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Denitrifying phosphorus removal for simultaneous nitrogen and phosphorus removal from wastewater with low C/N ratios and microbial community structure analysis

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ABSTRACT

Simultaneous nitrogen and phosphorus removal by denitrifying phosphorus accumulating organisms (DPAO) from domestic wastewater with a low C/N was studied in a pilot-scale A_2N/BAF process. Through long-term investigation for more than 820 d, it was evidenced that the A_2N/BAF performed well on COD, NH_4^+-N and TP removals with efficiencies averaged at 84, 97 and 90%, respectively. Phosphorus removal in the system was mainly attributed to the function of DPAO through the analysis of phosphorus uptake and nitrate consumption, and the relative phosphorus uptake activity of DPAO/PAO was approximately 70.3%. Moreover, the microbial diversity and dominant bacterial community in the A_2N/BAF was investigated with high-throughput sequencing. It was revealed that the A_2N/BAF sludge had comparatively less diversity than seed sludge and shared several common dominant taxonomic bacteria with seed sludge, while long-term operation also shifted the microbial community. The A_2N/BAF system was feasible for the treatment of low C/N wastewaters without any additional carbon source, which gave a new insight for efficient removal of organic and nutrients simultaneously.

Keywords: A_2N system; BAF; Denitrifying phosphorus removal; Low C/N wastewater; Community structure

1. Introduction

Biological nutrients removal is proved as the most cost-effective and environment-friendly approach for nitrogen and phosphorus removal [1]. During the N and P removal processes as the electron donor, the biodegradable carbon source is a key factor affecting the treatment efficiency. However, domestic sewage in some cities is characterized as low carbon to nitrogen ratios (C/N for short) ranged from two to six,

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indicating the insufficient carbon source for efficient nitrogen and phosphorus removal biologically, yet dosage of external carbon source inevitably results in an increase of operational cost. Therefore, it is of great significance to develop an effective treatment process with less carbon source demand and energy consumption to remove organic and nutrients simultaneously.

Recently, a group of microorganisms, termed as denitrifying phosphorus-accumulating organisms (DPAO) which could perform denitrification and phosphorus removal simultaneously, was recognized [2,3]. Afterwards, great efforts were made for their characterization [4,5]. Numerous reports evidenced that the capability of DPAO in phosphorus removal was even as high as that of PAO [6] and hence, various treatment processes were proposed or modified to involve DPAO for phosphorus removal [3,7]. However, most of these processes were operated in single-sludge system, where nitrifying bacteria and DPAO coexisted in the system; the treatment performances, especially the TP removal, were unstable or constrained by the key wastewater composition parameters and operational parameters [8], such as sludge retention time (SRT), hydraulic retention time (HRT) and availability of carbon source.

Anaerobic–anoxic/nitrification (A_2N) was a promising two-sludge process involving DPAO for N and P removal simultaneously from wastewater, in which nitrifying bacteria and DPAO grew in independent reactors to overcome the SRT contradiction and to improve the treatment performance [9]. As the internal carbon source (typically PHA) would be stored and utilized by DPAO for phosphorus accumulation and denitrification, the insufficiency of carbon source for nitrogen and phosphorus removal could be resolved. A_2N process had been proved as an energy-saving and cost-reducing process, which could save on aeration by 30% and carbon source by 50%, and also minimize the waste sludge. Although a great number of experimental studies on A_2N process were carried out in bench-scale with synthetic influent wastewaters [10,11], there were only several studies conducted with real domestic wastewater [12–14]. It was reported that DPAO biomass could be characterized with classical or molecular approaches; however, the comprehensive understanding of the denitrifying phosphorus removal system, including the optimal carbon source [5,15,16] and identification of dominant phosphorus removal organisms [17–19] was still unclear.

In this study, an A_2N/BAF system was developed to treat real domestic wastewater with low C/N ratios. A biological aerated filter (BAF) was employed to combine with A_2N, by which ammonia could be oxidized to nitrate effectively and suspended solids (SS) were also intercepted that both favoured the stability of the system [20]. The A_2N/BAF process was continuously operated in pilot scale for more than 820 d, and the aims in this work were: (i) to investigate treatment performance in terms of organic, nitrogen and phosphorus removal efficiencies, (ii) to evaluate the denitrifying phosphorus removal in the process and (iii) to specify the dominant functional bacterial community by means of high-throughput sequencing. The results herein will be of significance to understand the A_2N process and its real application for low C/N wastewater treatment.

2. Materials and methods

2.1. Experimental set-up

The pilot-scale A_2N/BAF process comprised of an A_2N system with BAF as the nitrification unit and a secondary BAF with a flow rate of 1.0 m^3/h. As shown in Fig. 1, raw wastewater flew into the primary sedimentation tank to achieve the rough separation of suspended solids. Afterwards, the treated water was introduced to the A_2N process that consisted of an anaerobic tank, a middle sedimentation tank, the primary BAF and an anoxic tank. It was to be remarked that a post-aeration zone was included in the end of anoxic tank for the residue phosphorus uptake with oxygen as electron acceptor, and simultaneously reviving the P-removal sludge. Finally, water–solid separation was accomplished in the secondary sedimentation tank, and the supernatant flew into the secondary BAF to achieve a high effluent quality. There were two sludge lines in this system: the bypass line from middle sedimentation tank to the anoxic tank and the recirculation line from the secondary sedimentation tank to the anaerobic tank, and flow rate of the two lines were both kept around 0.3 m^3/h. Both the primary and secondary BAF were packed with porcelain granule (3–5 mm) as media for the growth of nitrifying bacteria. Mixers were installed in anaerobic tank and anoxic tank for a better mixing at a rate of about 60 r/min.

2.2. Process operational design

The A_2N/BAF system was operated for more than 820 d. According to the absence or presence of secondary BAF, the operational time was divided into two

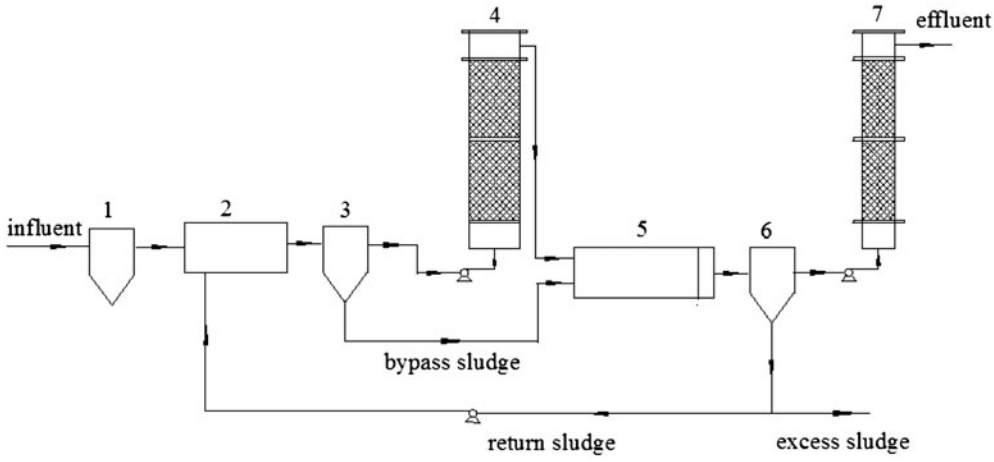

Fig. 1. Schematic diagram of the pilot-scale A_2N/BAF system (1—primary sedimentation tank; 2—anaerobic tank; 3—middle sedimentation tank; 4—primary BAF; 5—anoxic tank; 6—secondary sedimentation tank; 7-secondary BAF).

phases, termed as Phase I (from day 1 to 391) and II (from day 392 to 820). The overall HRT was kept about 11.5 h with HRT in anaerobic tank, middle sedimentation tank, primary BAF, anoxic tank, secondary sedimentation tank and secondary BAF of 2.0, 1.75, 1.5, 4.0, 1.75 and 0.5 h, respectively, and HRT of post-aeration zone was 0.5 h. Gas to water ratio for primary BAF and secondary BAF was 3:1 and 1:1, respectively, while the DO was controlled around 1.0 mg/L in the post-aeration z one. Ambient temperature during the whole operational period was averaged at 14.4˚C in winter and 27.7˚C in summer. MLSS was maintained in the range of 3,000–4,000 mg/L and SRT was around 15–20 d.

2.3. Characterization of influent wastewater and sludge of the system

The activated sludge inoculated for the A_2N/BAF system was taken from a municipal wastewater treatment plant (Luofang WWTP, Shenzhen). Influent for the system was the domestic wastewater in Shenzhen university town, and its major components are displayed in Table 1.

For evaluation of denitrifying P-removal in the pilot-scale process, batch experiments of anoxic and aerobic phosphorus uptakes were conducted. Sludge from the anaerobic tank in the A_2N/BAF was withdrawn and washed for three times using physiological saline. Then, the sludge was separated into two identical columns with phosphorus-containing water supplemented to an equal mixture volume to achieve a final phosphorus concentration of 20 mg/L. One column was dosaged with nitrate (25 mg/L) for denitrifying phosphorus uptake and the other one was supplied with oxygen for aerobic phosphorus uptake. The phosphate uptake and nitrate denitrifying rates were estimated from the slope of the line describing the change of phosphate and nitrate concentrations with time.

2.4. DNA extraction, PCR amplification and high-throughput sequencing

To characterize the microbial community in the A_2N/BAF system, the sludge samples were subjected to DNA extraction, PCR amplification and then high-throughput sequencing. Upon the steady state of the system around day 100, sludge sample was taken for total DNA extraction using FastDNA® SPIN Kit for Soil (MP Biomedicals, Illkirch, France). The primer set of 967F (CAACGCGAAGAACCTTACC) and 1046R

Table 1
Components of the influent into the A_2N/BAF system

	COD (mg/L)	NH_4^+-N (mg/L)	NO_3^--N (mg/L)	TN (mg/L)	TP (mg/L)	Alkalinity (mg/L)
Range	93–298	15.6–52.2	0–1.23	20.9–57.4	1.12–4.95	155.0–320.6
Average	196	35.2	0.24	40.7	2.90	225.4

(CGACAGCCATGCANCACCT) was adopted for DNA amplification of bacterial 16S rRNA gene fragments [21–23]. For sequencing, barcodes that allow sample multiplexing during sequencing were incorporated into both the forward and backward primers. Then, amplicons from the two samples were mixed together ensuring equal mass concentration in the final mixture, which was used for sequencing on the Illumina-HiSeq 2000 at the BGI of Shenzhen with the strategy of Paired-end sequencing (2 × 100 bp).

2.5. Analytical methods

The influent and effluent of each chamber of the A_2N/BAF system were sampled regularly for the analysis of COD, N-components, and TP (as for domestic wastewater, soluble phosphorus concentration in liquid phase would be often overestimated, which affected the accuracy of system treatment performance. Therefore, it was reasonable to use TP concentration instead of soluble phosphate concentration to investigate the P-content in system liquid phase and solid phase, which was important to assess the P conversion biologically.) in liquid phase. COD was measured by potassium dichromate method [24]; total phosphorus (TP) was quantified by molybdate colorimetric method using a spectrophotometer (Shimadzu, UVmini-1240) after decomposition with potassium persulphate under 121˚C for 30 min [25], while MLSS was determined by gravimetric method [24]. NH_4^+-N, NO_3^--N and alkalinity were measured with the Clever-chem200 (DeChem-Tech. Gmbh, Germany) [16].

3. Results and discussion

3.1. Treatment performance of A_2N during Phase I

Profiles of COD in each chamber of A_2N process were demonstrated in Fig. 2(a). More than 70% of COD was degraded in the anaerobic tank, major attributing to the hydrolization of particulate and colloidal organics to soluble organic matters and thereby utilization by P-removal bacteria. It was noted that the DPAO utilized the carbon source, especially in the form of intracorporal polymer (PHA), to help for excess denitrifying phosphorus uptake in anoxic tank [26]. After degradation in the anaerobic tank, the residue organic matters into the primary BAF was rather low, resulting in its high-quality effluent with an average concentration of 38 mg/L.

Fig. 2(b) displayed the removal performance of NH_4^+-N in the system. Decrease in NH_4^+-N concentration from 35 mg/L in the primary sedimentation tank to 29 mg/L in the anaerobic was mainly due to the dilution of returned sludge. After that, more than 88.5% of NH_4^+-N was oxidized in the primary BAF and effluent was as low as of 3.36 mg/L on average. The complete nitrification in the primary BAF favoured the effluent of secondary sedimentation tank.

A fairly low NO_3^--N concentration in the effluent of A_2N process was stably observed throughout the experimental phases (Fig. 2(c)). For the anaerobic tank, the NO_3^--N variation was consistently insignificant since NO_3^--N in the raw wastewater was no more than 0.5 mg/L. Afterwards, NO_3^--N concentration increased up to about 26 mg/L by the nitrification in the primary BAF, which could almost be denitrified in the following anoxic zone. Thereafter, a high-quality effluent with NO_3^--N concentration averaged as low as 0.24 mg/L was achieved.

Fig. 2(d) showed the variation of TP concentrations in different units of A_2N process. The TP concentration in the raw wastewater ranged from 1.12 mg/L to 4.95 mg/L with an average value of 2.90 mg/L. However, it was observed that a significant high TP concentration in anaerobic tank was around 7.24 mg/L, which was believed to be caused by the phosphorus release by DPAO. Thereafter, luxurily phosphorus uptake occurred in the anoxic zone, resulting in a low TP concentration around 0.6 mg/L. With assistance of the post-aeration next to the anoxic zone, the TP concentration in the effluent could be stably achieved to around 0.4 mg/L.

To sum, the excellent effluent quality in A_2N process through a long-term experiment evidenced that this system was capable of treating domestic wastewater with a high stability and effectiveness.

3.2. Effectiveness of the secondary BAF during Phase II

Although there were rather great fluctuations in the influent organic and nutrients concentrations, the effluent qualities, in terms of COD, NO_3^--N and TP concentrations, after the secondary sedimentation tank were relatively stable (Fig. 2). However, it should be figured out that NH_4^+-N concentration in the effluent from secondary sedimentation tank was still comparatively high since NH_4^+-N in the bypass sludge into the anoxic phase almost couldn't be further removed. Besides, the TP concentration in the effluent was correlated in a certain level with the abundance of SS in the secondary clarifier upon the performance of liquid–solid separation (data was not shown). Therefore, a secondary BAF was introduced in the Phase II following the secondary sedimentation tank, to ensure the complete nitrification and as well as to minimize the influence of SS on TP removal. The variations of

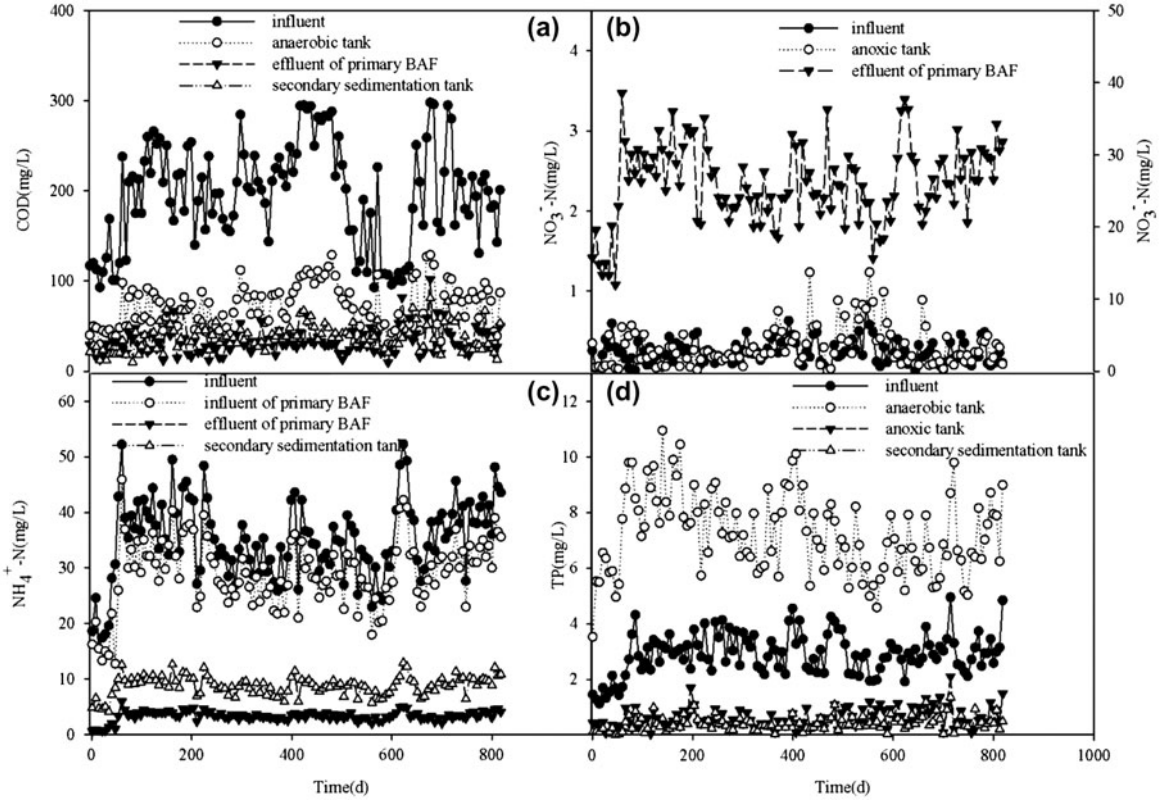

Fig. 2. Profiles of COD, NH_4^+-N, NO_3^--N and TP variation in major units of A_2N (Phase I). (a) COD, (b) NH_4^+-N, (c) NO_3^--N and (d) TP.

the pollutants before and after secondary BAF treatment are presented in Fig. 3. With a HRT as short as 0.5 h and gas to water ratio as low as 1:1, the secondary BAF could achieve approximately 84, 97 and 90% removals of COD, NH_4^+-N and TP, respectively, and the high-quality effluent, especially the TP concentration, as stably low as 0.3 mg/L was obtained.

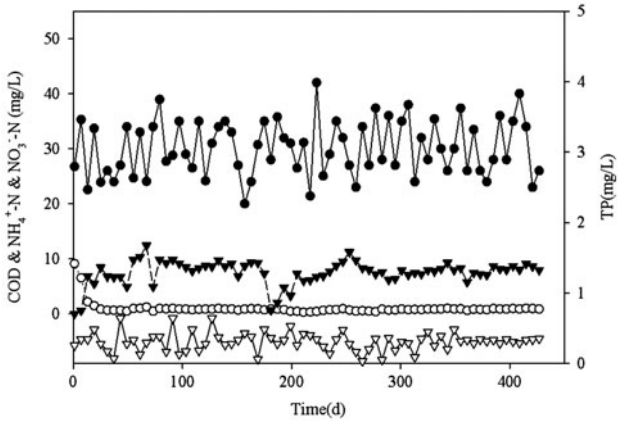

Fig. 3. Profiles of COD, NH_4^+-N, NO_3^--N and TP variation of secondary BAF.

3.3. Analysis of denitrifying phosphorus removal in the system

Through the monitoring and calculation of phosphorus and NO_3^-–N profiles in different chambers, it was observed that the consumption of the NO_3^-–N in anoxic zone displayed an obvious linear dependency on the abundance of the phosphorus uptake (Fig. 4). Using linear regression method, the mathematical relationship between NO_3^--N consumption (noted as ΔNO_3^--N) and the phosphorus uptake (noted as ΔTP) was obtained as $\Delta(NO_3^-\text{-N}) = 2.0257\ \Delta TP + 2.8574$, with R^2 of 0.8589. As for the whole experimental phases, the ratio between $\Delta(NO_3^-$-N) and ΔTP ranged from 1.76 to 3.59 mgN/mgP with an average value of 2.59 mgN/mgP. This N/P ratio was higher in contrast to that in previous reports, where the N/P values ranged from 0.476 to 2.38 mgN/mgP [6,27,28], and N/P ratio was influenced by pH [29], carbon source [30] and electron acceptor type as well as concentration [31]. Moreover, the obtained N/P in

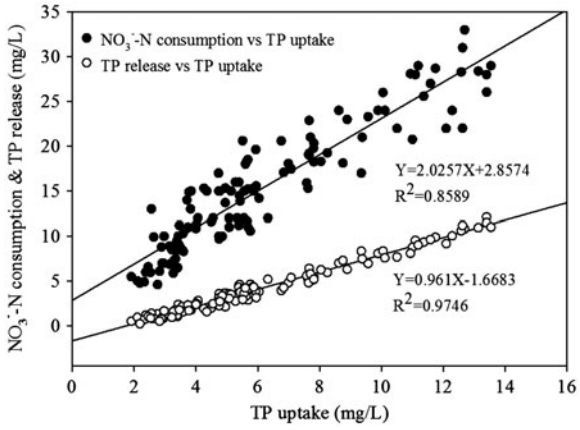

Fig. 4. Quantitative relationships between denitrifying P uptake and NO_3^--N consumption as well as anaerobic-P release.

A_2N/BAF system was higher than that obtained in the batch experiments, as that a portion of carbon source was recirculated to anoxic tank, whose nitrate ought to be preferentially consumed by denitrification [32]. In the current study, biodegradable COD, TP and NO_3^-–N concentrations into the anoxic tank were approximately 12, 7.24 and 18.2 mg/L, respectively, and about 2.86 mg/L COD was consumed per NO_3^-–N denitrified by ordinary heterotrophic organisms (OHOs) in anoxic tank. It was then deduced that the total NO_3^-–N denitrified by OHOs was about 4.20 mg/L, and the residual NO_3^-–N for denitrifying phosphorus removal was 14.0 mg/L. Therefore, the N/P by DPAO was 1.93 mgN/mgP on average.

In addition, as shown in Fig. 4, a linearly quantitative correlation between the amount of phosphorus release in the anaerobic zone and that uptake under anoxic condition could also be obtained with R^2 as high as 0.9746. All these results indicated that the phosphorus removal in the A_2N system was indeed owing to the function of DPAO.

Batch experiments were also conducted to evaluate the denitrifying P-removal and aerobic P-removal with NO_3^-–N and O_2 as electron acceptors, respectively. As shown in Fig. 5(a), phosphorus was mainly removed within 2.0 h and NO_3^--N concentration decrease corresponded with the decrease of TP concentration. The highest denitrifying P-removal rate was achieved during the first 15 min with phosphorus uptake rate and denitrification rate of 11.5 mg P/g-MLSS h and 10.4 mg NO_3^-–N/g-MLSS h, respectively, which was comparable to the A_2N system in previous reports [9,11]. Moreover, $\Delta(NO_3^-$-N) vs. ΔTP ratio for anoxic denitrifying phosphorus removal was 1.28 which was slightly lower than that obtained by linear regression

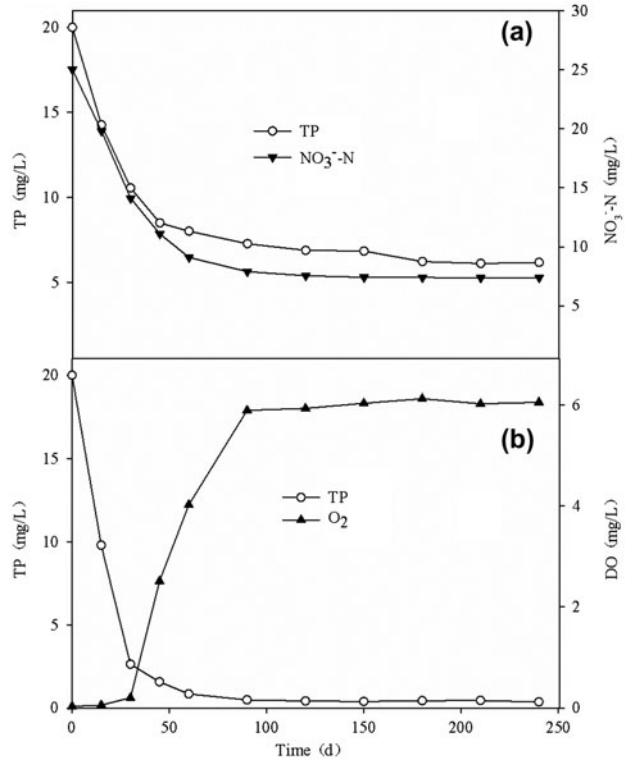

Fig. 5. Anoxic and aerobic phosphorus uptake efficiency of the sludge of the process.

in Fig. 4. This might attribute to the residual carbon source of anaerobic phase that was washed out directly using physiological saline rinse in batch experiment, which avoided the additional nitrate consumption by carbon source [28]. As for aerobic phosphorus uptake in Fig. 5(b), it took about 1 h to achieve the complete phosphorus removal, and highest denitrifying P-removal rate during the first 15 min was 20.4 mg P/g-MLSS h which was almost twice that of denitrifying P-removal, and this was corresponding to the result of Murnleitner et al. [33] deduced from the dynamical equation. In addition, DO in the column was almost zero during the initial 30 min due to the rapid consumption of oxygen resulted from aerobic phosphorus uptake. Despite the low DO level, the aerobic phosphorus uptake was still dominated for phosphorus removal sludge and anaerobic phosphorus release could be effectively inhibited. Then, DO increased obviously when the phosphorus uptake rate decreased and reached to a relative steady level.

Based on denitrifying and aerobic phosphorus uptake profiles, the relative phosphorus uptake activity of DPAO vs. PAO calculated by $\Delta P_{anoxic}/\Delta P_{aerobic}$ was approximately 70.3%, where ΔP_{anoxic} and $\Delta P_{aerobic}$ were the phosphorus uptake rates under anoxic and

aerobic conditions, respectively. This result implied that denitrifying phosphorus removal organisms had high phosphorus removal efficiency in this pilot-scale system, which was comparable with previous experimental result [6].

3.4. Microbial diversity and dominant taxonomy

High-throughput sequencing of the sludge from the pilot-scale A_2N/BAF and seed sludge generated 11,367 and 16,323 effective sequences after filtering the low quality reads using the RDP (Ribosomal Database Project) platform [34], respectively. After denoising, filtering out chimeras, and removing the archaeal sequences, the sequences of each sample were normalized to 9,495 which was the lowest sequence number of the two samples for downstream analysis.

Microbial diversity in terms of OTU, Chao1, Chao2 and abundance-based coverage estimator (ACE) at cut-off level of 3 and 5% computed by EstimateS software [35] are summarized in Table 2. On the basis of OTU number, A_2N sludge had a less rich diversity compared with the seed sludge, and the two samples shared 296 and 318 OTUs at cut-off level of 3 and 5%, respectively. Other indices including Chao1, Chao2, ACE and GOOD's coverage showed the similar patterns, reflected by the 1.3–1.4 more diversity of seed sludge. The diversity results indicated the microbial selection under anaerobic–anoxic circulation condition in the A_2N/BAF process and a proportion of microbes were screened out of the system after a long-term operation.

The comprehensive phylogenetic bacterial taxa information of those normalized 9,495 sequences at different levels were confirmed via global alignment for sequence taxonomy , and abundance was presented as percentage in total effective sequence of each sample. Fig. 6 depicted the relative bacterial community abundances on the phylum level of the two samples. Similar with the community structure analysis of globally distributed WWTP [34], *Proteobacteria* were the most abundant phylum, accounting for 46.0 and 60.1% of the total effective sequences for the seed sludge and A_2N/BAF sludge. Other dominant phyla

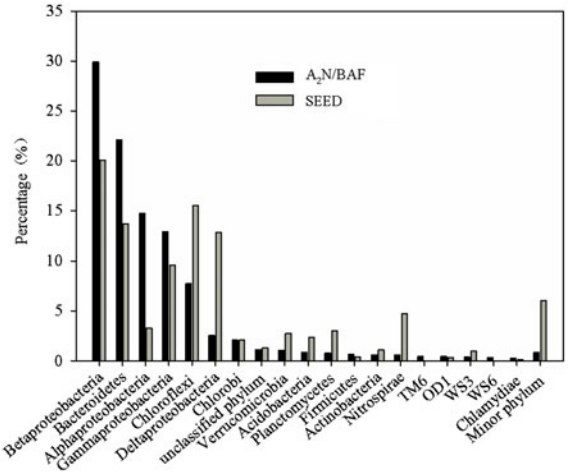

Fig. 6. Abundances (%) of the major phylum in the samples of seed sludge and A_2N/BAF. The phyla less than 1% in all the samples were classified as minor phyla.

(abundance > 1% in both samples) were *Bacteroidetes*, *Chloroflexi*, *Chlorobi* and *Verrucomicrobia*. Moreover, several phyla including *Acidobacteria*, *Actinobacteria*, *Planctomycetes* and *Cyanobacteria* only majored in the seed sludge. Moreover, the abundance of *Nitrospirae* that was related to nitrification in A_2N/BAF (0.57%) decreased significantly compared with the seed sludge (4.73%), which could be attributed to the absence of aeration in the denitrifying phosphorus removal sludge since the nitrification was achieved in BAF. Moreover, within the most dominant *Proteobacteria*, *β-Proteobacteria* was the most dominant class in both the seed sludge (34.7%) and the A_2N sludge (44.5%), followed by *γ-*, *α-*, *δ-* and *ε*-subvisions. Among them, *α-*, *γ-* and *β-Proteobacteria* in A_2N/BAF were more abundant than in the A_2N sludge, while *δ-* and *ε-Proteobacteria* were comparatively more abundant in the seed sludge, especially the *δ-Proteobacteria*.

Currently, *Candidatus Accumulibacter* (henceforth referred to as *Ca. Accumulibacter*) within family *Rhodocyclaceae* was the most accepted phosphorus removal bacteria candidates [36–38]. Based on the elaborate online survey on National Center for Biotechnology Information and phylogenetic analysis of reported 16S

Table 2
Microbial diversity of the sludge samples from A_2N/BAF and seed sludge

	3% cutoff				5% cutoff			
	OTUs	Chao1	Chao2	ACE	OTUs	Chao1	Chao2	ACE
Seed	1,547	3,906	1,546	3,891	1,189	2,253	1,146	2,220
A_2N/BAF	1,466	2,830	1,173	2,941	890	1,543	821	1,596

rRNA gene sequence from both full scale WWTP and bench scale reactors, four major clades were assigned by Kim et al. [39]. With the reference sequences from Kim's phylogenetic tree, in the present study, local basic local alignment search tool was employed for PAO quantification. Results showed that 0.71 and 1.01% of bacterial sequences from the seed and A_2N/BAF sludge could be assigned to *Ca*. *Accumulibacter*-like sequences at a similarity level of 97%. However, these values were below the average reported PAO percentage that was of 6–22% [40,41]. Therefore, the bacteria, other than *Ca*. *Accumulibacter*, must have performed the observed EBPR under anoxic conditions. Agreed well with the results in the previous study, *Dechloromonas*-related OTU outside the four known *Ca*. *Accumulibacter* clades were considered as the denitrifying PAO under anoxic conditions [42,43]. Considering the aerobic PAO and denitrifying PAO as a whole community, the PAO percentages in seed and A_2N/BAF sludge were estimated to be 1.44 and 4.96%, respectively, indicating a significant enrichment of DPAO after long-term operation of A_2N/BAF process.

In summary, the sludge from the A_2N/BAF had comparatively less microbial diversity than the seed sludge, and several common dominant bacteria were shared by the two processes. Besides, the microbial community of the A_2N/BAF process after long-term operation had significant shift compared with the seed sludge, indicating the diverse dominant bacteria enrichment in different treatment processes.

4. Conclusions

An innovative two-sludge system, A_2N/BAF, was developed to treat the domestic wastewater with low C/N ratios. The long-term operation in pilot scale for more than 820 d showed that final effluent of A_2N/BAF could meet the discharge standards satisfactorily, with average concentrations of COD, NH_4^+-N and TP as low as 30, 1.02 and 0.32 mg/L, respectively. The A_2 N/BAF system was feasible for the treatment of low C/N wastewaters without any additional carbon source.

Phosphorus removal in the system was believed to mainly attribute to the function of DPAO and phosphorus uptake exhibited excellent linear relationship with nitrate consumption. Denitrifying phosphorus removal sludge was of high efficiency, and phosphorus uptake rate and denitrification rate under anoxic condition were 11.5 mg P/g-MLSS h and 10.4 mg NO_3^--N/g-MLSS h, respectively, at the initial stage, and the relative phosphorus uptake activity of DPAO/PAO was approximately 70.3%.

Microbial diversity was decreased after acclimatization under the denitrifying phosphorus removal condition in the A_2N/BAF process. Sludge in the A_2N/BAF shared several common dominant bacteria with the seed sludge and the long-term operation also shifted the microbial community compared with the seed sludge. Abundance of phosphorus removal sludge was increased from 1.44% in seed sludge to 4.96% in the A_2N/BAF sludge.

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