PHOSPHORUS RELEASE AND UPTAKE OF A DENITRIFYING PHOSPHORUS-ACCUMULATING BACTERIUM WITH DIFFERENT ELECTRON ACCEPTORS

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Abstract. The phosphorus release (anaerobic) and phosphorus uptake (anoxic) capacity of one denitrifying phosphorus-accumulating bacterial strain, identified as *Acinetobacte*r by 16S rRNA sequence, and the influence of electron acceptors (NO_3^- and NO_2^-) were explored. Batch experiments with different environmental factors including pH. The types and levels of electron acceptors were performed. The results showed that anaerobic phosphorus release was greater in neutral and alkaline environments. Both nitrate and nitrite can act as electron acceptors to enable anoxic phosphorus uptake. In addition, the phosphorus uptake in the nitrate system was relatively higher than in the nitrite system in a neutral-alkaline environment (pH = 7 and 9), the average percentage of phosphorus uptake in the nitrate system reached 15.0 mg/L. For nitrite, the uptake percentage slowly increased with the increase of pH, from 9.5 mg/L (pH = 3) to 14.3 mg/L (pH = 7 and 9). The optimal stoichiometric concentration of electron acceptors and pH for phosphate removal in nitrate and nitrite systems appeared at ρ(nitrate) = 30 mg L^{-1}, pH = 9, and ρ(nitrite) = 5 mg L^{-1}, pH = 7, respectively. Our results indicated that *Acinetobacter* N-8 had a strong capacity for denitrifying phosphorus- accumulation and the utilization of oxygen, nitrate, and nitrite as electron acceptors, but the efficiency is significantly impacted by environmental pH.
Keywords: *activated sludge, Acinetobacter, pH, nitrate, nitrite*

Introduction

Phosphorous and nitrogen pollutants compounds are the primary causes of eutrophication in rivers, inland lakes and reservoirs (Chislock et al., 2014). In recent years, forty percent of reservoirs and lakes worldwide have faced varying degrees of eutrophication, approximately (Fu, 2016). Previous researches reported that nitrogen and phosphorus are the main factors affecting water quality improvement during the prevention and control of water pollution (Achieng et al., 2017; Yu et al., 2017). Therefore, the primary means to mitigate the problem is through efficient nitrogen and phosphorus removal. At present, the main conventional nitrogen and phosphorus removal technologies are physical, chemical (Fukahori et al., 2015; Srithep and Phattarapattamawong, 2017) and biological methods (Emara et al., 2014; Simsek et al., 2012). Recently, microbiologically based bioremediation technology is of increasing interest because of its advantages, including low cost, convenient operation, significant treatment effect, and low secondary pollution (Singh et al., 2019). The increasing number of wastewater treatment plants (WWTPs) removed nitrogen and phosphorus pollutions adopted biological methods for decades (Joshi et al., 2018; Zhang et al., 2018). Biological phosphorus removal from wastewater is based on the activity of phosphorus-accumulating bacteria, while biological nitrogen removal primarily relies on the nitrate

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reduction provided by denitrifying bacteria under anoxic conditions. However, denitrifying bacteria and phosphorus-accumulating bacteria compete for carbon sources.

The discovery of denitrifying phosphorus-accumulating bacteria (DPAB) and further research on the theory of denitrifying nitrogen and phosphorus removal provide new concepts and perspectives for the improvement and innovation of advanced treatment technology enabling simultaneous nitrogen and phosphorus removal. Denitrifying nitrogen and phosphorus removal technology overcomes the competition for carbon sources encountered in traditional treatment technologies and enables the full utilization of the carbon source (Lee et al., 2001). DPAB are regarded as one kind of facultative anaerobic bacteria that can simultaneously use O_2 and NO_3^--N or NO_2^--N as the final electron acceptors to assimilate phosphorus under anoxic or aerobic conditions (Li and Huang, 2013; Xie et al., 2016). Recently, a lot of previous researches has reported the progress of isolation and biological characteristics of DPAB (Ling et al., 2015; Miao et al., 2016), with the isolation results showed the DPAB mainly include *Acinetobacter* (Tsuneda et al., 2006), *Aeromonas* (Qiang et al., 2008) *Paracoccus*, *Planctomycetes* (Liu et al., 2013) and so on. Regarding biological characteristics, the influences on the growth and degradation of DPAB have also been studied to optimize the function of DPAB to removal efficiency of nitrogen and phosphorus pollutants via a biological denitrifying-phosphorus process. Xie et al. (2016) isolated one denitrifying phosphorus-accumulating strain from activated sludge, and the results show this DPAB strain has high removal capacity for both NO_3^--N and PO_4^{3-}-P (87% and 75%, respectively). Wan et al. (2017) also showed that DPAB have good performance in denitrification and phosphorus removal and can overcome the competition for the carbon source between anaerobic phosphorus release and denitrification.

For efficiency improvement purpose, many previous works have investigated the influencing factors of denitrifying phosphorus-accumulating bacteria. Researchers believed that pH (Li et al., 2018), temperature (Figdore et al., 2018), dissolved oxygen (Yuan and Oleszkiewicz, 2011), sludge retention time (Merzouki et al., 2001) and carbon source (Sun et al., 2016) could affect the metabolism of denitrifying phosphorus-accumulating microorganisms. Among them, pH is regarded as an important control index which indicates the potential of biological phosphorus removal, and it is a crucial factor which impacts the surface chargeability and permeability of microbial cell (Zafiriadis et al., 2011). However, few reports have investigated the effects of types of electron acceptors on the anaerobic phosphorus release and anoxic phosphorus uptake activity of DPAB. In the present study, the anaerobic phosphorus release and the effects of two key electron acceptors (NO_3^- and NO_2^-) on anoxic phosphorus uptake were investigated to obtain a better understanding of the metabolic behavior of DPAB by using a strain screened from activated sludge. In addition, the influences of environmental factors, including pH and electron acceptor types and levels, on the activity of removal efficiencies were studied.

Materials and methods

Media

All biochemical reagents were of analytical grade. The pH was adjusted using 0.1 mol L^{-1} HCl and 0.1 mol L^{-1} NaOH. Basically, phosphorus-accumulating organism isolation medium, beef-peptone medium, phosphorous-limited medium and phosphorous-rich medium used in this work were prepared as shown in *Table 1*.

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Table 1. *The constituents of the media adopted in this work*

Media	Constituents	Quantity	pH	Reference
Phosphorus-accumulating organisms isolation medium (1000 mL)	$CH_3COONa·3H_2O$	3.6 g	7.0	Xie et al. (2016)
	$MgSO_4·7H_2O$	0.132 g		
	$Na_2HPO_4·2H_2O$	0.0287 g		
	K_2SO_4	0.027 g		
	NH_4Cl	0.0573 g		
	$CaCl_2·2H_2O$	0.017 g		
	Agar	20 g		
	HEPES buffer	12 mL		
	Trace element solution	2 mL		
Trace element solution (1000 mL)	$FeCl_3·6H_2O$	1.5 g	7.0	Sun et al. (2015)
	H_3BO_3	0.15 g		
	$CuSO_4·5H_2O$	0.03 g		
	$Na_2MoO_4·2H_2O$	0.06 g		
	KI	0.03 g		
	$MnCl_2·4H_2O$	0.12 g		
	$ZnSO_4·7H_2O$	0.12 g		
	$CoCl_2·2H_2O$	0.12 g		
Beef-peptone medium (1000 mL)	Peptone	10 g	7.2	Xie et al. (2016)
	Beef	3 g		
	NaCl	5 g		
	Agar	20 g		
Phosphorous-limited medium (1000 mL)	CH_3COONa	3.32 g	7.0	Merzouki et al. (1999)
	$Na_2HPO_4·2H_2O$	0.023 g		
	$MgSO_4·7H_2O$	0.081 g		
	K_2SO_4	0.018 g		
	NH_4Cl	0.153 g		
	$CaCl_2·2H_2O$	0.011 g		
	HEPES buffer	7 mL		
	Trace element solution	2 mL		
Phosphorous-rich medium (1000 mL)	CH_3COONa	3.32 g	7.0	Xie et al. (2016)
	KH_2PO_4	0.025 g		
	$MgSO_4·7H_2O$	0.091 g		
	$CaCl_2·H_2O$	0.026 g		
	NH_4Cl	0.305 g		
	PIPES buffer	8.5 mL		
	Trace element solution	2 mL		
Nitrate reduction medium (1000 mL)	KNO_3	1 g	7.0	Sun et al. (2015)
	K_2HPO_4	2.42 g		
	$C_6H_{12}O_6$	1 g		
	Agar	1 g		
	Peptone	20 g		

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Analysis methods

The final data were presented in terms of mean ± standard deviation (SD) from all the replicates. Statistical analysis, such as correlation analysis and paired-samples T-test was carried out using Statistical Product and Service Solutions (SPSS, version 20.0). Bacterial growth was measured with a Finland Bioscreen Automatic Growth Curve Analyzer. The absorbance was represented by optical density (OD) at 600 nm. The pH value was measured with a METTLER FE20-FiveEasy Plus™ pH meter, and PO_4^{3-}-P was detected using Mo-Sb spectrophotometry. NO_3^--N and NO_2^--N concentrations were determined by using the phenoldisulfonic acid method and α-naphthylamine spectrophotometry, respectively. Each treatment was performed in triplicate.

Enrichment culture of DPAB

The activated sludge used in this study was inoculated in an operating activated sludge reactor located in Beijing Normal University, Beijing, China. Ten grams of active sludge, which had been cleaned three times with distilled water at 200 rpm for 3 min, was added to the beef-peptone medium (200 mL), followed by 2 days' incubation at 30 °C and 140 rpm min^{-1}. One milliliter of culture was subsequently added to the triangle bottle with 9 mL sterile water and thus formed the 10^{-1} diluent solution. Serial dilutions of bacterial suspensions from 10^{-1} to 10^{-8} were obtained using the same method, and the phosphate-accumulating organisms (PAO) isolation mediums were inoculated with 0.1 mL of each dilution in duplicate and cultured in a 30 °C incubator for 2-3 days. Single colonies were sub-cultured by picking and streaking three times to obtain isolated pure colonies (Sun et al., 2015).

Screening of strains

Screening for phosphate uptaker: single colonies were isolated and streaked on phosphorous-limited medium (100 mL) followed by 2-days incubation at 30 °C, 140 rpm min^{-1}. These cultures were centrifuged at 10^4 rpm for 2 min, after which the supernatant was removed. Then, the cultures were re-suspended in 200 mL of phosphorus-rich medium and incubated under the same conditions for 2 days to an OD_{600} of 0.1. The absorbance of PO_4^{3-}-P in the culture supernatant was measured via spectrometry as described. The phosphorus removal rates were calculated from the changes in absorbance. The following equation was used to determine phosphorus removal:

$$R=(C_0-Ct)/C_0\times 100\% \quad \text{(Eq.1)}$$

where C_0 represents the initial concentration, and C_t indicates that at time t.

The strains with higher phosphorus uptake rate (more than 50%) were chosen for the following Poly-P and poly-β-hydroxybutyrate (PHB) staining. Positive strains were chosen as DPAB. All the strains were preserved in beef-peptone medium slant cultures at 4 °C. Then screening for DPAB has been carried out on the denitrification medium with and without (Blank) KNO_3 as a substrate. Strains were inoculated on nitrate reduction medium and incubated at 30 °C for 7 days with observation every day.

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Identification of strains

Strain characteristics were determined according to previously described methods (Sun et al., 2015). Bacterial genomic DNA was used as a template to amplify 16S rRNA with a pair of universal primers: forward primer (F), CCTCCTACGGGNBGCASCAG, reverse primer (R), GACTACNVGGGTATCTAATCC. All PCR reactions were carried out in 30 μL reactions with 15 μL of Phusion High-Fidelity PCR Master Mix (New England Biolabs), 0.2 μmol of forward and reverse primers, and approximately 10 ng of template DNA. Thermal cycling consisted of initial denaturation at 98 °C for 1 min followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s, and elongation at 72 °C for 60 s, with a final cycle at 72 °C for 5 min. The same volume of 1 × loading buffer (containing SYB green) was mixed with PCR products and electrophoresed on 2% agarose gel for detection. Samples with a bright main strip between 400 and 450 bp were chosen for further experiments. PCR products were mixed in equidensity ratios. Then, the combined PCR products were purified with the GeneJET Gel Extraction Kit (Thermo Scientific). As a template to build the library, a computer test was conducted.

The screened strain was placed in beef-peptone medium (200 mL) with the initial $OD_{600} \approx 0$ and cultured at 30 °C for 48 h. Then, the bacterial growth curve was drawn based on the measured OD_{600}.

Effects of different electron acceptors

Denitrifying phosphorus-accumulating bacteria has been enriched via alternative anaerobic and anoxic operations. The batch experiments of anaerobic phosphorus release and anoxic phosphorus uptake were performed with different environmental factors in order to examine the influence of the initial pH and the types and levels of electron acceptors on the efficiency of denitrifying-phosphorus removal.

The entire experiments were operated with a cycle of 7 h, consisting of a 4-h anaerobic stage and a 3-h anoxic stage. During the initial 4 h anaerobic stage, 160 μL of phosphorus-rich medium with different pH values and 40 μL of bacterial suspension (OD_{600} = 0.4) were fed into the 96-well plates, forming suspensions. During the anoxic stage, 50 μL of different concentrations of electron acceptor solution (nitrate or nitrite solution) was added. Specifically, the concentration levels of electron acceptors are set as follows: 10, 20, 30, 40 and 50 mg L^{-1} for nitrate and 1, 2, 3, 4 and 5 mg L^{-1} for nitrite. The environmental pH values were set as 3, 5, 7, 9 and 11. The initial phosphate concentration in the system was 25 mg L^{-1}. Each treatment was performed three times. The following equation was used to determine phosphorus release and uptake amount:

$$R_{releace}=(C_{4h}-C_0)\times 100\% \quad \text{(Eq.2)}$$

$$A_{uptake}=C_{4h}-C_{7h} \quad \text{(Eq.3)}$$

where $R_{release}$ is phosphorus release rate, A_{uptake} is phosphorus uptake amount, C_0 represents the original concentration of PO_4^{3-}-P, C_{4h} represents the concentration of PO_4^{3-}-P at the end of the anaerobic stage (4 h), and C_{7h} represents the concentration of PO_4^{3-}-P at the end of anoxic stage (7 h).

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Results and discussion

Strain enrichment, screening and identification

Strain enrichment and screening

The screening results show that 21 colonies with various morphologies were selected and sub-cultured from selected mediums of 10^{-6} culture. The isolation of single-colony was carried on with phosphorous-limited culture medium, and then phosphate uptakers were screened according the growing conditions.

Eleven colonies were selected because they exhibited phosphorus removal rates exceeding 50% (*Fig. A1* in the *Appendix*). Staining for Albert's metachromatic granules, staining for PHB granules and screening for denitrifiers among these 11 strains resulted in one all positive strain, as shown in *Table A1*, named N-8. Thus, we identified this strain as DPAB (Sun et al., 2015). In other words, N-8 could obtain denitrification and phosphorus removal, simultaneously. We then stored the strains in a refrigerator at 4 °C for the subsequent experiments.

Morphological characteristics and 16S rRNA sequence analysis

Round milk-white colonies with neat edges were been formed by N-8. The colonies were non-transparent, and had a smooth, moist surface. The gram staining experiment results showed that the bacteria were gram-negative. A scanning electron microscope image of N-8 is shown in *Figure 1*. These germ cells were catenary, and with dimensions of 1.0-1.5 μm × 1.0-1.2 μm. Granule staining results illustrated the accumulation of PHB and Poly-P particles present in these cells (*Fig. A3*).

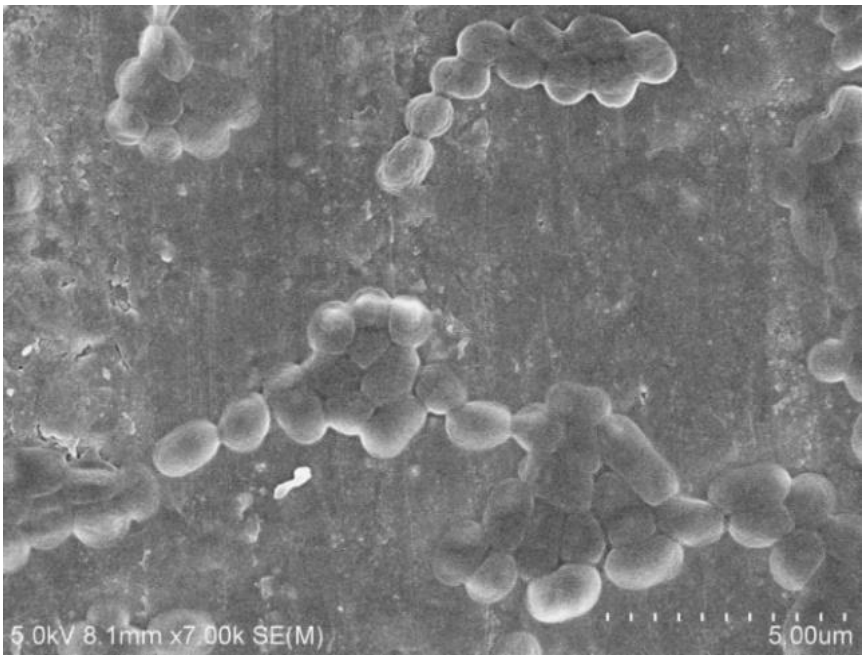

Figure 1. *Scanning electron microscope image of denitrifying phosphorus-accumulating bacteria strain N-8*

The 16S rDNA sequences of N-8 were submitted to the GenBank database and analyzed against known gene sequences using BLAST. The N-8 strain rDNA exhibited

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more than 90.15% identity with ID 219151. Through the comparison and annotation of the GreenGenes database, strain N-8 was determined to be *Acinetobacter*. The top 20 multi-sample operational taxonomic units (OTUs) and the phylogenetic trees are shown in *Figure 2*, including N1, N2, N3 from three parallel experiments. The results for 16S rDNA of DPAB in Sun's experiments showed that the strain rDNA exhibited 99.1% similarity to the known *Thauera linaloolentis* (Sun et al., 2015). Tsuneda et al. (2006) demonstrated that one strain of DPAB, *Acinetobacter*, could be found in a sequencing batch reactor and could have high average nitrogen and phosphorus removal efficiencies.

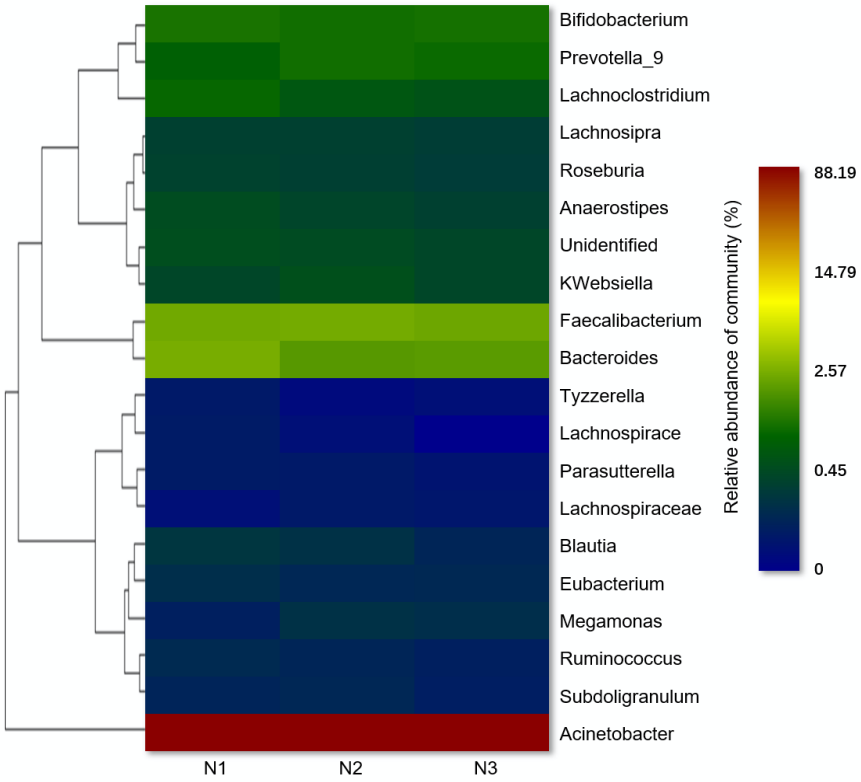

Figure 2. *The heatmap-phylogenetic trees of the top 20 multi-sample OTUs*

Growth curve of bacteria

Using the absorbance of bacteria at 600 nm to estimate bacterial growth was the principal method. The results of bacterial growth in beef-peptone medium are shown in *Figure 3*. Within the 6 h adaption period from inoculation, the population of the N-8 strain grew slowly. From 6 h to 25 h, logarithmic growth occurred, during which time N-8 grew rapidly, and the OD_{600} increased from 0.042 to 1.003. After 25 h, the growth rate of the N-8 strain starting to decreased, and the OD_{600} remained stable at 1.0, approximately. The results (*Fig. 3*) showed that the population of the N-8 strain varied with time during the logarithmic period, significantly.

Anaerobic phosphorus release

The anaerobic phosphorus release experiment was carried out with initial phosphate concentration was 25 mg L^{-1}. The phosphorus variation of the systems during the

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anaerobic period is shown in *Figure 4*. In this part, we used the phosphorus release rate follow *Equation 2* to characterize whether phosphorus release occurs. The $R_{release}$ greater than 1 indicated the occurrence of phosphorus release, whereas a value less than 1 indicated the absence of phosphorus release. The results show that phosphorus release will occur under anaerobic conditions and only in neutral and alkaline environments. *Figure 4* shows that the phosphorus contents in the neutral and alkaline environments (pH = 7, 9 and 11) were all higher than 1.0 and have the significantly different with that in the acid conditions (pH = 3 and 5) at $p < 0.05$ level based on the paired-samples T-test. The average values of phosphorus release rate at pH = 7, 9, 11 were 1.21 (±0.23), 1.19 (±0.20) and 1.17 (±0.18), respectively. In contrast, the contents under acidic conditions (pH = 3 and 5) were less than 1, meaning that no phosphorus release occurred. Thus, phosphorus release was more likely to occur in neutral and alkaline environments for DPAB, which may occur because the acidic conditions are not suitable for the growth of these microorganisms (Tsuneda et al., 2006). The phenomenon of phosphorus release may be caused by the microbes' poly-P degradation (Thwaites et al., 2018) to maintain their own activities in the absence of electronic receptors and HAc.

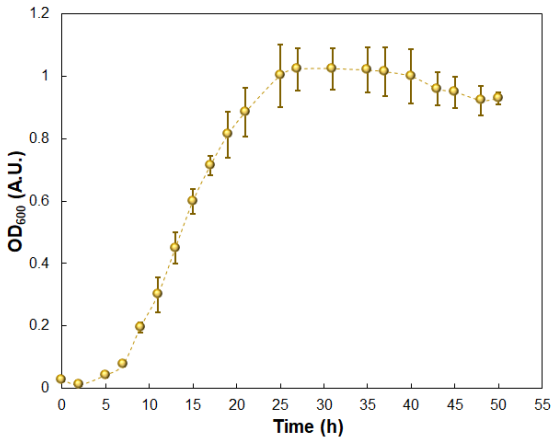

Figure 3. *Growth curve of denitrifying phosphorus-accumulating bacteria strain N-8 in 48 h*

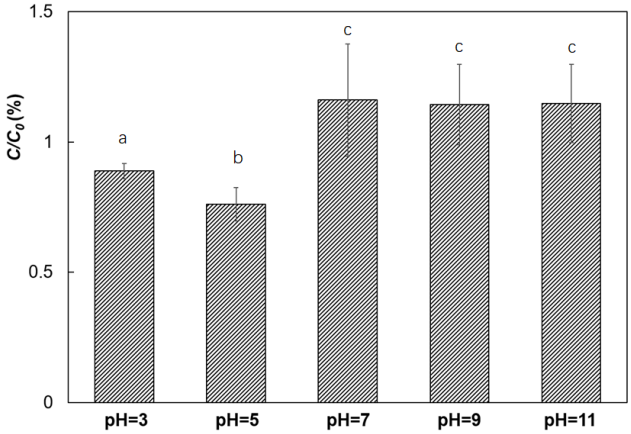

Figure 4. *The phosphorus release rate after the anaerobic period (4 h). Data are mean ± standard deviation and the maximum extent of their errors is shown as the error bar. Bars with the same letter are not significantly different at $p < 0.05$*

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Anoxic phosphorus uptake

Batch experiments in this part were performed to investigate the effect of different types and concentrations of electron acceptors (NO_3^- and NO_2^-) on the anoxic phosphorus uptake of DPAB under different pH conditions. The batch experiments were defined as A, B, C, D and E, with nitrate concentrations of 10, 20, 30, 40, and 50 mg L^{-1} or nitrite concentrations of 1, 2, 3, 4 and 5 mg L^{-1}, respectively. The pH values were 3, 5, 7, 9 and 11. At the beginning of the anoxic stage, electron acceptors (nitrate or nitrite) in different concentrations were added to start the phosphorus uptake of DPAB.

The concentrations of phosphate at the end of a cycle (C_{7h}) are shown in *Figure 5a*. According to *Equation 3*, the values and changes of phosphorus uptake with each sample are shown in *Figure 5b*. The results show that the phosphorus contents of all systems at the end of anoxic stage decreased. So, we can conclude anoxic phosphorus uptake did occur with both nitrite and nitrate as electron acceptors. And from *Figure 5b* we could speculate the average phosphorus uptake of nitrite system (12.5 ± 2.3 mg/L) was higher than nitrate (10.3 ± 4.8 mg/L, $p < 0.05$). But when pH = 7 and pH = 9, the amount of nitrate system (15.0 ± 2.7 mg/L) slightly higher than that of nitrite system (14.3 ± 1.7 mg/L) with no significant difference ($p > 0.05$). Zhou et al. (2010) demonstrated that anoxic phosphorus uptake could be achieved successfully by using nitrate or nitrite as the electron acceptor, and the relatively small amount of anoxic phosphorus was taken up by microorganisms with nitrite for one DPAB. There was a slight difference in activity between the two types of electron acceptor systems, and the amount of phosphorus uptake under anoxic conditions also had significant relationships with pH and the electron acceptor concentration.

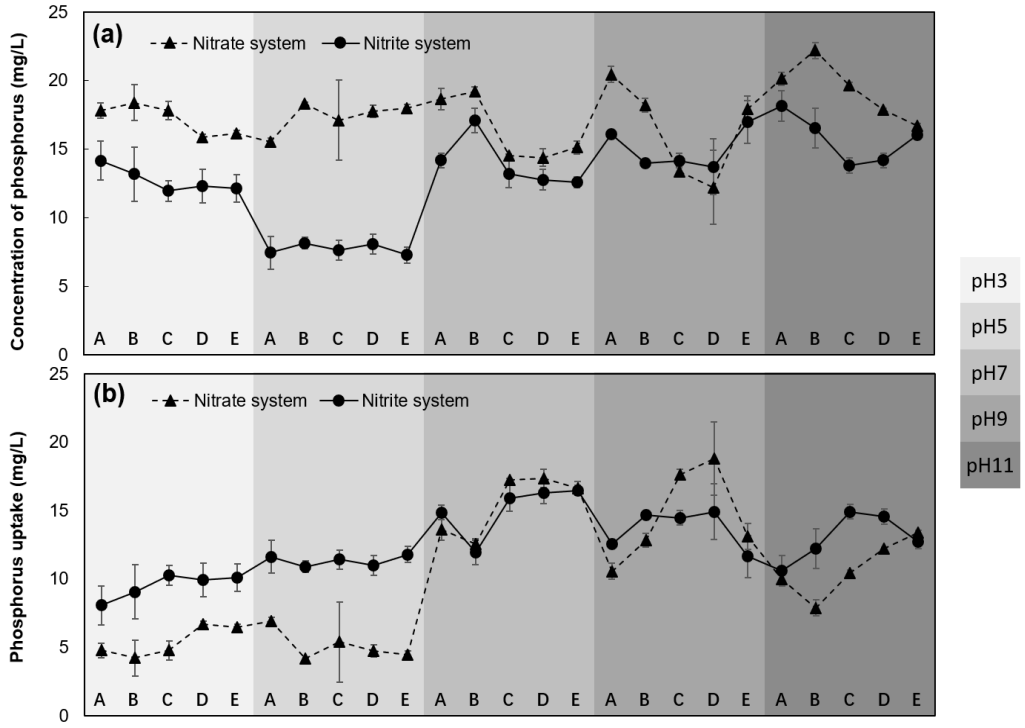

Figure 5. *Concentration of phosphate (a) at the end of the anoxic stage (C_{7h}) and the amount of phosphate uptake (b) during the anoxic stage. From A to E represent the concentration of nitrate (10 mg L^{-1}, 20 mg L^{-1}, 30 mg L^{-1}, 40 mg L^{-1} and 50 mg L^{-1}, respectively) and nitrite (1 mg L^{-1}, 2 mg L^{-1}, 3 mg L^{-1}, 4 mg L^{-1} and 5 mg L^{-1}, respectively)*

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Phosphorus uptake among different pH systems exhibited obvious differences, with better efficiency in neutral-alkaline environments. The changes in the nitrate system were more significant than those in the system with nitrite as the electron acceptor. For nitrate as the electron acceptor, the amount of uptake in neutral-alkaline environments was significantly higher than that in acidic environments, with average uptake amount of 13.6 ± 3.2 mg/L and 5.2 ± 1.0 mg/L, respectively. In contrast, with nitrite as the electron acceptor, the increased uptake with the increase of pH was from 9.5 ± 0.8 mg/L (pH = 3) to 15.1 ± 1.7 mg/L (pH = 7), stabilized at 13.6 ± 1.3 mg/L (pH = 9) and 13.0 ± 1.6 mg/L (pH = 11). The level of phosphorus uptake under acidic conditions (pH = 3 and 5) with nitrite as the electron acceptor was better than that in the nitrate system ($p < 0.01$). One reason for this phenomenon may be that the nitrite aqueous solution is alkaline (Mack and Bolton, 1999), and it can neutralize a portion of the hydrogen ions.

Phosphorus uptake had been influenced significantly by the initial concentration of nitrate or nitrite. According to paired-samples T-test, A level vs. C, D and E level, and B level vs. C, D, and E level showed a significant difference ($p < 0.05$), but C, D and E did not show that with each other. The highest average phosphorus uptake showed at C level whether for nitrate (15.5 mg/L) or nitrite (15.1 mg/L), then decreased with the increased or decrease of electron acceptor concentration. Therefore, within a certain range, a higher electron acceptor concentration could promote the activity of anoxic phosphorus uptake. However, if the concentration rose beyond the optimal value, it would be inhibitory to phosphorus removal. Zhou et al. (2010) showed that phosphorus uptake was impacted by the limitation of electron acceptor when limited concentration of nitrate present under anoxic conditions, but when the concentration out of the range of 60 to 120 mg L^{-1}, the activity was not further promoted. Thus, ρ(nitrate) = 30 mg L^{-1} and ρ(nitrite) = 3 mg L^{-1} are proved to be the optimal concentration of electron acceptor for the phosphorus uptake efficiency in this study.

Phosphorus removal efficiency

The profiles of denitrifying-phosphorus removal are shown in *Figure 6a*. The removal rate was evaluated as *Equation 1*. The phosphorus removal rates of the nitrite systems were higher than those of the nitrate systems ($p < 0.01$). The average rates of the nitrite systems were $49.2 \pm 3.3\%$ (pH = 3), $69.2 \pm 1.4\%$ (pH = 5), $44.2 \pm 6.7\%$ (pH = 7), $40.1 \pm 5.2\%$ (pH = 9) and $49.2 \pm 3.3\%$ (pH = 11), respectively, while for the nitrate systems, they were $31.2 \pm 4.0\%$ (pH = 3), $30.7 \pm 4.0\%$ (pH = 5), $34.6 \pm 8.4\%$ (pH = 7), $34.3 \pm 12.5\%$ (pH = 9) and $22.8 \pm 7.6\%$ (pH = 11), respectively. Similarly, Zeng et al. (2013) showed that phosphorus removal efficiency under a nitrite system was higher than that in a completely nitrifying environment. Moreover, the denitrifying-phosphorus removal rate increased with the increased initial concentration at low conditions of both electron acceptors, and high concentrations will inhibit the effect, such as pH = 9, ρ(nitrate) = 50 mg L^{-1}, ρ(nitrite) = 5 mg L^{-1} and pH = 11 ρ(nitrite) = 4 and 5 mg L^{-1}. The optimal stoichiometric concentration of electron acceptor and pH for the phosphate removal by microorganisms in the nitrate and nitrite systems appeared at ρ(nitrate) = 30 mg L^{-1} at pH = 9 and ρ(nitrite) = 5 mg L^{-1} at pH = 7, respectively.

The influence of pH on phosphorus removal was somewhat complicated (Li et al., 2018) and could be divided into two circumstances. The microorganism played a significant role on removal rates under neutral and alkaline conditions. However, the removal rate was mainly controlled by the physical and chemical processes under acidic conditions because the most suitable pH for DPAB was pH = 7~9 (Sun et al., 2016).

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Another reason for this phenomenon was the physical properties of nitrite, for which the aqueous solution was alkaline (Mack and Bolton, 1999). Under acidic environments, greater H^+ is consumed with the increased pH.

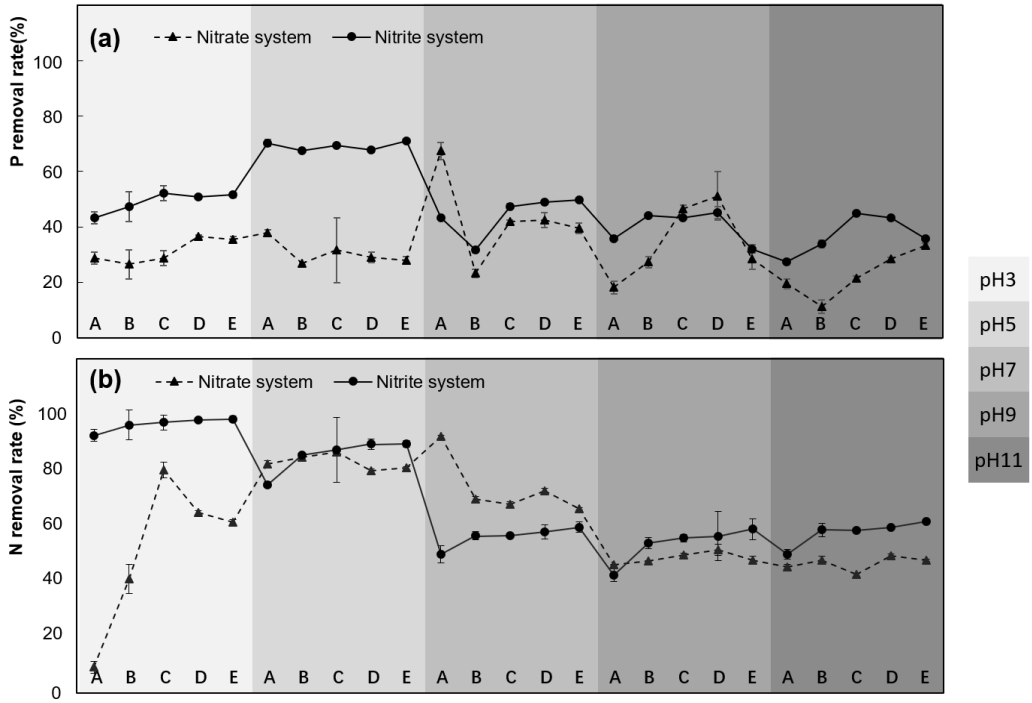

Figure 6. *Removal rate of phosphate (a) nitrogen (b) during the cycle. From A to E represent the concentration of nitrate (10 mg L^{-1}, 20 mg L^{-1}, 30 mg L^{-1}, 40 mg L^{-1} and 50 mg L^{-1}, respectively) and nitrite (1 mg L^{-1}, 2 mg L^{-1}, 3 mg L^{-1}, 4 mg L^{-1} and 5 mg L^{-1}, respectively)*

Nitrogen removal efficiency

The results of nitrogen removal in the two systems obtained from the tests are shown in *Figure 6b*. Regarding the variation of nitrogen removal, there was no significant difference between nitrate and nitrite systems with various environmental factors, except at pH = 3. The removal rates in the nitrite systems were obviously higher than those in the nitrate systems at pH = 3. The influence of pH on nitrogen removal was similar to its influence on phosphorus removal, with a higher removal rate in nitrite systems in acidic environments (pH = 3 and 5) and higher removal rates in nitrate systems in neutral-alkaline environments (pH = 7, 9 and 11). The highest nitrogen removal rates for nitrate and nitrite were observed around ρ(nitrate) = 10 mg L^{-1} at pH = 7 and ρ(nitrite) = 5 mg L^{-1} at pH = 3, respectively. Additionally, nitrogen removal in nitrite systems slowly increased with an increased electron acceptor concentration in neutral-alkaline environments, whereas no such changes were observed in nitrate systems, as shown in *Figure 6*. The higher nitrogen removal by microorganisms appeared at pH = 5, with removal rate of 82.69% (±2.66%) for nitrate and 85.36% (±6.13%) for nitrite.

Correlation and comparison

The correlation analysis of phosphate removal rate (R_P) and nitrate removal rate (R_N) of the entire operation was investigated, as shown in *Table 2*. Pearson's correlation

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analysis results revealed considerable differences between the two types of systems under different conditions. In the nitrate system, the Pearson's correlation coefficient (PCC) between R_P and R_N with all pH levels showed no obvious correlation, with PCC = 0.37 (P = 0.068). In the nitrite system, there was a significant positive correlation between the removal of PO_4^{3-} and the removal of NO_2^-, with PCC = 0.624 (P = 0.001). The removal of nitrogen-phosphate at all pH levels in the nitrite system appears to be more complicated than that in the nitrate system. Moreover, under neutral-alkaline conditions, the correlation coefficient in the nitrate system, PCC = 0.686 (P = 0.005), was much higher than that for nitrite.

The experimental results discussed above reveal that both nitrate and nitrite as electron acceptors were effective for DPAB in denitrifying-phosphorus removal. Phosphorus removal was mainly completed by denitrifying-phosphorus removal occurring under anoxic conditions. The level of phosphorus uptake by microorganisms in the nitrite system was higher than that with nitrate as the electron acceptor. Furthermore, the results demonstrated that there is a certain relationship in both nitrite and nitrate systems between the activities of anoxic phosphorus uptake and the initial concentration of electron acceptors. The optimal environmental factors of nitrate and nitrite for denitrifying-phosphorus removal were ρ(nitrate) = 30 mg L^{-1} at pH = 7 and ρ(nitrite) = 3 mg L^{-1} at pH = 7, respectively. Other stoichiometric studies on the denitrification processes showed that nitrite is, compare to nitrate, more active and sensitive electron acceptor for achieving nitrification (Zhou, 2010, 2001).

Table 2. *Pearson's correlation coefficients analysis of different electron acceptors*

Condition		R_P **v.s.** R_N	
		PPC	**p**
All pH	Nitrate	0.37	0.068
	Nitrite	0.624**	0.001
pH = 7.9 and 11	Nitrate	0.686**	0.005
	Nitrite	0.249	0.371

*Significant correlation at .05 level (bilateral)
**Significant correlation at .01 level (bilateral)

Conclusion and recommendations

To further improve the efficiency of sewage treatment and management, it is important to understand the metabolic behavior of denitrifying phosphorus-accumulating bacteria, which play a crucial role in the removal of nitrogen and phosphorus elements in wastewater treatment. In this work, the screened strain of DPAB, named N-8, Acinetobacter sp., could adopt both nitrate and nitrite as electron acceptors and has high synchronous nitrogen and phosphorus removal efficiency under anoxic condition. The types and concentrations of electron acceptors, as well as the initial pH of the systems, have significant effects on the removal of nitrogen and phosphorus. There were a stronger phosphorus and nitrogen removal efficiency by DPAB in the nitrite system than that in the nitrate system. Moreover, the effect of DPAB was greater in neutral and alkaline environments. The optimal stoichiometric concentrations of electron acceptor and pH for phosphate removal in the nitrate and nitrite systems were ρ(nitrate) = 30 mg L-1, pH = 9, and ρ(nitrite) = 5 mg L-1, pH = 7,

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respectively. Based on previous studies, almost all of DPAB are able to utilize oxygen (Hu et al., 2002; Tsuneda et al., 2006) as electron acceptors to remove phosphorus, we speculate that strain N-8 also can utilize oxygen with a different pattern from nitrate and nitrite, but the detail is not investigated in this work. Therefore, further research on the utilization patterns of DPAB for three types of electron acceptors, oxygen, nitrate and nitrite, is strongly recommended.

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APPENDIX

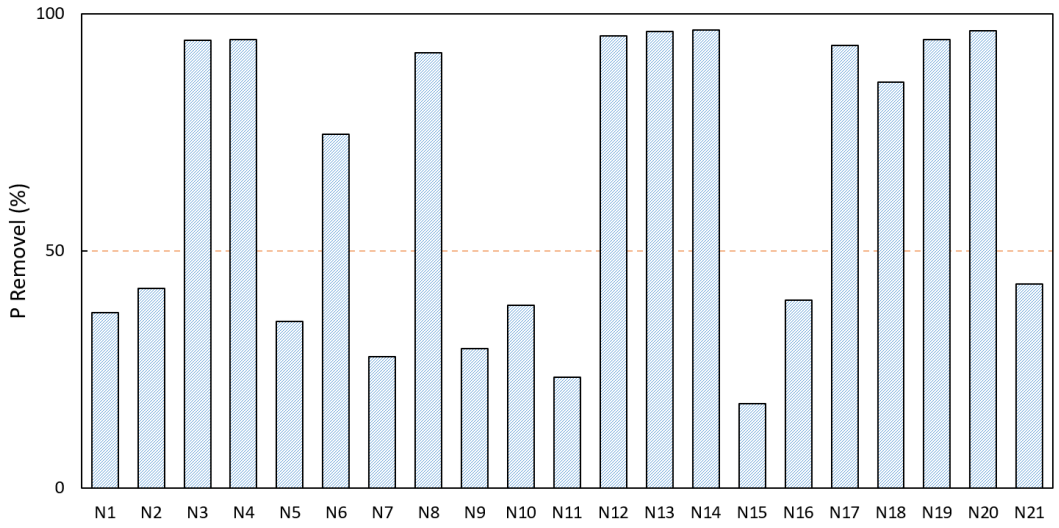

Figure A1. *Phosphorus removal rates of 21 colonies that can survive on the isolation medium. These colonies that showed a greater than 50% ratio will be further tested*

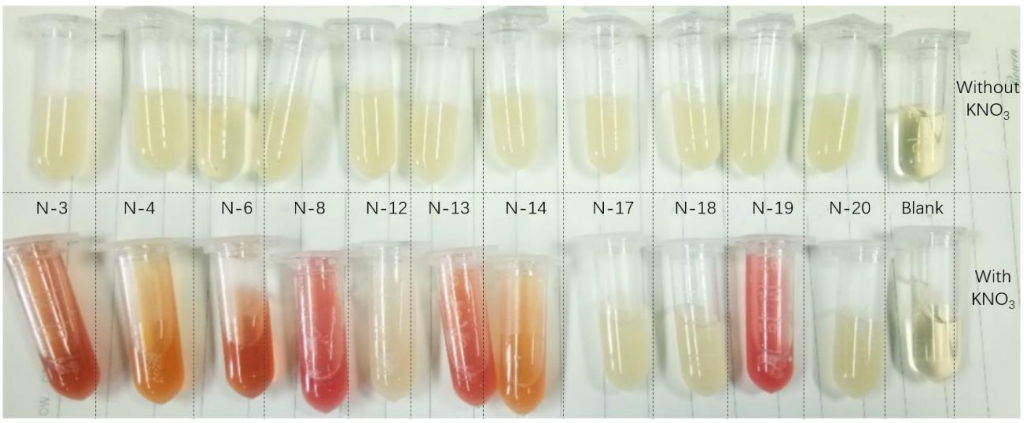

Figure A2. *Nitrate reduction test results of 11 colonies whose phosphorus removal rates exceeding 50%*

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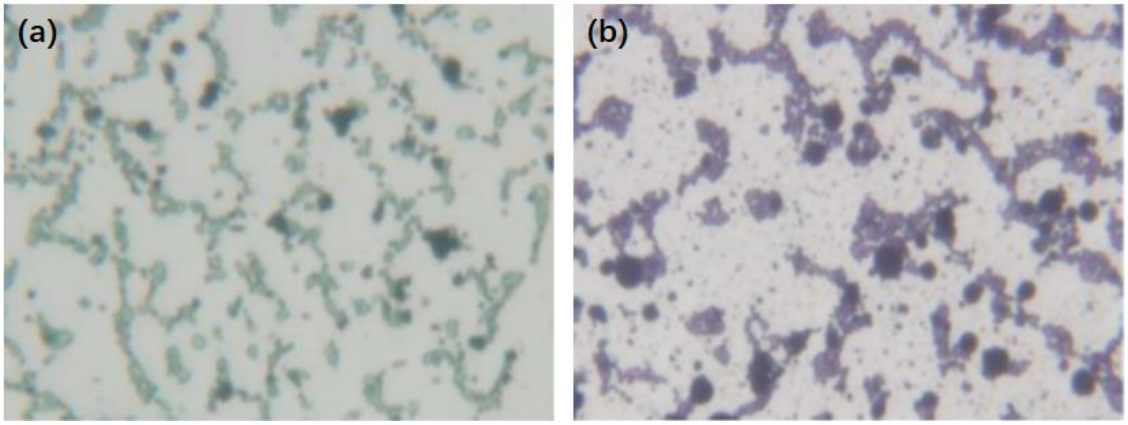

Figure A3. *Albert's metachromatic granules (a) and PHB granules (b) staining results for N-8*

Table A1. *Results of denitrifying phosphorus accumulating bacteria screening test*

	N-3	N-4	N-6	N-8	N-12	N-13	N-14	N-17	N-18	N-19	N-20
P-removel (%)	94.5	94.6	74.6	91.8	95.4	96.3	96.9	93.4	85.7	94.6	96.4
Albert Staining	+	+	-	+	+	+	-	+	+	-	+
PHB Staining	-	-	+	+	+	-	-	+	-	+	+
Nitrate reduction test	+	+	+	+	-	+	+	-	-	+	-

" + " indicates positive, "-" indicates negative

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