs Branch, Denitrification

INTRODUCTION

Eutrophication of water bodies caused by excessive nitrogen and phosphorus discharge is one of the most concerning environmental problems. To reduce its impact, nitrogen and phosphorus in sewage must be removed, especially phosphorus [1,2]. There are chemical and biological methods for phosphorus removal from wastewater, among which the activated sludge method is prominently used [3-8]. The activated sludge system provides suitable environmental conditions to cultivate and domesticate PAOs, which releases phosphorus, synthesizes poly-β-hydroxy-alkanoates (PHA), and absorbs phosphorus under aerobic conditions using oxygen as an electron acceptor to transfer phosphorus from sewage to activated sludge. In 1985, Hascote et al. [9] reported that PAOs can absorb phosphorus with NO_3^--N as an electronic receptor, which was the first report on denitrifying phosphorus absorption. The denitrifying phosphorus removal principle is similar to traditional biological phosphorus removal. The phosphorus release process in the anaerobic section is consistent with the traditional process. In the anoxic section, denitrifying phosphorus removal bacteria uses nitrate as the electronic acceptor to absorb phosphorus. In 1987, Comeau and Gerber [10,11] also reported that PAOs were not specific aerobic bacteria, and some PAOs could take NO_3^--N as the electronic receptor. In 1993, Kuba et al. observed through batch-test that PAOs with denitrification function were easily enriched under the environmental conditions of anaerobic/anoxic alternation. Øtgaard et al. [12] found that about 60% of chemical oxygen demand (COD) was absorbed in the form of PHA in the anaerobic tank in the study on the operation of the actual sewage plant of the University of Cape Town (UCT) process.

In the subsequent anoxic tank, one-third of the COD was consumed in the form of PHA, accompanied by phosphorus absorption. Therefore, the anoxic section in the UCT process played an essential role in enhancing biological phosphorus absorption. As a result, about 30% of phosphate was removed from the anoxic tank. Other research reports pointed out that the improved UCT process strengthened the alternation of an anaerobic/anoxic environment, eliminated other bacteria, and was conducive to the growth of PAOs with denitrifying function [13-15]. Kuba et al. [13] studied the UCT phosphorus removal process in the Holten wastewater treatment plant (WWTP). They found that about 30-50% of the mixed liquid volatile suspended solids (MLVSS) in the efficiently operating plant contained PAOs, and 50% of them had the ability to denitrify.

Numerous studies have reported the existence of denitrification phosphorus absorption, but the performances were quite different, mainly affected by the following factors: NO_3^--N concentration, sludge age, carbon source type and carbon to phosphorus ratio (C/P) and pH value, etc. [16-27]. As an electron acceptor, NO_3^--N greatly influences the denitrification and phosphorus absorption process.

Given the widespread phenomenon of denitrification and phosphorus absorption in the synchronous nitrogen and phosphorus removal system, in 1999, the International Water Association (IWA) expert group introduced the activated sludge reaction kinetic model

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2d (ASM2d). This was based on activated sludge reaction kinetic model 2 (ASM2), thus adding two processes of PAOs storing phosphorus under anoxic conditions and using PHA to grow [28]. $\frac{S_{NO_3^-}}{K_{NO_3^-}+S_{NO_3^-}}$ is one of the "switching functions" of the denitrification process of common heterotrophic bacteria and the anoxic phosphorus uptake of PAOs, where $S_{NO_3^-}$ is the NO_3^--N concentration in the anoxic section, and $K_{NO_3^-}$ is the semi-saturated constant (the recommended value of IWA is 0.5 mg/L). This switching function controls the anoxic section's denitrification and phosphorus accumulation storage rate [28]. In addition, Londong [29] showed that to use the denitrification potential fully, the NO_3^--N concentration in the anoxic section can be controlled within a non-zero low-value range in the continuous-flow single-sludge synchronous nitrogen and phosphorus removal system. Musvoto et al. [30] showed that in the modified University of Cape Town (MUCT) process, the NO_3^--N concentration in the main anoxic section is the main influencing factor in the denitrifying phosphorus absorption process. Wang et al. [31] showed that using the NO_3^--N concentration in the main anoxic section as the optimal operation parameter of the continuous-flow single-sludge synchronous nitrogen and phosphorus removal system can reduce the effluent nitrogen and phosphorus concentration. The relationship between the flow and distribution of carbon, nitrogen and phosphorus under different concentrations of NO_3^--N was found based on material balance and chemometrics principles.

Furthermore, the reduction effects on carbon source, power consumption and excess sludge production were analyzed and evaluated. Finally, the treatment effect and theoretical principle proved the feasibility of NO_3^--N as an operation control parameter. With the help of engineering technology, the above research results analyzed the relationship between NO_3^--N and denitrifying phosphorus absorption from a macro perspective but lacked the support of microbiological principles.

It has been pointed out that PAOs mainly belong to the Rhodochlus Group in Beta proteobacteria and are named Candidatus Accumulibactor phosphatis [32]. It is the dominant phosphorus removal microorganism [33,34] that is the most widely used and deeply researched PAOs in urban domestic sewage treatment. Modern molecular biology technology does not rely on pure culture, which is an effective means for qualitative and quantitative analysis of PAOs. For quantitative analysis of PAOs, 16S rRNA is a widely used gene marker quantified by real-time fluorescent quantitative PCR (qPCR) technology. However, 16S rRNA is highly conservative and cannot distinguish the evolutionary branches of Accumulibacter [35]. Therefore, using 16S rRNA as a gene marker, molecular biology can only quantify the abundance of all PAOs and cannot quantify different evolutionary branches. Macrogenomic analysis shows that Accumulibacter has a single copy of the ppk1 encoding gene [36], which plays an essential role in the polyphosphate (poly-P) transformation in Accumulibacter. Therefore, the ppk1 functional gene has become a gene marker for studying the evolutionary branch of Accumulibacter.

There are many evolutionary branches of Accumulibacter, and the utilization ability of NO_3^--N of each evolutionary branch is significantly different [37,38]. This paper uses the A/ASBR to measure the phosphorus accumulating microbial population with the configured sewage as the treatment object. The fluorescence real-time quantitative PCR technology is used to study the relationship between the number and composition of the activated sludge microbial population, the number and composition of Accumulibacter IA, IIA, IIB, IIC, IID, and IIF, and the change of NO_3^--N concen-

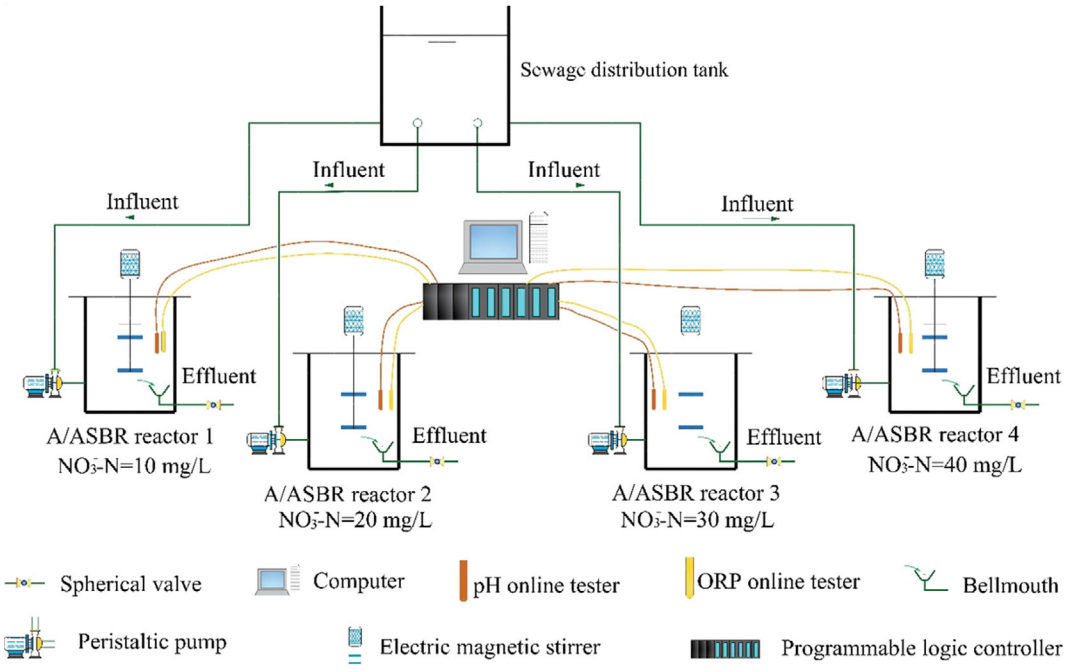

Fig. 1. Test device.

Table 1. Composition and water quality characteristics of simulated wastewater

Compound composition	Dosage	Trace element composition	Concentration g/L
Acetic acid	3 mL	$FeCl_3$	0.9
KH_2PO_4	0.6 g/L	H_3BO_4	0.15
$NaHCO_3$	5 g/L	$CoCl_2{\cdot}7H_2O$	0.15
$CaCl_2$	0.2 g/L	$CuSO_4{\cdot}5H_2O$	0.03
$MgSO_4$	1.0 g/L	KI	0.18
Trace element solution	12 L	$MnCl_2{\cdot}4H_2O$	0.06
		$Na_2Mo{\cdot}2H_2O$	0.06
		$ZnSO_4{\cdot}7H_2O$	0.12

tration, to determine the microbial population structure and its driving factors, and to combine the microbiological community with the macro function of the system. Furthermore, to achieve the purpose of effectively regulating the stability of the system and optimizing its functions.

MATERIALS AND METHODS

1. Test Device

The test device and process are shown in Fig. 1. A batch test was carried out in four A/ASBR reactors with an effective volume of 4.5 L. The reactor diameter was 15 cm, the height was 35 cm, and the superelevation was 10 cm. A drainage bell was set 5 cm away from the bottom of the reactor. The WTW inolab pH probe and oxidation-reduction potential (ORP) probe were set in the A/ASBR device, and the stirrers were set in the reactors to ensure the uniform mixing of sludge and water. To prevent the aerobic phosphorus absorption process, the top of the reactor was covered. A sewage distribution tank channelled water as influent into the A/ASBR reactor. The effective volume of the sewage distribution tank was 16.4 L (thus, L×B×H=20 cm×20 cm×50 cm), of which the superelevation was 9 cm. One water outlet was set at the middle position 5 cm away from the bottom of the four sides of the tank, which was connected with the peristaltic pumps of the four reactors (A/ASBR reactcor 1, A/ASBR reactcor 2, A/ASBR reactcor 3 and A/ASBR reactcor 4) for inlet water, respectively. The four A/ASBR reactors were independent.

During the test, the sewage in the water distribution tank entered the reactors through the peristaltic pumps. The treated water was discharged from the system through the outlet bell mouths after the biochemical reactions and precipitation processes were completed. In the reaction processes, the values of pH and ORP were measured in real-time and transmitted to the PLC (programmable logic controller) system in the computer to monitor and control the operating environments.

2. Experiment Scheme

Synthetic sewage was used in the experiment; acetic acid was added as a carbon source, and KH_2PO_4 was added as a source of phosphorus salt in the influent. $NaHCO_3$ was added to maintain the pH value in the neutral range during the reaction, and $MgSO_4$ and $CaCl_2$ were added to meet the requirements of Mg^{2+} and Ca^{2+} ions in the process of phosphorus uptake by PAOs. In addition, trace element solutions were added to meet the nutritional requirements of the growth and reproduction of activated sludge microorganisms. See Table 1 for the dosage and water quality characteristics of each component of simulated wastewater.

The inoculated sludge introduced in the A/ASBR reactor was transferred from the aerobic section of the MUCT reactor with an effective volume of 90 L operated in the laboratory. The system has been running for nearly two years, treating domestic sewage with COD/total nitrogen (TN) lower than 6. When extracting the inoculated sludge, the operating parameters of the reactor were as follows: the treated water volume was 10 L/h, the sludge load was (0.253±0.071) kg/(kg·d), the sludge reflux ratio was 0.5, the mixed liquid reflux ratio was 1.0, the sludge age was 12 days, and the NO_3^--N concentration in the main anoxic section was 2.5 mg/L. At this time, the system's denitrification and phosphorus removal effect were good, and the phenomenon of phosphorus absorption in the anoxic zone was evident. Furthermore, the material balance calculation results showed that the anoxic phosphorus absorption rate of the system (the percentage of anoxic phosphorus absorption in the total phosphorus absorption) was stable at about 47% [31].

After the sludge was taken out, it was inoculated into the four A/ASBR reactors, and the initial mixed liquor suspended solids (MLSS) concentration was 1,500 mg/L. The reactor was operated in cycles, 8 h per cycle, including water inflow 5 min, anaerobic 2 h, anoxic 4 h, sedimentation 1.5 h, and drainage/sludge 25 min; thus, one cycle per day. The water inflow of each reactor was 3.6 L per cycle. To ensure that the inlet water quality was identical, the wastewater to be treated was configured in one sewage distribution tank. After mixing evenly, the wastewater was pumped into their respective A/ASBR reactor using a longer peristaltic pump (driver bt600-2j, pump head yz2515x, flow range 0.07 mL/min-3,000 mL/min, Baoding, China), one for each reactor. The hydraulic method was adopted to control the sludge age to 15 d [39]. To prevent phosphate precipitation caused by increased pH value in the phosphorus absorption process (phosphate precipitation could occur when the pH value was more significant than 8.5). The reaction process was monitored online with a pH meter, and the pH value of the reaction process system was maintained within the range of 7.0± 0.1 by adding dilute HCl or NaOH solution.

After the anaerobic phosphorus release reaction, different amounts of potassium nitrate (0.30, 0.60, 0.90, 1.20 g) were added to the reactor to enter the anoxic phosphorus absorption section. The four A/ASBRs were named A/ASBR1, A/ASBR2, A/ASBR3, and A/

ASBR4 according to the different dosages of potassium nitrate. The reactors were operated for 30 cycles, the effects of phosphorus release, phosphorus absorption, and denitrification remained stable, and samples were taken for experimental research.

3. Conventional Water Quality Indexes and Testing Methods

Water samples were taken from the drainage bell set at 5 cm from the bottom of the A/ASBR reactor during the test. After the influent volume in the reactor reached its target, sampling in the anaerobic section started and the reaction time was recorded as 0 min. Samples were taken at intervals of 15 min within 0-60 min; 30 min intervals within 60-120 min; sampling at 15-min interval within 120-180 min; sampling at 30-min interval with 180-240 min; sampling at 60-min interval within 240-360 min. Among them, the water sample at 0 min was the influent water sample and the water sample at 360 min was the effluent water sample. The water samples were centrifuged at 4,500 rpm for 7 min, and the supernatant was collected. The indexes of COD, total phosphorus (TP), NH_4^+-N, NO_3^--N, and NO_2^--N were determined by the standard method [40].

4. Deoxyribonucleic Acid (DNA) Extraction and Purification

At the end of the experiment, a certain amount of evenly mixed mud water mixture was extracted from the four A/ASBR reactors. After high-speed freezing and centrifugation to remove excess water, it was freeze-dried at −80 °C and stored for inspection. About 0.1 g of sample was weighed and DNA was extracted using MP Soil DNA Rapid Extraction Kit (Bio 101, Vista, CA, USA) regarding the instructions for the kit for specific methods. DNA concentration was determined using a nanodrop spectrophotometer ND-1000 (Thermo Fisher Scientific, USA) microspectrophotometer. The subsequent analysis stage was entered when the extraction quality met the requirements.

5. Real-time Quantitative PCR Test

The fluorescent real-time quantitative reaction was performed on the Mx3005P real-time quantitative PCR amplification instrument (Agilent Technologies, USA), using a 25 μL reaction system (Brilliant II SYBR Green qPCR (real-time fluorescent quantitative PCR) Master Mix, Agile Technologies, USA). The reaction system is shown in Table 2 [37]. Bacterial 16S rRNA gene, total Candidatus accumulibacter 16S rRNA gene and Candidatus accumulibacter specific functional gene ppk were used to quantify the absolute abundance of whole bacteria, total Accumulibacter and Accumulibacter IA, Accumulibacter IIA, Accumulibacter IIB, Accumulibacter

Table 2. qPCR reaction mixture

Reaction component	Volume	Final concentration
Brilliant II SYBR Green qPCR master mix (2x)	12.5 μL	1X
Forward primer 10 mM	1 μL	400 nM
Forward primer 10 mM	1 μL	400 nM
ROX	0.5 μL	40 nM
Sterile water	8 μL	-------
DNA template	2 μL	-------

Table 3. Specific primer sequences and programs of qPCR

Primer	Sequence	Amplification size (bp)	Target	Program
1055f 1392r	ATGGCTGTCGTCAGCT ACGGGCGGTGTGTAC	323	Bacterial 16S rRNA	95 °C 10 min; (95 °C 30 s, 50 °C 60 s, 72 °C 20 s)×45
518f PAO-846r	CCAGCAGCCGCGGTAAT GTTAGCTACGGCACTAAAAGG	351	Acc 16S rRNA	95 °C 3 min; (94 °C 30 s, 60 °C 45 s, 72 °C 30 s)×35
Acc-ppk1-763f Acc-ppk1-1170r	GACGAAGAAGCGGTCAAG AACGGTCATCTTGATGGC	408	Acc-IA	95 °C 3 min; (94 °C 30 s, 63 °C 45 s, 72 °C 30 s)×45
Acc-ppk1-893f Acc-ppk1-997r	AGTTCAATCTCACCGAGAGC GGAACTTCAGGTCGTTGC	105	Acc-IIA	95 °C 3 min; (94 °C, 30 s, 61 °C 45 s, 72 °C 30 s)×45
Acc-ppk1-870f Acc-ppk1-1002r	GATGACCCAGTTCCTGCTCG CGGCACGAACTTCAGATCG	133	Acc-IIB	95 °C 3 min; (94 °C 30 s, 61 °C 45 s, 72 °C 30 s)×45
Acc-ppk1-254f Acc-ppk1-46Or	TCACCACCGACGGCAAGAC CCGGCATGACTTCGCGGAAG	207	Acc-IIC	95 °C 3 min; (94 °C 30 s, 66 °C 45 s, 72 °C 30 s)×45
Acc-ppk1-1123f Acc-ppk1-1376r	GAACAGTCCGCCAACGACC ACGATCATCAGCATCTTGGC	254	Acc-IIC (ppkl excluding OTU NSD3)	95 °C 3 min; (94 °C 30 s, 63 °C 45 s, 72 °C 30 s)×45
Acc-ppk1-375f Acc-ppk1-522r	GGGTATCCGTTTCCTCAAGCG GAGGCTCTTGTTGAGTACACGC	148	Acc-1ID	95 °C 3 min; (94 °C 30 s, 63 °C 45 s, 72 °C 30 s)×45
Acc-ppk1-355f Acc-ppk1-600r	CGAACTCGGCGAAAGCGAGTA ATCGCCTCCGAGCAACTGTTC	246	Acc-IIF	95 °C 3 min; (94 °C 30 s, 70 °C 45 s, 72 °C 30 s)×45

IIC, Accumulibacter IID and Accumulibacter IIF, respectively. See Table 3 for primer sequence, reaction system and temperature rise procedure. To ensure the validity of the data results, the standard curve was established by ten times gradient dilution of plasmid DNA with known concentration. The standard and tested samples were set three parallel and the negative control was set. The linear values of the fluorescence quantitative standard curve all satisfied R^2>0.98 and the amplification efficiency was 80%-120%. The single peak curve was specific amplification. According to the known copy number of the standard sample, the quantitative standard curve was obtained through the fluorescence threshold and cycle number Ct value of the standard sample in the quantitative process. Then the copy number of the DNA sample to be tested was calculated according to the Ct value of the sample to be tested given by the system [37,41,42].

RESULTS AND DISCUSSION

1. Analysis of Phosphorus Removal Performance of A/ASBR

After the successful start-up of the reactor, samples were taken for analysis. During stable operation, the water quality indicators such as COD, TP and NO_3^--N of the influent and removal rates of the reactor effluent are shown in Table 4.

According to the principle of denitrifying phosphorus absorption, the phosphorus removal efficiency of the A/ASBR reactor was mainly affected by NO_3^--N. According to the principle of simultaneous nitrogen and phosphorus removal and denitrifying phosphorus absorption. The NO_3^--N added in the A/ASBR reactor was mainly removed through the common denitrification process with COD as the carbon source and the denitrifying phosphorus absorption process with poly-β-hydroxy-butyrate (PHB) stored in the PAOs as the carbon source. According to the research report, the reaction rate of the ordinary denitrification process was significantly higher than that of the denitrification phosphorus absorption process; that is, when there is COD in the reactor, the ordinary denitrification process will be carried out preferentially [43]. It can be seen from Table 4 that with the increase of NO_3^--N dosage in the four A/ASBR reactors, the ratio of influent COD and NO_3^--N in the anoxic section decreased from 17.42 to 4.44. Denitrifying bacteria are heterotrophic bacteria, so no matter which method is used to remove NO_3^--N, its removal rate is related to the COD/NO_3^--N ratio; that is, with the reduction of this ratio, the removal rate of NO_3^--N decreases. When the ratio dropped to 4.44, the denitrification carbon source was insufficient, so the concentration of NO_3^--N effluent increased and the removal rate decreased.

In the A/ASBR system, NO_3^--N was the only electron acceptor in the phosphorus absorption process. Therefore, the larger the dosage, the more sufficient electron acceptors were provided, and the more suitable it was for cultivating and domesticating denitrifying phosphorus accumulating bacteria. Therefore, in A/ASBR1 and A/ASBR2, the dosing concentration of NO_3^--N was low and the electron acceptor of denitrifying phosphorus uptake was limited, resulting in the reduction of glycogen storage during phosphorus uptake, affecting the PHB synthesis during phosphorus release. Consequently, the phosphorus removal effect was poor. TP removal rates were only 48.86% and 66.34%. However, when the NO_3^--N concentration in the anoxic section was increased to 30 mg/L and 40 mg/L, the electron acceptor was sufficient and the total phosphorus removal effect was excellent, increasing to 86.62% and 92.00%, respectively.

The four A/ASBR reactors were operated for about 80 cycles. See Fig. 2 for the variation of TP, COD and NO_3^--N concentration with reaction time in the 75 th typical operation cycle. It can be seen that the degradation of COD in the four reactors presented the following variation law: it stopped/slowed after early rapid consumption and then increased the removal rate after the addition of NO_3^--N. The activated sludge degraded pollutants mainly in the early stage of the biosorption process at a fast rate, so the COD concentration in the sewage in the early stage decreased rapidly. Then, the adsorbed COD was absorbed and transformed by phosphorus accumulating bacteria and denitrifying bacteria as a nutrient, showing a slight decrease in COD concentration. In the

Table 4. Quality characteristics of influent and effluent water during stable operation

Reactor	Indicator (mg/L)	Influent (mg/L)	Effluent (mg/L)	Removal rate (%)	Influent COD/NO_3^--N
A/ASBR1	COD	175.06±5.56	38.09±4.78	78.24±4.21	17.42±1.98
	TP	8.31±0.98	4.25±1.01	48.86±0.88	
	NO_3^--N	10.05±0.97	0.98±0.45	90.25±0.75	
A/ASBR2	COD	167.31±6.01	34.56±4.21	79.34±5.05	8.23±1.54
	TP	8.17±1.12	2.75±0.86	66.34±1.04	
	NO_3^--N	20.32±1.04	2.86±0.98	85.93±1.03	
A/ASBR3	COD	182.43±5.32	36.54±4.97	79.97±4.53	6.35±2.17
	TP	7.85±1.23	1.12±0.43	86.62±0.87	
	NO_3^--N	28.72±0.95	4.97±0.76	82.69±0.91	
A/ASBR4	COD	175.43±6.12	35.75±4.59	76.62±5.87	4.44±1.97
	TP	8.37±1.09	0.67±0.76	92.00±1.04	
	NO_3^--N	39.47±1.16	11.54±1.07	70.76±1.15	

Note: The concentration data of each indicator in the table is mean value±standard deviation (n=60, p=0.683).

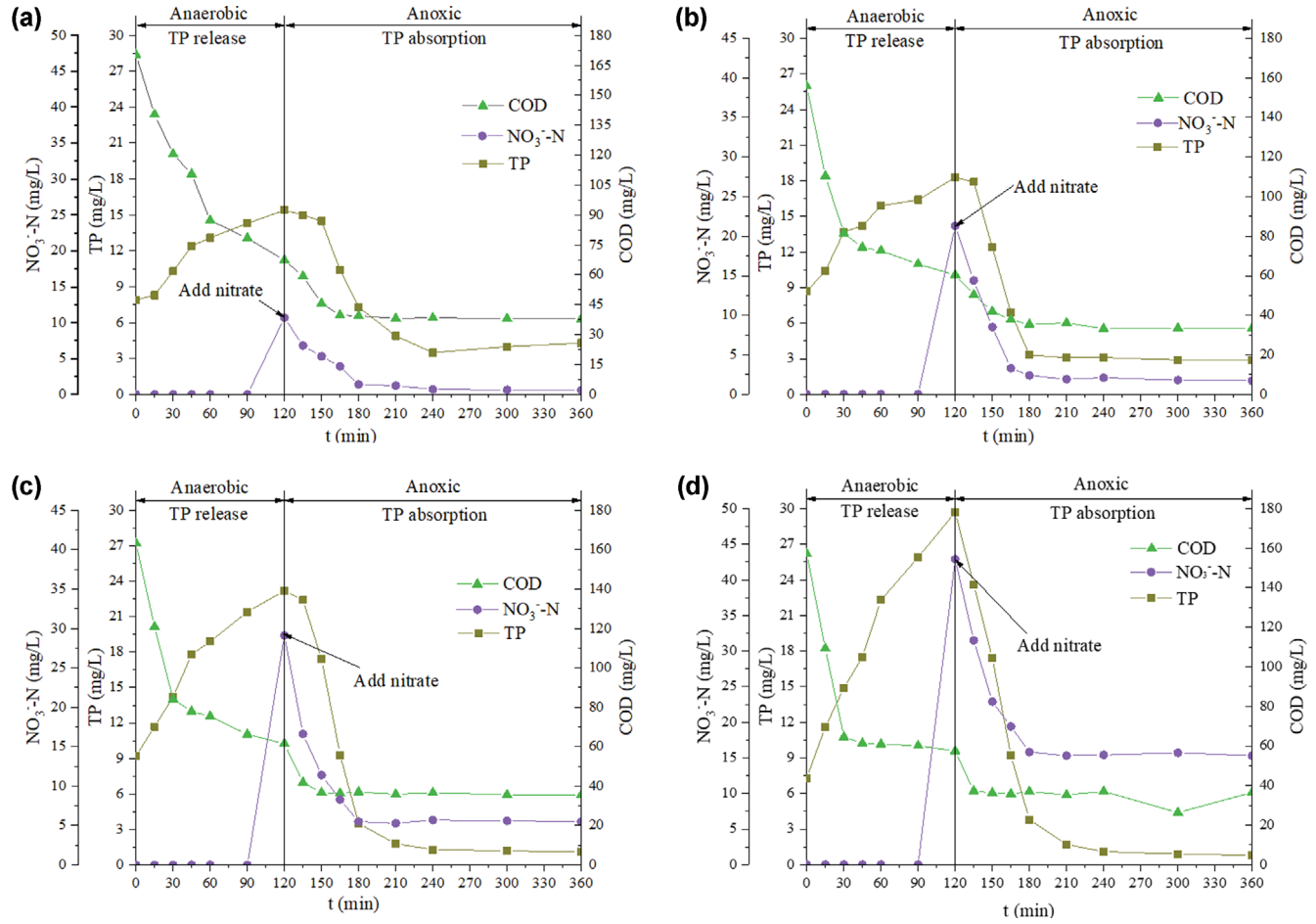

Fig. 2. The typical variation law of each index with the variation of NO_3^--N dosage. (a) NO_3^--N=10 mg/L; (b) NO_3^--N=20 mg/L; (c) NO_3^--N= 30 mg/L; (d) NO_3^--N=40 mg/L.

anoxic section, because NO_3^--N was used as an electron acceptor, the denitrification rate was more significant than the rate of COD conversion to PHA and the anaerobic digestion rate, so the COD degradation rate in sewage increased at this time.

Phosphorus absorption and nitrate reduction in the four reactors were carried out simultaneously (Fig. 2), indicating that denitrifying phosphorus absorption reaction had taken place, which proved that there were PAOs with denitrifying function in the activated sludge. However, the phosphorus release and absorption of the four reactors were different and increased with the increase of NO_3^--N concentration; the phosphorus release was 7.52, 9.61, 15.00 and 22.31 mg/L, respectively, and the phosphorus absorption was 11.17, 15.40, 22.01 and 28.97 mg/L, respectively. Furthermore, from Fig. 2(a), when the NO_3^--N dosing concentration was 10 mg/L, the NO_3^--N concentration in the reactor was very low after 210 min of the reaction, resulting in an anaerobic state and endogenous respiratory phosphorus release reaction, i.e., invalid phosphorus release, resulting in a slight increase in the TP concentration of subsequent effluent. On the other hand, when the NO_3^--N concentration was increased to 20 mg/L, 30 mg/L and 40 mg/L, respectively, the electron acceptor was sufficient, the reactor was always anoxic state, and no invalid phosphorus release occurred.

It can also be seen from Fig. 2 that the phosphorus release rate and NO_3^--N reaction rate of each reactor at the initial stage of anaerobic and anoxic were high, and then with the extension of reaction time at each stage the reaction rates of phosphorus release and NO_3^--N gradually slowed. In addition, the initial phosphorus release rate increased with the increase of nitrate concentration. In the anoxic section, the denitrifying phosphorus uptake rate changed considerably under different NO_3^--N concentrations, increasing from 2.00 mg/(L·h) of 10 mg/L NO_3^--N to 24.42 mg/ (L·h) of 40 mg/L NO_3^--N. The reason may be that the dosing concentration of NO_3^--N in A/ASBR1 was low for a long time, the abundance of phosphorus accumulating bacteria in activated sludge was low, the quantity of COD converted into PHA in influent was small, and part of nitrate in sewage was degraded with COD as carbon source. Therefore, the denitrifying phosphorus absorption rate was low or even at zero in the early stage of anoxia. However, the NO_3^--N concentration in A/ASBR3 and A/ASBR4 was relatively high, because the reactors constantly alternated in the anaerobic/anoxic environment. Many phosphorus-accumulating bacteria with denitrifying functions were cultivated and domesticated. As a result, the quantity of COD in the influent that was converted into PHA increased. Most of the NO_3^--N added was denitrified with PHA as the carbon source. Therefore, the phosphorus absorption rate was high in the early stage of anoxia.

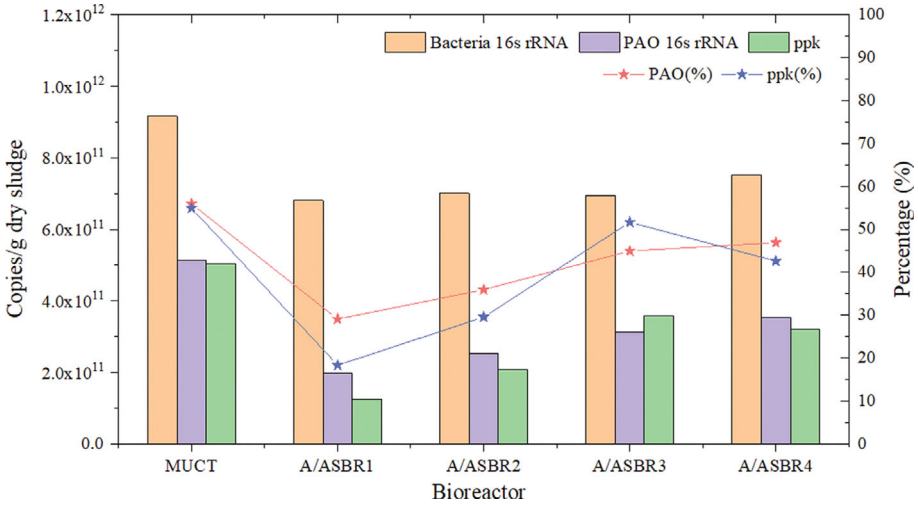

Fig. 3. The number and percentage of 16S rRNA and ppk genes of A/ASBR PAOs.

2. The variation of absolute abundance of PAOs in the A/ASBR system driven by NO_3^--N

During the test, the real-time quantitative PCR determination of bacteria inoculated with sludge and activated sludge of A/ASBR reactor, 16S rRNA of PAOs and ppk gene of PAOs is shown in Fig. 3. In the figure, the ppk gene was the sum of ppk genes of all branches of PAOs. PAOs (%) and ppk (%) refer to the percentage of 16S rRNA of PAOs and the sum of ppk genes in 16S rRNA of bacteria.

The real-time quantitative PCR detection showed that the gene numbers of inoculated sludge bacteria 16S rRNA, PAOs 16S rRNA and ppk were 9.17×10^{11} copies/g dry sludge, 5.13×10^{11} copies/g dry sludge and 5.04×10^{11} copies/g dry sludge, respectively. The percentages of PAOs 16S rRNA and ppk genes were 55.94% and 54.95%, respectively, with a slight difference. Previous research calculated the cell material production of PAOs and the total bacteria by taking the MUCT reactor as the system boundary, based on the principles of material balance and stoichiometry and according to carbon distribution and flow law, nitrogen, and phosphorus. Among them, the production of PAOs accounted for 57.15% of the total bacteria, similar to the result of gene quantity determination of PAOs. In Fig. 3 the number of 16S rRNA genes in the four A/ASBR reactors was basically the same, between 6.81×10^{11} -7.53×10^{11} copies/g dry sludge, but they were all lower than that of the inoculated sludge. On the other hand, with the increase in NO_3^--N concentration, PAOs amount (absolute abundance) in the A/ASBR reactor increased, and the proportion gradually increased. For example, when the NO_3^--N concentration was 10 mg/L, the quantity of PAOs 16S rRNA and ppk genes was 1.98×10^{11} copies/g dry sludge and 1.25×10^{11} copies/g dry sludge, respectively, and the percentage was 29.07% and 18.32%, respectively. However, when the NO_3^--N dosage was 30 mg/L, they were 3.12×10^{11} copies/g dry sludge and 3.59×10^{11} copies/g dry sludge, and the percentages were 44.95% and 51.07%, respectively.

It can also be seen from Fig. 3 that when the NO_3^--N dosage was about 40 mg/L, the number of Acc 16S rRNA genes of phosphorus accumulating bacteria increased to 3.53×10^{11} copies/g dry sludge, but the increase rate decreased. As a result, the number of ppk genes decreased to 3.21×10^{11} copies/g dry sludge. The increase in the number of Acc 16S rRNA genes of phosphorus accumulating bacteria was due to the increased concentration of NO_3^--N, which provided more electron receptors and promoted the growth and reproduction of more PAOs. However, the decrease in the ppk gene can be attributed to more unmeasured PAO branches being cultivated and grown with the increase of NO_3^--N concentration in the system. Mao et al. [43] reported that, besides the seven branches detected in this paper, there are also Accumulibacter IB, IC, ID, IE, IIE, IIG, IIHand other branches. He et al. [35] also reported that phosphorus accumulating bacteria could be divided into 12 branches. Therefore, it can be inferred that NO_3^--N and its dosage were the main driving factors for changing PAOs quantity and composition in the A/ASBR reactor.

3. Variation in the Number and Composition of Each Branch of PAOs Driven by NO_3^--N

According to the real-time quantitative PCR results of the ppk gene in each branch of PAOs, all seven branches were present in each activated sludge sample, indicating that the MUCT system and A/ASBR system maintained a high diversity of branches. The detection results are shown in Fig. 4. The percentage in the figures was the percentage of each branch content in the total ppk gene. It can be seen from Fig. 4(a) that Accumulibacter IIC is dominant in all reactors under different NO_3^--N concentrations and its content is much higher than that of other branches. When the NO_3^--N concentration is 30 mg/L, the quantity of Accumulibacter IIC and IIC(ppk) is the highest. In addition, IIC and IIC (ppk1 excluding OTU NS D3), as two subspecies of the IIC branch, have different abundance changes in the whole system. IIC, compared with IIC (ppk1 excluding OTU NS D3), has an apparent competitive advantage, and its quantity increases with the increase of NO_3^--N concentration, thus accounting for 84.53% of the total Accumulibacter. The number of IA and IID branches also increased with the NO_3^--N concentration, but the percentage of the two branches changed little, close to that of the inoculated sludge.

Previous studies pointed out that when the actual domestic sew-

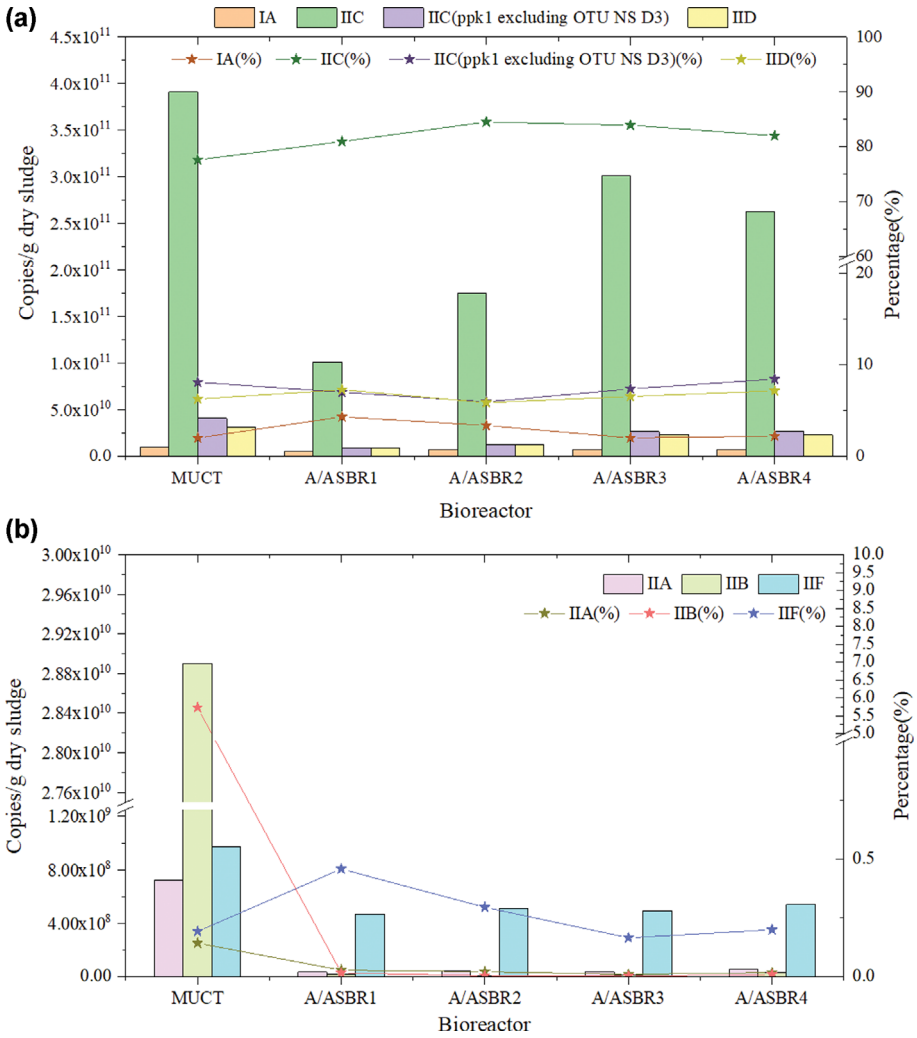

Fig. 4. Absolute abundance and content of PAOs branches. (a) Absolute abundance and content of Accumulibacter IA, IIC and IID; (b) Absolute abundance and content of Accumulibacter IIA, IIb and IIF.

age was treated in the continuous flow process, the Accumulibacter IIC and IID branches had strong denitrifying phosphorus removal capacity, which is similar to the results of this study [44,45]. Another research report pointed out that in the pilot sequencing batch reactor (SBR), the enriched Accumulibacter IIC showed strong NO_3^--N removal capacity. Nevertheless, the literature also points out that not all denitrification contributions can be attributed to this branch [46]. According to the genome sketch results of Accumulibacter IIC, the bacterium lacked nitric oxide reductase (nor) and nitrous oxide reductase (nos) genes and did not have a complete denitrification metabolic pathway. Therefore, it was speculated that other flanking denitrifying bacteria might be involved in denitrifying phosphorus uptake [47]. This may also be why the ppk gene copy number of Accumulibacter IIC decreased when the NO_3^--N dosage increased to 40 mg/L. Therefore, the synergistic mechanism of denitrifying functional flora needed to be further studied. When Accumulibacter IID existed in activated sludge and its content was high, NO_3^--N, NO_2^--N and oxygen could be used as electron receptors to achieve good nitrogen and phosphorus removal effect [27], which is consistent with the results of this study. This was the reason for the high absolute abundance of IID branches in activated sludge.

It has been proved that the Accumulibacter IA branch can use NO_3^--N for denitrifying phosphorus absorption, and it can be seen from the above Fig. 4(a) that the number of this branch gradually increased with the increase of NO_3^--N concentration from 5.37×10^9 copies/g dry sludge increased to 7.17×10^9 copies/g dry sludge. Furthermore, Chao et al. showed that when NO_3^--N was the electron acceptor, the proportion of PAOs in the whole bacteria in the SBR reactor decreased from the initial 2.8% to 0.6%. However, the phosphorus removal performance of the system was enhanced, which may be because Accumulibacter IA with NO_3^--N as the electron acceptor was enriched. Hence, consistent with the research result, IA accounted for a relatively high proportion [48].

Therefore, with the increase of NO_3^--N dosing concentration, the number of copies of Accumulator IA and Accumulator IID genes increased, respectively. In contrast, the number of Accumulator IIC and Accumulator IIC (ppk1 excluding OTU NS D3) showed an overall increasing trend (decreased at 40 mg/L). This indicates that NO_3^--N gradually provided sufficient electron recep-

tors for A/ASBR, promoted the alternative circulation of activated sludge in an anaerobic/anoxic environment, and domesticated more PAOs branches with denitrifying functions. This was consistent with the above experimental conclusion that the higher the NO_3^--N concentration, the lower the total phosphorus concentration in the effluent, and the better the phosphorus removal effect. Therefore, the change in the number and composition of PAOs was the microbiological explanation for the effect of denitrifying phosphorus removal.

It can be seen from Fig. 4(b) that the absolute abundance of branches IIA, IIB and IIF was statistically significantly lower than that of other branches, indicating that they contributed less to the whole process (not the core genus). Metagenomic analysis showed that the IIA branch could not utilize NO_3^--N because it did not have nitrate reductase (nar) [48]. Flowers [49] designed a 16S rRNA fluorescence in situ hybridization (FISH) probe to analyze two different types of branches of Candidatus Accumulibacter. It was considered that the IA branch hybridized with probe Acc-I-444 had NO_3^--N removal ability and the corresponding cell shape was rod type. However, the IIA branch hybridized with probe Acc-II-444 did not have nitrogen removal ability and was spherical. This result is consistent with the previous metagenomic results and Carvalho et al. [50].

It can also be seen from Fig. 4(b) that the number of IIB branches in the inoculated sludge was 2.89×10^{10} copies/g dry sludge and a percentage of 5.74% of the total ppk gene of PAOs. However, the proportion gradually decreased during the later culture. The quantity of the four A/ASBRS was 2.08×10^{7} copies/g dry sludge, 9.65×10^{6} copies/g dry sludge, 1.07×10^{7} copies/g dry sludge, 3.12×10^{7} copies/g dry sludge, respectively. The proportion was very low, close to zero. This may be due to the physiological and biochemical characteristics of the strain itself. Some studies have suggested that branch IIB can use NO_3^--N as an electron acceptor, which may be the reason why the branch had not been wholly elutriated out of the system. Branch IIF contained the periplasmic nitrate reductase gene (nap), which can use NO_3^--N as an electron acceptor. However, it only accounted for a small proportion of PAOs in the inoculated sludge and A/ASBR system and had never become a dominant bacterium.

To sum up, the inoculated sludge of the four A/ASBR reactors in this study was from the same reactor simultaneously, and the treated wastewater was from the same distribution tank. Except that the NO_3^--N concentration was different at the end of anaerobic and the initial stage of anoxia, other operating parameters and cycles were precisely the same. Therefore, it can be inferred that the main driving factor for the change in the quantity and composition of activated sludge microbial PAOs and each branch was the concentration of NO_3^--N.

In this study, the response law of the number and composition of phosphorus accumulating bacteria and their branches to the change of NO_3^--N dosing concentration was consistent with the macro research results of the impact of NO_3^--N on denitrifying phosphorus absorption performance in the earlier stage [14-16,31]. The combination of the two can provide a scientific basis for developing macro-control parameters and strategies for the simultaneous nitrogen and phosphorus removal system and achieving the stability of the effective control system and the purpose of functional optimization.

CONCLUSIONS

This study determined whether the concentration of NO_3^--N is the driving factor of PAOs bacterial population structure under anoxic conditions. The A/ASBR reactor was operated to conduct a fluorescence real-time quantitative PCR test. In addition, the composition and structure changes of phosphorus accumulating bacteria and their branches under different NO_3^--N concentrations were investigated. The test conclusions are as follows:

1. The absolute abundance of the 16S rRNA gene in the A/ASBR reactor had nothing to do with the NO_3^--N, which was at 6.81×10^{11} -7.53×10^{11} copies/g dry sludge.
2. With the increase of NO_3^--N concentration, the amount (absolute abundance) of PAOs in the A/ASBR reactor increased, and the proportion gradually increased. The NO_3^--N dosage increased from 1.98×10^{11} copies/g dry sludge at 10 mg/L to 3.59×10^{11} copies/g dry sludge at 40 mg/L.
3. With the increase of NO_3^--N concentration, the quantity and composition of the denitrifying Accumulator IA, Accumulator IIC, Accumulator IIC (ppk1 excluding OTU NS D3) and Accumulator IID in the branches of phosphorus accumulating bacteria changed significantly. The proportion of Accumulator IIA, IIB and IIF in A/ASBR activated sludge was small, even close to zero, but they were not scoured out of the system.
4. NO_3^--N and variation in concentration affected the number and composition of PAO and its branches in the A/ASBR reactor; thus, it is the main driving factor of the PAO flora structure.

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NOMENCLATURE

A/ASBR : anaerobic/anoxia sequencing batch reactor
ASM2 : activated sludge reaction kinetic model 2
ASM2d : activated sludge reaction kinetic model 2d
COD : chemical oxygen demand
DNA : deoxyribonucleic acid
FISH : fluorescence in situ hybridization
IWA : International Water Association
MLSS : mixed liquor suspended solids
MLVSS : mixed liquor volatile suspended solids
MUCT : modified university of cape town
nap : nitrate reductase gene
nar : nitrate reductase
nor : nitric oxide reductase
nos : nitrous oxide reductase
ORP : oxidation-reduction potential
PAOs : polyphosphate accumulating organisms
PCR : polymerase chain reaction

PHA : poly-β-hydroxy-alkanoates
PHB : poly-β-hydroxy-butyrate
PLC : programmable logic controller
poly-P : polyphosphate
ppk : polyphosphate kinase
qPCR : real-time fluorescent quantitative PCR
SBR : sequencing batch reactor
TN : total nitrogen
TP : total phosphorus
UCT : university of cape town
WWTP : wastewater treatment plant

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