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Operation performance and microbial community dynamics of phosphorus removal sludge with different electron acceptors

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Abstract Operation performances of phosphorus removal sludge with different electron acceptors in three parallel SBRs were firstly compared in the present study, and the effect of post-aeration on denitrifying phosphorus removal was also studied. Moreover, community dynamics of different phosphorus removal sludge was systematically investigated with high-throughput sequencing for the first time. TP removal rates for nitrate-, nitrite-, and oxygen-based phosphorus removal sludge were 84.8, 78.5, and 87.4 %, with an average effluent TP concentration of 0.758, 0.931, and 0.632 mg/l. The average specific phosphorus release and uptake rates were 20.3, 10.8, and 21.5, and 9.43, 8.68, and 10.8 mgP/(gVSS h), respectively. Moreover, electron utilization efficiency of denitrifying phosphorus removal sludge with nitrate as electron acceptor was higher than nitrite, with P/e^- were 2.21 and 1.51 mol-P/mol-e^-, respectively. With the assistance of post-aeration for nitrate-based denitrifying phosphorus removal sludge, settling ability could be improved, with SVI decreased from 120 to 80 and 72 ml/g when post-aeration time was 0, 10, and 30 min, respectively. Moreover, further phosphorus removal could be achieved during post-aeration with increased aeration time. However, the anoxic phosphorus uptake was deteriorated, which was likely a result of shifted microbial community structure. Post-aeration of approximately 10 min was proposed for denitrifying phosphorus removal. Nitrate- and nitrite-based denitrifying phosphorus removal sludge exhibited similar community structure. More phosphorus accumulating organisms were enriched under anaerobic–aerobic conditions, while anaerobic–anoxic conditions were favorable for suppressing glycogen-accumulating organisms. Significant differences in pathogenic bacterial community profiles revealed in the current study indicated the potential public health hazards of non-aeration activated sludge system.

Keywords Phosphorus removal sludge · Electron acceptor · Post-aeration · Bacterial community dynamics

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Introduction

Enhanced biological phosphorus removal (EBPR) has been widely applied all over the world and proved to be a cost-effective and environmentally friendly process to remove phosphorus from wastewater [29, 30]. The EBPR process is based on the enrichment of poly-phosphorus accumulating organisms (PAO) by recirculating the activated sludge through alternating anaerobic–aerobic conditions. PAO are favored against other microorganisms

unable to uptake organic substrates under anaerobic conditions, and phosphorus is ultimately removed by discharging excess sludge rich in poly-phosphorus. Besides alternating anaerobic–aerobic conditions, phosphorus can also be removed under anaerobic–anoxic cycling [1, 20, 33, 37]. The organisms with anoxic phosphorus removal phenotype are referred to as denitrifying PAO (DPAO), the exploitation of DPAO for biological nutrient removal is highly desirable as phosphorus removal and denitrification can be simultaneously achieved in the same process, which is of great significance for wastewater treatment with carbon shortage [10].

Most of the research on denitrifying phosphorus removal has been conducted with nitrate as the electron acceptor [30, 35]. Results indicate that nitrate is a suitable electron acceptor for DPAO and denitrifying phosphorus removal process can save the aeration and carbon source required for denitrification [10, 26]. On the other hand, as the denitrification intermediate, nitrite has been proven to be an alternative for denitrifying phosphorus uptake [21, 33, 37] despite the report of inhibition of nitrite on phosphorus removal [25]. Therefore, many treatment processes are proposed or optimized to maximize denitrifying phosphorus removal [22, 30], which can be of great value for EBPR systems innovations [33].

Great advances have been achieved in characterizing phosphorus removal sludge. However, few studies have been parallel conducted to compare the phosphorus removal performances of sludge with different electron acceptors, and the effect of coupled electron acceptors for phosphorus removal is also insufficient. Moreover, a detailed understanding of phosphorus removal sludge with different electron acceptors from the microbiological point of view is far from enough since the previous studies are often based on the traditional molecular biotechnologies. In the current study, phosphorus removal sludge with nitrate, nitrite, and oxygen as the sole electron acceptor, respectively, were acclimatized in three parallel lab-scale sequencing batch reactors (SBRs) under strict anaerobic–anoxic (A–A) or anaerobic–oxic (A–O) conditions. Phosphorus removal performance and community structure between different phosphorus removal sludge was firstly compared with the aid of pyrosequencing, which exhibit overwhelming superiority on profiling complex bacterial community for its unprecedented sequencing depth [34, 36]. Moreover, the effect of short-time post-aeration on anaerobic–anoxic phosphorus removal and community dynamics was then systematically investigated. Results obtained in the current study will eventually help to improve the understanding of denitrifying phosphorus removal and process efficiency.

Materials and methods

Apparatus and methods

Phosphorus removal sludge with different electron acceptors was enriched in three parallel sequencing batch reactors (SBRs) under room temperature (averaged at 14.4 °C in winter and 27.7 °C in summer). The SBRs fed with nitrate, nitrite, and oxygen as electron acceptors were referred to as R_{NO_3}, R_{NO_2} and R_{O_2}, respectively. R_{NO_3} and R_{NO_2} were operated under strict anaerobic–anoxic (A–A) conditions, and nitrate or nitrite was continuously supplied for the initial 1.0 h of the anoxic phase with a peristaltic pump to avoid the rapid consumption of electron acceptors at the initial stage and the possible secondary phosphorus release at the end of anoxic phase [17]. Initial nitrate and nitrite concentrations were designed at approximately 20 and 25 mg/l, respectively. During acclimation and operation, dosing flow of nitrate and nitrite stock solution was timely adjusted (±5–10 %) to achieve an effluent nitrate and nitrite concentration of 2–7 mg/l, thus to satisfy the complete anoxic phosphorus uptake and avoid excessive nitrate or nitrite at the beginning of anaerobic phase. R_{O_2} was operated under anaerobic–oxic (A–O) conditions and air was supplied with an aerator during the oxic phase. The SBRs, with an effective working volume of 6.5 l, were initially operated for 6 months in fill-and-draw mode with the cycle of 8 h, consisting of five phases: filling (15 min), anaerobic phase (2.5 h), anoxic or oxic phase (4.0 h), settling (1.0 h), and withdrawing (0.25 h). During anaerobic and anoxic phases, the SBRs were continuously aerated with nitrogen gas to achieve an oxygen-free condition.

Later, the nitrate-based denitrifying phosphorus removal reactor (R_{NO_3}) was then gradually acclimatized from strict anaerobic–anoxic (A–A) to anaerobic–anoxic–oxic (A–A–O) conditions by stepwise decrease of the 4-h anoxic time and increase in oxic (post-aeration) time (from 0 to 10 and 30 min), and DO during the post-aeration phase was maintained at 1–2 mg/l by gassing air with an aerator.

At the end of the settling phase, 4 l of clarified supernatant was exchanged with equal volume of influent, which consisted of a mixture of real primary settled municipal wastewater and synthetic wastewater in volumetric proportion of 1:10. The tap water-based synthetic wastewater contained (g/l): CH_3COONa (0.2), NH_4Cl (0.014), NaH_2PO_4 (0.02), and 0.3-ml trace element solution which consisted (g/l) of EDTA (10), $FeCl_3$ (1.0), $CoCl_2{\cdot}6H_2O$ (0.15), KI (0.18), H_3BO_3 (0.15), $ZnSO_4{\cdot}7H_2O$ (0.12), $MnSO_4{\cdot}7H_2O$ (0.12), $CuSO_4{\cdot}5H_2O$ (0.03), and $(NH_4)_2Mo_4O_{13}{\cdot}2H_2O$ (0.06). Moreover, Allylthiourea (ATU) of about 4 mg/l was additionally supplemented in the influent of the A–O SBR to inhibit the nitrification activity and to keep the A–O reactor

free of nitrite and nitrate. SRT of the A–A and A–O SBRs were approximately 20 and 15 days, respectively. Relevant components were periodically analyzed to monitor the reactor operation performance during the whole study.

Chemical analysis

TP concentration was determined by molybdate colorimetric method with a spectrophotometer (Shimadzu, UVmini-1240). Concentrations of NO_3^-–N and NO_2^-–N were measured automatically by colorimetric methods with a Cleverchem200 (DeChem-Tech. GmbH, Germany). MLSS was determined periodically with the gravimetric method.

Sludge sample selection for microbial analysis

The three SBRs were seeded with sludge from a local municipal wastewater treatment plant (WWTP). Firstly, seed sludge (noted as seed) was taken to provide the initial microbial information of the SBRs before acclimation. After running for 100 days when the SBRs had been in stable operation for a long time, the sludge samples from the three SBRs were taken for comparison of their community structure. For the post-aeration, sludge samples were taken on the 25th and 56th day to represent the acclimation and stable operation periods for aeration time of 10 min, and on the 34th and 63rd day for aeration time of 30 min, respectively.

DNA extraction, PCR amplification, and high-throughput sequencing

Total DNA of the sludge samples were extracted using the FastDNA® SPIN Kit for soil (MP Biomedicals, Illkirch, France) according to the manufacturer's instructions. PCR primer sets of 967F and 1046R [31], which had been incorporated with barcodes for each primer pair [27], were chosen to target the V6 hypervariable region of bacterial 16S rRNA gene. Each 30-μl PCR reaction system contained 15 μl of 2 × buffer, 1.5 μl of each primer (1 nmol), 0.75 μl of MightyAmp® DNA Polymerase (TaKaRa, Dalian, China), and 20–50 ng of genomic DNA. The amplification was conducted in an i-Cycler (Bio-Rad, Hercules, CA, USA) under the following thermo steps: initial denaturation at 98 °C for 2 min, followed by 28 cycles at 98 °C for 20 s, 55 °C for 20 s, 68 °C for 30 s and a final extension step at 68 °C for 5 min. Three parallel amplifications of each DNA were conducted simultaneously and then mixed together for further analysis. The quality of amplification products were examined by agarose (2.0 %) gel electrophoresis. Finally, the PCR amplicons were purified with quick Midi Purification Kit (Tiangen), and quantified using a spectrometry (NanoDrop-1000).

Finally, amplicons of different samples were mixed together by ensuring equal mass concentrations in the final mixture, and the composition of the PCR products of V6 region of 16S rRNA was determined by library construction and sequencing on the Illumina-HiSeq 2000 sequencer at BGI of Shenzhen with the strategy of paired-end sequencing (2 × 100 bp).

The raw reads have been deposited into the NCBI short read archive (SRA) database (accession number: SRR948565).

Sequence analysis

Following sequencing, routine processing of primer removal, low-quality sequence screening, and sample sort were firstly carried out with the sequencing pipeline (PP) tool of the Ribosomal Database Project (RDP) [4]. The Align tool of RDP's PP was used to align the sequences, and a cluster file was generated with the RDP's complete linkage clustering tool. With the cluster file, operational taxonomic units (OTUs) were obtained through two self-written C++ programs. Then OTU-based cluster analysis (CA) and principal coordinate analysis (PCoA) was performed using PAST software [16, 24] to evaluate the community similarity and dynamics of sludge samples. The significant OTUs (>1.0 %) obtained were first BLAST on NCBI, then related full-length 16S rRNA GenBank records with identical or highly similar V6 region were selected and used for phylogenetic tree construction.

Results and discussion

Reactor operation performance

Operation performances of sludge with different electron acceptors

Operation performances of the three SBRs from acclimation to stable period are shown in Fig. 1. It took approximately 39, 54, and 27 days, respectively, for R_{NO_3}, R_{NO_2} and R_{O_2} to reach the stable-state operation characterized by stable effluent phosphorus concentrations and phosphorus removal rates. Comparatively speaking, R_{NO_2} sludge took a longer time for acclimation, perhaps due to the biological toxicity of nitrite [23] at the initial acclimation stage. At stable operation period, effluent TP concentration of R_{NO_3}, R_{NO_2} and R_{O_2} were 0.758, 0.931, and 0.632 mg/l, respectively, corresponding to TP removal rates of 84.8, 78.5, and 87.4 % on average.

Moreover, specific phosphorus release and uptake rates, which were evaluated by increased or decreased phosphorus

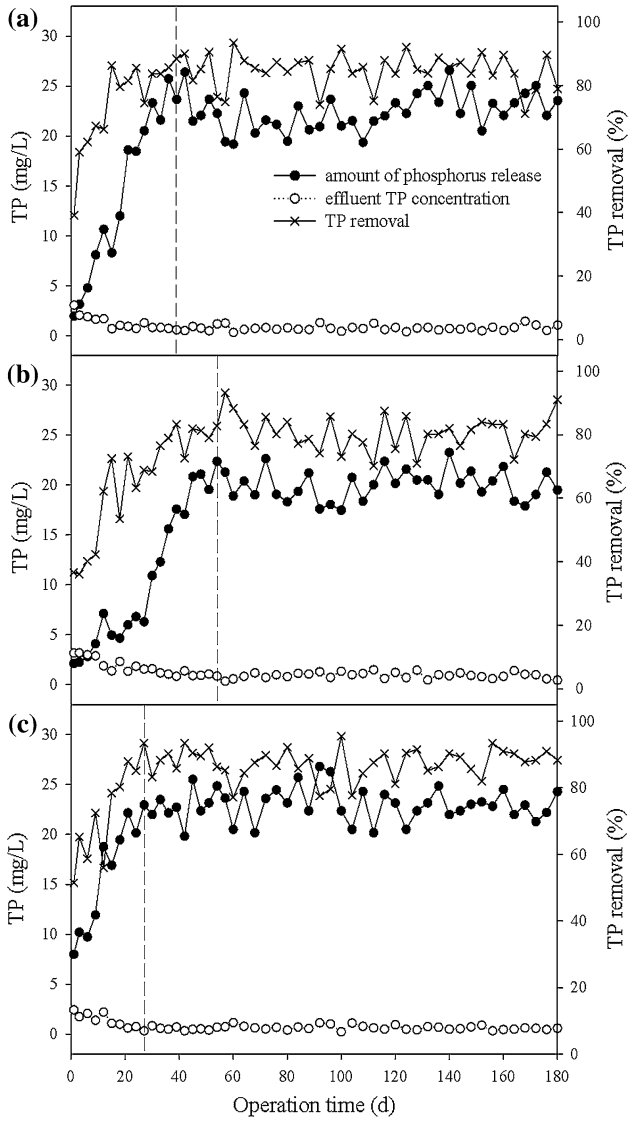

Fig. 1 Operation performance of phosphorus removal sludge with different electron acceptors. **a** NO_3^-–N; **b** NO_2^-–N; **c** O_2. The *dotted line* separates the operation into acclimation period and stable period

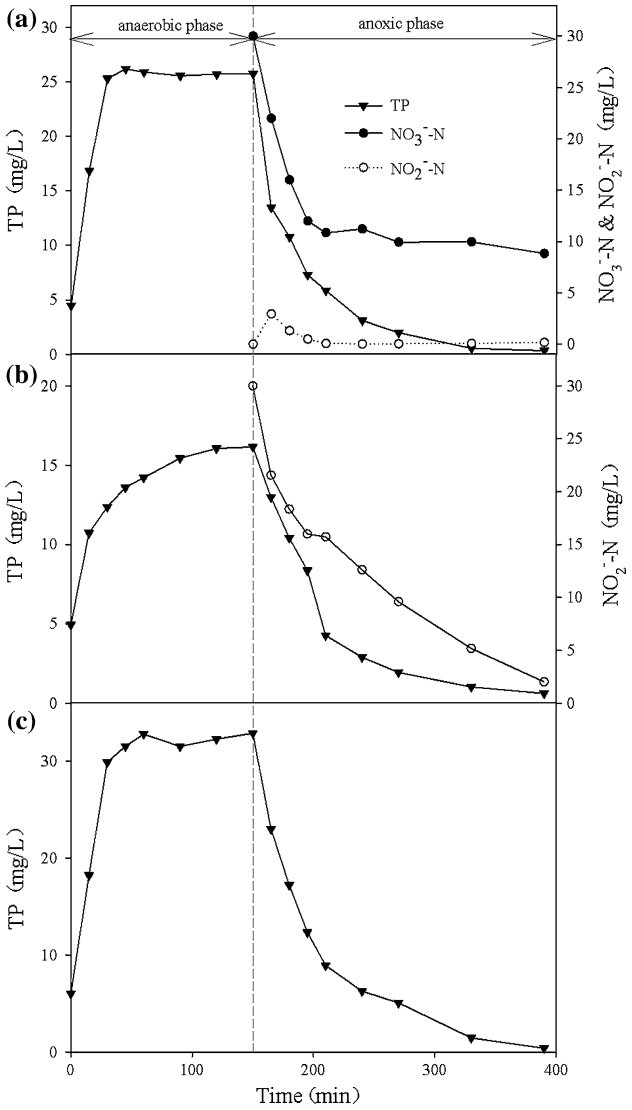

Fig. 2 Complete cycle profiles of phosphorus removal sludge with different electron acceptors. **a** NO_3^-–N; **b** NO_2^-–N; **c** O_2

concentration per gram VSS per hour (ΔTP/gVSS h), of sludge with different electron acceptors were investigated by phosphorus concentration profiles during the complete operation cycle, as shown in Fig. 2. During anaerobic phosphorus release, it took 30, 120, and 30 min for R_{NO_3}, R_{NO_2} and R_{O_2} to reach stable TP concentrations, and the average phosphorus release rates during the initial 30 min were 20.3, 10.8, and 21.5 mgP/(gVSS h), respectively. The average phosphorus uptake rates were 9.43, 8.68, and 10.8 mgP/(gVSS h) for R_{NO_3}, R_{NO_2} and R_{O_2}, and the denitrification rates for R_{NO_3} and R_{NO_2} were 9.59 and 10.4 mgN/(gVSS h) for the initial 60 min of anoxic phase. Moreover, the P/N (phosphorus uptake/nitrogen denitrified) ratios for R_{NO_3} and R_{NO_2} were 0.98 mgP/mgNO_3^-–N and 0.82 mgP/mgNO_2^-–N, namely, 2.21 and 1.51 mol-P/mol-e^- when converting to P/e^- (phosphorus uptake/electrons transferred), indicating a higher electron utilization efficiency of denitrifying phosphorus removal sludge with nitrate as electron acceptor compared with nitrite. However, the P/N ratios were still comparatively lower than previous studies [8, 35] and the theoretical value [10].

The above results indicated that R_{NO_3} demonstrated similar phosphorus removal efficiency with R_{O_2}, which was congruent with the result of previous report [8], yet phosphorus removal performance of R_{NO_2} was comparatively weaker [37].

Figure 3 shows the settling ability of different phosphorus removal sludge. Sludge volume index (SVI) of the seed sludge was 105 ml/g. At acclimation period, SVI of R_{NO_3} and

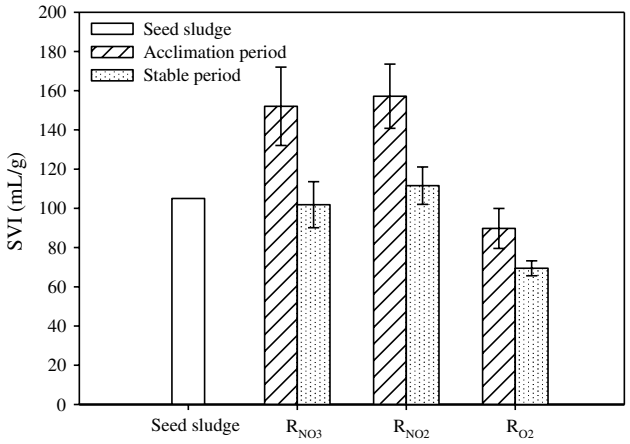

Fig. 3 Settling ability of sludge with different electron acceptors

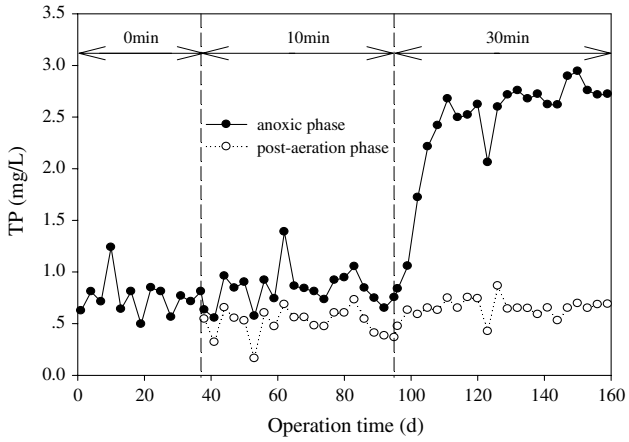

Fig. 4 Phosphorus removal performances with different post-aeration times

R_{NO_2} sludge increased to 152 and 157 ml/g on average, while SVI of R_{O_2} sludge decreased to 90 ml/g. At stable period, the settling ability was improved for all the sludge, indicated by SVI values that decreased to 102, 112, and 69 ml/g for R_{NO_3}, R_{NO_2} and R_{O_2}, respectively. Moreover, R_{O_2} sludge grew as granules of about 1.0–3.0 mm in diameter and thus resulted in the better settling ability [6, 13], indicating that A–O condition might be more favorable toward improving the sludge settling ability, compared with A–A operation.

Effect of post-aeration on denitrifying phosphorus removal

During denitrifying phosphorus removal, plenty of small bubbles were observed on the surface of denitrifying phosphorus removal sludge, which often resulted in sludge floating during selling phase. Therefore, short-time post-aeration was proposed for bubble stripping, further phosphorus uptake and sludge activity regeneration, and the impact of long-term operation with post-aeration on denitrifying phosphorus removal performance was investigated in the current study.

During the operation with post-aeration, sludge settling ability was indeed improved, with SVI decreased from 120 to 80 and 72 ml/g corresponding to post-aeration times of 0, 10, and 30 min, respectively. Fig. S1 demonstrates the settling profiles of denitrifying phosphorus removal sludge with different post-aeration times, and the results indicated that a post-aeration of 10 min was sufficient for improving the settling ability of denitrifying phosphorus removal sludge. It was worth noting that the improved settling ability seemed to be unrelated to the stripping of oxygen during post-aeration since the small bubbles produced during denitrification had been stripped off with the assistance of continuous aeration of nitrogen gas for the whole anoxic phase. Then the differences in microbial community structure should be a more likely cause of better settling ability of aerobic phosphorus removal sludge, which will be discussed in the following section.

TP concentrations during phosphorus uptake during anoxic and post-aeration phases are depicted in Fig. 4. When the nitrate-based anaerobic-anoxic SBR was operated without post-aeration, TP concentration at the end of anoxic phase was about 0.756 mg/l. With the post-aeration time increased from 0 to 10 min, TP concentration at the end of anoxic phase still remained almost the same, and the concentration at the end of post-aeration phase decreased to 0.511 mg/l with the assistance of further aerobic phosphorus uptake during post-aeration. However, when post-aeration time was further extended from 10 to 30 min, TP concentration at the end of anoxic phase obviously increased to as high as 2.42 mg/l and concentration after post-aeration was decreased to 0.646 mg/l on average.

The above results suggest that the sludge-settling ability could be improved and phosphorus could be further removed during post-aeration phase, however, the anoxic phosphorus uptake was deteriorated despite the similar final effluent TP concentration. Therefore, the bacterial community structure was further investigated.

Microbial community characteristics

Community structure of sludge with different electron acceptors

Cluster analysis (CA) on the basis of OTUs number was employed to evaluate the similarity of microbial community among the phosphorus removal sludge with different electron acceptors as well as the seed sludge. As shown in Fig. S2, CA plotted with OTUs at 3 % cut-off level based on Bray–Curtis distance calculated with the PAST software [36] revealed that R_{NO_3} and R_{NO_2} sludge were clustered

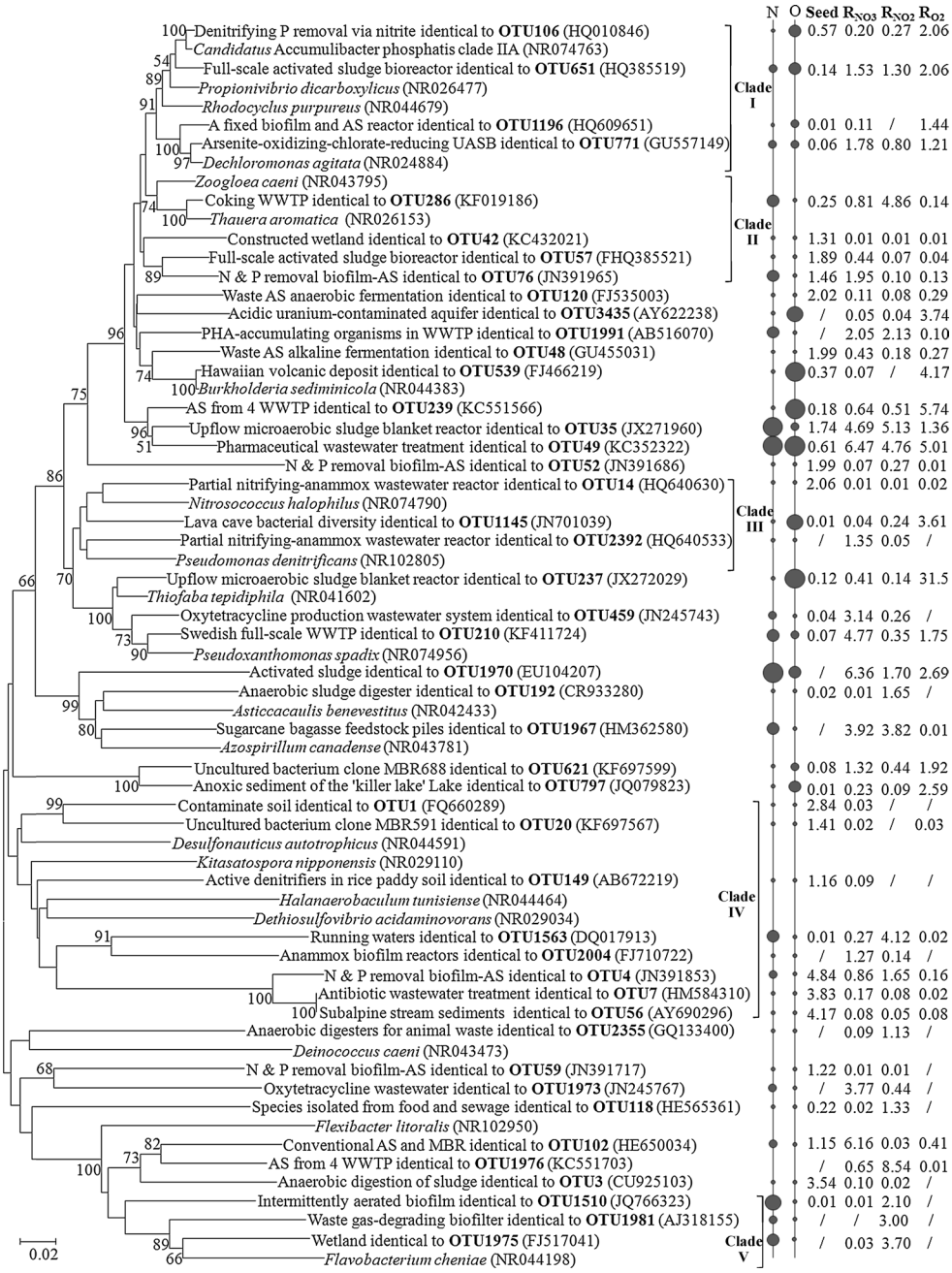

Fig. 5 The phylogenetic relationship between the significant OTUs (>1.0 % of each sample) of the four sludge samples and their close relatives retrieved from NCBI GenBank. The tree was constructed using the minimum-evolution algorithm with 1,000 bootstrapping. *Numbers at the nodes* are the bootstrap values. The abundances were <1, 1–2, 2–3, 3–4, and >4 %, respectively, for the cycles with increased sizes. The N* were the averaged OTU abundance of nitrate- and nitrite-based denitrifying phosphorus removal sludge, and the O are the OTU abundance of aerobic phosphorus removal sludge

together, while R_{O_2} sludge and seed sludge were on other separate branches. This result suggested a similar microbial community structure of denitrifying phosphorus removal sludge with nitrate and nitrite as electron acceptors.

The phylogenetic relationship between the related NCBI GenBank sequences that share highly similar V6 regions with the significant OTUs (abundance >1.0 % of each sample) of the four sludge samples are shown in Fig. 5. These significant OTUs covered 41.4, 52.4, 51.5, and 69.0 % of the entire community, respectively, for seed sludge, R_{NO_3}, R_{NO_2} and R_{O_2} sludge. Each of the four samples had unique OTUs distribution patterns, while R_{NO_3} and R_{NO_2} sludge shared more significant OTUs compared with the R_{O_2} sludge, which might be attributed to the similar bacterial community structure resulting from their similar redox conditions.

Currently, *Candidatus* "Accumulibacter" was highlighted as one of the most important phosphorus-removal organism candidates [5, 11, 20]. Through the metagenomic study of EBPR sludge from two different lab-scale systems, Martin et al. [14] found that *Candidatus* "Accumulibacter" did not contain the gene for respiratory nitrate reductase (Nar), while the genes responsible for nitrite reduction to nitrogen were present. It was known that nitrate reduction

to nitrite could be carried out by flanking microorganisms with nitrate reductase, and then nitrite was further utilized as an electron acceptor for DPAO. Consequently, it was possible that the same denitrifying phosphorus removal organisms were responsible for anoxic phosphorus uptake under nitrate- and nitrite-based denitrification conditions. The quite similar phosphorus removal organisms related OTUs (OTU106, 239, 651, 771, 3435, and 120) distribution on the phylogenetic tree (Fig. 5) could support the above speculation. Nevertheless, phosphorus removal efficiency of sludge with nitrite as electron acceptor was lower than nitrate-based denitrifying phosphorus removal. This was mainly due to that specific functional process, such as denitrifying phosphorus removal, usually required various types of organisms for participation and cooperation, and the energy generated from nitrate reduction may facilitate the denitrifying phosphorus uptake with nitrite as electron acceptor.

The averaged OTUs abundances of R_{NO_3} and R_{NO_2} sludge (N*) as well as the R_{O_2} sludge (O) were demonstrated with circles of different sizes, as shown in Fig. 5. There were five major clades (I, II, III, IV, and V) for these sludge samples: Clade I was similar with *Candidatus* "Accumulibacter" or *Rhodocyclus purpureus* [38], which were phosphorus removal-related organisms. As for the clade I, their abundances in aerobic phosphorus sludge was higher than that in denitrifying phosphorus removal sludge, despite that the phosphorus removal performance were similar. Clade II was related with *Zoogloea caeni* or *Thauera aromatic*, which were important organisms for formation of sludge flocs, and these similar sequences were frequently reported in nutrient removal systems. Clade III was related with *Nitrosococcus halophilus*, which was the important functional organisms for nitrification. Obviously, their abundances in aerobic phosphorus removal sludge and the seed sludge were quite similar while they were far higher than that in denitrifying phosphorus removal sludge due to the oxygen limitation under anaerobic–anoxic conditions. Clade IV involved several organisms in wastewater treatment, such as *Desulfonauticus autotrophicus*, *Halanaerobaculum tunisiense*, etc. In addition, there was also clade V that was similar with *Flavobacterium cheniae* within *Flavobacteria*, which were a group of facultative anaerobic commensal bacteria and opportunistic pathogens [2]. Abundances of *Flavobacteria*-related sequences abundances in nitrite-based phosphorus removal sludge were far higher than all other sludge, indicating that under nitrite-based A–A system, certain influent bacteria may leak from the activated sludge system possibly due to the insufficient redox stress.

For further investigation of potential pathogenic bacteria abundance in different phosphorus removal sludge, the Basic Local Alignment Search Tool (BLAST) was employed based on the local database of representative pathogenic bacteria sequences (Table S3) summarized by Ye and Zhang [34]. The sequences assigned to pathogenic bacteria at similarity of 97 % are listed in Table 1. Respectively, 1.12, 0.80, 5.17, and 2.76 % of bacterial sequences of seed sludge and R_{NO_3}, R_{NO_2}, R_{O_2} sludge could be assigned as putative pathogenic bacteria. Moreover, different environmental conditions induced distinguishing features on pathogenic bacteria distribution, such as *Enterobacter aerogenes* and *Francisella tularensis* only harbored in denitrifying phosphorus-removal conditions. *Pseudomonas aeruginosa* was the most dominant pathogenic species for the three phosphorus removal sludge, followed by *Escherichia coli*. *Serratia marcescens* was most dominant in seed sludge and also dominant in nitrite- and oxygen-based sludge, and moreover, *Aeromonas veronii* was also dominant in nitrite-based sludge. Another interesting finding was that *Bordetella pertussis*, which was transmitted by the respiratory route, was also detected in sludge with different electron acceptors, with the abundances of 0.02–0.08 %. It should be noted that *B. pertussis* have also been detected in 14 geographically distributed wastewater treatment plants around the world, by PCR amplicon sequencing targeting the V4 hyper variable region of 16S rRNA gene [34]. What is more, *B. pertussis* have been detected in municipal wastewater treatment plants by high-throughput shotgun sequencing and subsequent metagenome-based virulence factor analysis [3]. These results as well as the finding achieved in the current study indicated the occurrence of non-fecal sources pathogen in WWTP, which need further attention.

Recently, the occurrence and fate of pathogenic bacteria has become one of the important concerns in the risk assessment of existing wastewater treatment facilities [3, 12, 15]. In the meantime, novel treatment processes (e.g., denitrifying phosphorus removal without any aeration; the

Table 1 Abundances of pathogenic bacteria in the seed sludge and phosphorus removal sludge with different electron acceptors

Pathogenic species	Abundances (%)			
	Seed	R_{NO_3}	R_{NO_2}	R_{O_2}
Aeromonas veronii	0.05	0.08	0.15	0.07
Aeromonas hydrophila	0.06	0.02	0.00	0.04
Arcobacter butzleri	0.03	0.00	0.00	0.00
Bordetella pertussis	0.07	0.06	0.08	0.02
Enterobacter aerogenes	0.03	0.01	0.02	0.00
Escherichia coli	0.12	0.24	0.22	0.59
Francisella tularensis	0.00	0.00	0.01	0.00
Pseudomonas aeruginosa	0.27	0.37	4.53	1.86
Serratia marcescens	0.47	0.01	0.16	0.17
Total abundances	1.12	0.80	5.17	2.76

Table 2 Abundances (%) of PAO and GAO for sludge with different electron acceptors and different post-aeration time

	Seed	R_{NO_3}	R_{NO_2}	R_{O_2}	10 min		30 min	
					$R_{10\text{-ACC}}$	$R_{10\text{-STA}}$	$R_{30\text{-ACC}}$	$R_{30\text{-STA}}$
PAO	0.716	1.78	2.54	4.81	9.96	1.63	1.73	0.990
GAO	0.368	1.44	1.32	30.9	8.05	1.38	2.79	2.68

ACC and STA indicated that the sludge samples were taken at the acclimation and stable phases

sulfate reduction, autotrophic denitrification, nitrification integrated (SANI) process [32]) have been continuously proposed to satisfy the requirement of sludge reduction, energy savings, and cost reductions. Researchers are often more concerned about the performance of the new processes, while function of pathogen control is neglected to a certain extent. Thus, profiling pathogenic bacteria among sludge using different accepters carried out in the current study not only identified the pathogens' tolerance to a certain process but also aimed to excite the researcher's interest of possible health risks of novel biological treatment processes.

PAO and GAO quantification of sludge with different electron acceptors

Quantifications of PAO and GAO (glycogen accumulating organisms) were relied on the sequence similarity against the existing identified sequences. As previously mentioned, *Candidatus* "Accumulibacter" was the mostly accepted phosphorus removal organism, and phylogenetically closely related to *Propionvibrio* or *Rhodocyclus* within the family *Rhodocyclaceae* [16]. Through the elaborate online survey and phylogenetic analysis of reported 16S rRNA gene sequence from both full-scale WWTP and bench-scale reactors, Kim et al. [9] had grouped the *Candidatus* "Accumulibacter"-like sequences into four major clades. Based on the reference sequences (Table S1) of Kim's phylogenetic tree [9] and the *Dechloromonas*-related sequence (unpublished data), PAO was then quantified through local BLAST. Results showed that 0.71, 1.78, 2.53, and 4.80 % of sequences from Seed, R_{NO_3}, R_{NO_2} and R_{O_2} could be assigned to *Candidatus* "Accumulibacter"-like sequences at similarity level of 97 % [7], as shown in Table 2. Results indicated that PAO in all the sludge were obviously enriched after acclimation in alternating anaerobic–anoxic or anaerobic–oxic conditions, reflected by the increased abundances. Comparatively speaking, PAO abundance in R_{O_2} sludge was much higher than that in R_{NO_3} and R_{NO_2} sludge, suggesting that anaerobic–aerobic condition might be more suitable for enrichment of PAO. Despite this, R_{NO_3} demonstrated similar phosphorus removal performance with R_{O_2}, which proved the phosphorus removal efficiency under alternating anaerobic–anoxic condition [8].

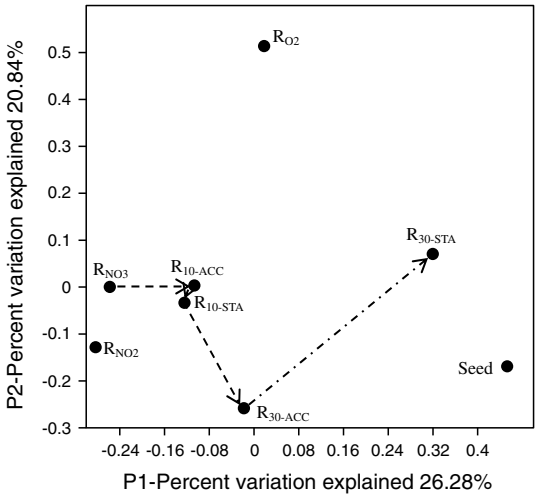

Fig. 6 Non-parametric multidimensional scaling plot of similarities among phosphorus removal sludge with different electron acceptors and nitrate-based denitrifying phosphorus removal sludge with different post-aeration time based on the Bray–Curtis similarity measure of 3 % cut-off OTU (ACC and STA indicated that the sludge samples were taken at the acclimation and stable phases)

Using the reference database of Table S2, a similar approach was used for quantification of GAO, which was often considered as the competitor of PAO [18, 19], in the phosphorus removal sludge with different electron acceptors. As shown in Table 2, 0.37, 1.44, 1.32, and 30.9 % of sequences of seed, R_{NO_3}, R_{NO_2} and R_{O_2} could be assigned to GAO-like sequences at a similarity level of 97 %. The result indicated that GAO might be prone to be enriched in aerobic phosphorus removal environment (A–O), which might be one reason for the above-mentioned similar phosphorus removal performance under comparatively higher PAO abundance. Alternating anaerobic–anoxic operation was favorable toward elimination of GAO [28], which might be beneficial for maintenance of stable operation.

Effect of post-aeration on microbial community

The principle coordinate analysis (PCoA) was employed to evaluate the similarity of the sludge microbial community among different sludge samples. PCoA based on

Bray–Curtis distances at 3 % cut-off OTUs is shown in Fig. 6. Similar to the result from CA (Fig. S2), R_{NO_3} and R_{NO_2} sludge were grouped together, with R_{O_2} sludge and seed sludge in independent groups. For the sludge samples with post-aeration on the PCoA plot, they moved from sample without post-aeration (R_{NO_3}) to seed sludge when aeration time was gradually increased. This result, together with the improved settling ability, suggested that the improvement of settling ability of post-aeration sludge resulted from the microbial community shift.

Additionally, the abundances of PAO and GAO were again quantified to evaluate the effect of post-aeration on denitrifying phosphorus removal system. As shown in Table 2, at acclimation stage of post-aeration for 10 min, both PAO and GAO abundances abruptly increased from 1.78 and 1.44 to 9.96 and 8.04 % respectively, which might be due to the environmental disturbance introduced by post-aeration. With the continuous operation and acclimation, their abundances at stable stage decreased to the level before post-aeration. When post-aeration time was extended to 30 min, there was a decrease of PAO abundance and increase of GAO abundance, which might be the reason for observed deterioration of denitrifying phosphorus removal performance. Therefore, post-aeration of approximately 10 min was proposed for denitrifying phosphorus removal process.

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