Environment Protection Engineering

Vol. 40 2014 No. 1

DOI: 10.5277/epe140106

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ENRICHMENT OF PAO AND DPAO RESPONSIBLE FOR PHOSPHORUS REMOVAL AT LOW TEMPERATURE

A new strategy of enrichment of polyphosphate accumulating organisms (PAO) and denitrifying polyphosphate accumulating organisms (DPAO) at low temperature ranging from 8 °C to 11 °C was demonstrated through two lab-scale reactors operated in sequential anaerobic-aerobic (AO) or anaerobic-anoxic (AA) conditions. It was found that the AO reactor is able to achieve a good phosphorus removal performance after 40 days of operation, while a similar stable phosphorus removal can be obtained in the AA reactor after 80 days. This result suggests that the enrichment of PAO was easier than that of DPAO at low temperature. Through switching batch tests, when DPAO is exposed to aerobic conditions, it can immediately exhibit a good phosphorus removal similar to that under anoxic conditions, while PAO can only present poor phosphorus removal when exposed to anoxic conditions, suggesting that two different types of *Accumulibacter* were enriched both in AA and AO reactors. Microbial analysis with fluorescence in situ hybridization (FISH) and DAPI (4′,6-diamidino-2-phenylindole) staining revealed that *Accumulibacter* was dominant both in the two reactors, accounting for 61.6% and 79.3% of all bacteria in AA and AO reactors, respectively. Although the different amount of *Accumulibacter* was enriched in the two reactors, the similar microbial morphologies were observed by using scanning electron micrograph (SEM), both presenting long-rod morphology. This kind of *Accumulibacter* may display affinities for sodium acetate used as the carbon sources here. This strategy proposed in this study was shown to be effective in achieving a very high enrichment of *Accumulibacter* at low temperature by linking chemical analysis with microbial observation.

1. INTRODUCTION

The discharge of nutrient materials (i.e., nitrogen and phosphorus) from wastewater to soil and waters may adversely affect water resources in some ways,

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especially potential contributions to eutrophication. Nitrogen and phosphorus polluted surface waters often need to be pretreated prior to use in drinking water systems such as those from Taihu Lake, Chaohu Lake and Dianchi Lake, China. As phosphorus is the limiting nutrient in algal blooms, phosphorus removal from wastewater, hence, has become an important need to protect public health and reduce ecological risk. In order to meet a stringent limit for phosphorus, enhanced biological phosphorus removal (EBPR) is generally regarded as an economical and environmentally friendly technology for the removal of phosphorus from wastewater due to its advantages relative to conventional chemical precipitation method such as using ferric chloride and aluminium oxide.

In EBPR process, a group of bacteria are generally enriched through sequential anaerobic-aerobic conditions, known as polyphosphate accumulating organisms (PAO) responsible for phosphorus removal. During the anaerobic period, PAO take up carbon sources, particularly volatile fatty acids (VFA) such as acetate and propionate, store them as poly-β-hydroxyalkanoates (PHA), supplied with energy from the hydrolysis of polyphosphate (poly-P) (resulting in the release of phosphorus from the cells of PAO) and glycolysis of glycogen. In the subsequent aerobic period, PAO take up phosphorus in excess of the anaerobic release to store them as poly-P, usually called luxury phosphorus uptake, simultaneously accompanying the growth of biomass and the regeneration of glycogen with the required energy from the oxidation of PHA stored in cells of PAO under the anaerobic condition [1]. The details of primary characteristics of EBPR systems have been thoroughly studied [2–4]. Although EBPR system has many advantages, it has difficulty of application to the treatment of wastewater with low C/N ratio under conventional anaerobic-aerobic conditions due to the competition for carbon sources between phosphorus release and denitrification [5]. Commonly, the most appropriate solution to the problem associated with the limitation of carbon sources is the introduction of denitrifying polyphosphate accumulating organisms (DPAO) responsible for phosphorus removal under anaerobic-anoxic condition into EBPR system [6]. Compared with conventional EBPR running under anaerobic-aerobic conditions, nitrate can be used as an electron acceptor instead of oxygen for simultaneous phosphorus removal and denitrification under anaerobic-anoxic conditions, which can save aeration (30%), minimize sludge production (50%) and reduce the demand for carbon sources (50%) [2].

In recent years, studies on DPAO have been performed in lab-scale or full-scale EBPR system [6,7], most of which have mainly focused on the electron acceptors, chemical parameters analysis and influence factors such as pH [8], carbon sources [9] and temperature [10,11]. In contrast, temperature, among all these factors mentioned above, seems to be the most beyond control, especially for practical operation [11]. In general, temperature has a strongly influence on the nutrient materials removal performance of EBPR system. When temperature varying from 15 °C to 30 °C, the lower temperature, the greater amount of microorganisms responsible for phosphorus re-

moval seems to be enriched in EBPR system [12]. However, there is no information available on the running performance of EBPR system at even lower temperature such as around 10 °C, often presenting in winter in China.

Thus, a new strategy for obtaining microorganisms responsible for phosphorus removal (i.e., PAO and DPAO) at 8–11 °C was developed in this study. This proposed strategy was performed using two lab-scale EBPR reactors, operating under both anaerobic-aerobic and anaerobic-anoxic conditions for 80 days. The effectiveness of the strategy was assessed by both monitoring carbon, nitrogen and phosphorus removal performance using chemical analysis and investigating the change of microbial community structure characteristics in each EBPR reactor using fluorescence in situ hybridization (FISH), DAPI (4′,6-diamidino-2-phenylindole) staining, and scanning electron micrograph (SEM). Comparison of PAO and DPAO was also carried out to investigate their activities in various supplied electron acceptors.

2. MATERIALS AND METHODS

Reactor setup and operation. Two lab-scale sequencing batch reactors (SBR) with the working volume of 3.3 dm^3 (Fig. 1) were conducted for phosphorus removal, one operated with a sequence of anaerobic-aerobic conditions (AO) for PAO enrichment and the other in anaerobic-anoxic cycling mode (AA) for DPAO enrichment.

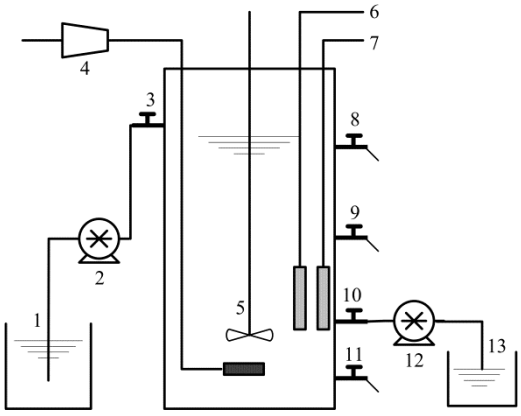

Fig. 1 Schematic diagram of experimental setup: 1 – influent tank, 2 – influent pump, 3 – water inlet, 4 – air pump, 5 – mixer, 6 – ORP/pH meter, 7 – DO meter, 8, 9 – sample points, 10 – water outlet, 11 – waste sludge outlet, 12 – effluent pump, 13 – effluent tank

Seed sludge was withdrawn from an aerobic basin of the Chengdong Municipal Wastewater Treatment Plant, Nanjing, China. The cycle time was 8 h and consisted of: a 0.5 h filling period, 2 h anaerobic period, 4 h aerobic or anoxic period, 1 h settling

period and 0.5 h decant period. In each cycle, 1.9 dm^3 of synthetic wastewater (composition detailed in Table 1) was fed to the reactor during the filling phase, resulting in a 13.9 h of hydraulic retention time (HRT) and an effluent of the same amount as influent (1.9 dm^3) was discharged at the end of one cycle. In the AA reactor, sodium nitrate solution was pumped in the first 1 min of the anoxic period to provide anoxic conditions. Volumes of 330 cm^3 and 115 cm^3 mixed liquor were removed per day from AO and AA reactors, to maintain the solids retention time (SRT) at 10 and 20 days, respectively. Air was supplied at the flow rate of 1.5 dm^3/min to maintain the dissolved oxygen (DO) concentration higher than 2 mg/dm^3 during the aerobic period. pH in the two reactors was maintained at 7.0±0.2, with the addition of 0.5 M HCl or 0.5 M NaOH when the pH was above or below this setpoint. Two reactors were operated at room temperature, ranging from 8 °C to 11 °C. The liquor in the reactors was completely mixed with an overhead stirrer (200 rpm) during both anaerobic and aerobic or anoxic period.

Table 1

Composition of wastewater used for experiment

Feeds	Concentration [g/dm^3]	Nutrient solution	Concentration [g/dm^3]
CH_3COONa	1.03	$FeCl_3 \cdot 6H_2O$	1.50
KH_2PO_4	0.18	H_3BO_3	0.15
$(NH_4)_2SO_4$	0.47	$CuSO_4 \cdot 5H_2O$	0.03
$CaCl_2$	0.02	KI	0.18
$MgSO_4 \cdot 7H_2O$	0.18	$MnCl_2 \cdot 4H_2O$	0.12
Nutrient solution	0.60 cm^3/dm^3	$Na_2MoO_4 \cdot 2H_2O$	0.06
		$ZnSO_4 \cdot 7H_2O$	0.12
		$CoCl_2 \cdot 6H_2O$	0.15
		EDTA	10.00

COD:P = 20:1, pH = 7.0±0.2.

Two reactors responsible for the PAO and DPAO enrichment were operated for 80 days. The entire operation strategy was divided into two phases:

- Phase 1 (0–30 days). The concentrations of COD and PO_4^{3-}-P in the feed were 800 mg/dm^3 and 40 mg/dm^3, respectively, with the addition of 50 mg NO_3^--N per dm^3 in anoxic phase, and the ratio of COD to P was 20:1, adopting high substrate load to promote rapid growth of microorganisms responsible for phosphorus removal; no waste sludge was discharged from the two reactors in this phase to maintain a large amount of biomass.
- Phase 2 (30–80 days). The concentrations of COD and PO_4^{3-}-P in the influent were decreased to 300 mg/dm^3 and 20 mg/dm^3, respectively, with the decrease of ni-

trate to 30 mg of NO_3^--N /dm^3 in anoxic phase, and their ratio was correspondingly decreased to 15:1. Waste sludge was regularly removed from the two reactors to maintain the concentration of MLSS in AA and AO reactor 4.5 g/dm^3 and 3.7 g/dm^3, respectively.

Batch tests. For the batch tests, 0.5 dm^3 of activated sludge was taken from both the AA and AO reactors at the end of aerobic and anoxic process at 80 days, and was immediately washed twice with the nutrient solution (Table 1) not containing the basic medium. The activated sludge treated from AA reactor responsible for anoxic phosphorus removal was divided into two parts and filled into two 1 dm^3 test devices (transformed triangular flask), One part was operated in an anaerobic-aerobic mode and the other under anaerobic-anoxic conditions. The AO sludge treated was performed following the same rule. AO sludge was exposed to anoxic conditions to monitor its capability of using nitrate as the electron acceptor, and similarly, AA sludge was exposed to aerobic conditions to monitor its capability of using oxygen as the electron acceptor. Before the anaerobic phase, 0.5 dm^3 of synthetic wastewater was added to achieve the initial concentration of 300 mg COD/dm^3, 20 mg PO_4^{3-}-P /dm^3. After a 2h anaerobic period, oxygen and nitrogen were supplied in the aerobic and anoxic conditions, respectively, with the addition of 30 mg NO_3^--N /dm^3 in anoxic conditions as well.

Chemical analysis. The phosphorus removal performance of PAO and DPAO from AO and AA reactors was monitored through chemical analytical techniques. Samples were taken regularly from the two reactors and four batch test devices throughout the study, sampled every two days during the PAO and DPAO enrichment period and every 15 minutes for batch test. PO_4^{3-}-P and NO_3^--N were analyzed by the segmented flow analysis (AutoAnalyzer3, SEAL, Germany). Chemical oxygen demand (COD), mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) were determined by the standard methods for the examination of water and wastewater (APHA, 2002). Dissolved oxygen (DO), pH and oxidation-reduction potential (ORP) were measured by a DO meter analyzer (YSI 5100-230, USA) and pH and ORP meter analyzer (YSI 100, USA).

Microbial analysis. Sludge samples collected from both the AA and AO reactors were divided into two parts. One part was fixed in 4% paraformaldehyde at 4 °C for 3 h for fluorescence in situ hybridization (FISH) and the other in 2.5% glutaric dialdehyde at 4 °C for 24 h for scanning electron micrograph (SEM). For comparison, seed sludge afore-mentioned was also investigated by FISH and SEM. FISH was performed according to the modified method of Kang et al. [13] to assess the evolution of microbial populations in both reactors throughout the study. SEM was chosen to study

changes in the microbial morphologies. The 16Sr RNA oligonucleotide probes adopted for FISH are listed in Table 2. PAO651, PAO462 and PAO846 were applied together (PAOmix comprising equal amounts of those three probes) to target the *Accumulibacter*, a known PAO. DAPI (4′,6-diamidino-2-phenylindole) staining was performed for targeting the entire all bacteria [14]. FISH images were obtained with the BioImaging Navigator fluorescence microscope (Olympus FSX100, Tokyo, Japan) using a software of Olympus FSX-BSW.

Table 2

FISH probes used in this study

Probe mix	Probe	Sequence (5′–3′)	rRNA	Target	Dye 5′
PAOmix	PAO651	CCCTCTGCCAAACTCCAG	16S	651–668	Cy3
	PAO462	CCGTCATCTACWCAGGGTATTAAC		462–485	Cy3
	PAO846	GTTAGCTACGGCACTAAAAGG		846–866	Cy3

Quantification of the PAO community relative to the entire bacterial populations was determined by THE FISH image analysis with the Image-Pro Plus software (version 6.0, USA), following the method reported by Gao et al. [15]. Fluorescence in situ hybridization was conducted following the below procedures:

- Samples fixation. Fixed sludge samples with a 4% paraformaldehyde as mentioned above were centrifuged (5000×g RCF for 5 min) and washed twice in 0.01 M PBS, and then resuspended in a PBS ethanol solution (1:1, v/v), being stored at –20 °C.
- Fixed samples dehydration. Before dehydration, the fixed sludge samples were homogenized using a homogenizer fr 3 times for 30 s. Homogenized samples were spotted onto slides treated with 3-aminopropyl-triethoxysilane (APES) acetone (1:50, v/v) and then dried for 30 min at 40 °C. Dried slides containing sludge samples were dehydrated for 3 min in 50%, 80% and 100% (v/v) ethanol and allowed to air dry, and then stored in a desiccator prior to hybridization within FISH experiments.
- Hybridization. Hybridization of the treated sludge samples was performed for 2.5 h at 46 °C in a 0.001 cm^3 probe (50 μg/cm^3, Table 1) and in a 0.009 cm^3 hybridization buffer solution (pH 7.2) containing 0.9 M NaCl, 20 mM tris-HCl, 0.01 % sodium dodecyl sulfate (SDS) and 35% formamide for PAOmix.
- Washing samples: when fluorescent in situ hybridization for sludge samples completed, a stringent washing step was performed 4 times for 5 min in a washing buffer solution (48 °C, pH 7.2) containing 20 mM tris-HCl, 5 mM EDTA, 0.01%SDS, 50 mM NaCl. And then these slides attached sludge samples were washed with double distilled water and immediately dried at 30 °C for 2 h, and then were observed under a fluorescence microscope.

For SEM observation, fixed AA and AO sludge samples were rinsed 3 times (for 15 min each time) with a 0.1 M phosphate buffer (PB), pH 7.0. For a better preserva-

tion of the cell structure within microorganisms, treated sludge samples were fixed again in 0.1% acetic acid for 2 h, and then rinsed using the same method mentioned above. Fixed samples were dehydrated in an ethanol series, 50%, 70%, 80%, 90%, 100% ethanol (v/v) for 15 min each. Ethanol in dehydrated samples was displaced by 1:1 (v/v) of ethanol to isoamyl acetate for 30 min with slightly shaking and then by 100% isoamyl acetate for 30 min. These treated samples dried by the CO_2 critical point drying. The treated sludge samples were observed after spray-gold treatment, using a scanning electron microscope (JSM-6360LV, Japan).

3. RESULTS

The EBPR performance of AO and AA reactors running under anaerobic-aerobic and anaerobic-anoxic conditions, respectively, was investigated throughout the enrichment to PAO and DPAO at low temperature. During this process, the change of microbial community structure in each EBPR reactor was observed. Moreover, comparison of PAO and DPAO was also carried out to investigate their activities through switching tests. For comparison, seed sludge was simultaneously conducted to investigate its capacity of phosphorus removal and microbial characteristics.

3.1. PERFORMANCE OF AA AND AO REACTOR

The phosphorus removal performance throughout the process of microorganisms acclimatization in the both EBPR reactors at low temperature (8–11 °C) over the 80 day time period is shown in Fig. 2. During the AO reactor operation under anaerobic-aerobic conditions, a stable phosphorus removal performance was presented after 40 days, as given by the variation of phosphorus concentration in the effluent, while the AA reactor reached the similar stable state phase after approximately 80 days running under anaerobic-anoxic conditions. At the same operation parameters, each reactor responded differently throughout the PAO and DPAO enrichment experiments, which DPAO acclimatization to attain stable state required one times more than PAO, indicating the higher activities of PAO at low temperature than DPAO. At the stable state phase, both the AA and AO reactors exhibited a good phosphorus removing performance and the effluent phosphorus concentrations were both lower than 0.5 mg P/dm^3. Consistently, the stable concentrations of MLSS, MLVSS, phosphorus release and uptake and the constant ratios of MLVSS to MLSS and phosphorus release to phosphorus uptake were also observed both in the AA and AO reactors.

The amount of phosphorus stored in the microorganisms such as PAO and DPAO can be generally implied according to the ratio of MLVSS to MLSS, and the lower ratio, the greater amount of phosphorus may be stored in microbes responsible for phosphorus removal. At the end of acclimatization of PAO and DPAO in the two reactors, the average MLVSS and MLSS concentrations were 2.6 g/dm^3 and 3.7 g/dm^3, 3.5 g/dm^3 and 4.5g/dm^3, respectively, exhibiting that their ratios were 0.70 and 0.78,

respectively. These results implied that a higher amount of phosphorus was stored in the AO sludge than in AA sludge per gram of biomass.

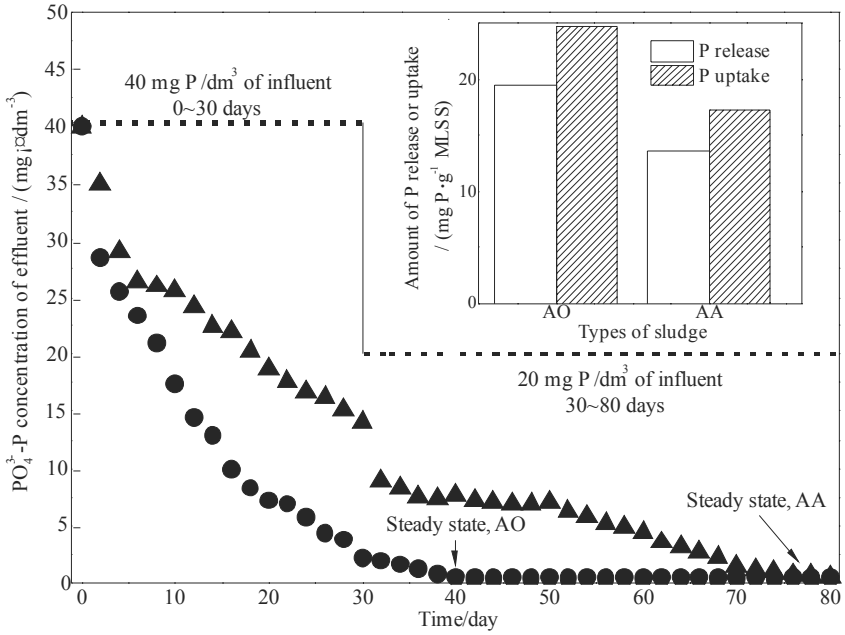

Fig. 2. Phosphorus profiles during the enrichment of PAO and DPAO

A typical key phosphorus biochemical transformation responsible for EBPR was observed both in the AA and AO reactors through a cycle batch test performed at the end of acclimatization study, as shown in Figs. 2–5, strongly suggesting that PAO and DPAO were predominant in their respective reactors proposed in this study. However, a significant difference in the amount of phosphorus release and uptake per MLSS between AO sludge and AA sludge was monitored (Fig. 2), probably due to the fact that *Accumulibacter* exhibits a different metabolic process based on the different running modes, namely anaerobic-aerobic and anaerobic-anoxic. For AO sludge, the anaerobic phosphorus release rate and aerobic phosphorus uptake rate were 19.46 mg P/(g MLSS) and 24.74 mg P/(g MLSS), respectively, both higher than the phosphorus release rate and anoxic phosphorus uptake rate of AA sludge, which were 13.56 mg P/(g MLSS) and 17.33 mg P/(g MLSS), respectively. These results demonstrated the PAO and DPAO phenotypes responsible for phosphorus removal from wastewater. Although a significant difference existing in the amount of phosphorus release/uptake between PAO and DPAO was observed, the ratio of the phosphorus release to the phosphorus uptake (0.786) in AO sludge was quite consistent with that in AA sludge (0.782), further suggesting that both PAO and DPAO were dominant in their respective reactor at the end of enrichment period. This explanation was also demonstrated by the linear

relationship between the amount of COD consumption and that of phosphorus release under anaerobic conditions, as given in Fig. 3.

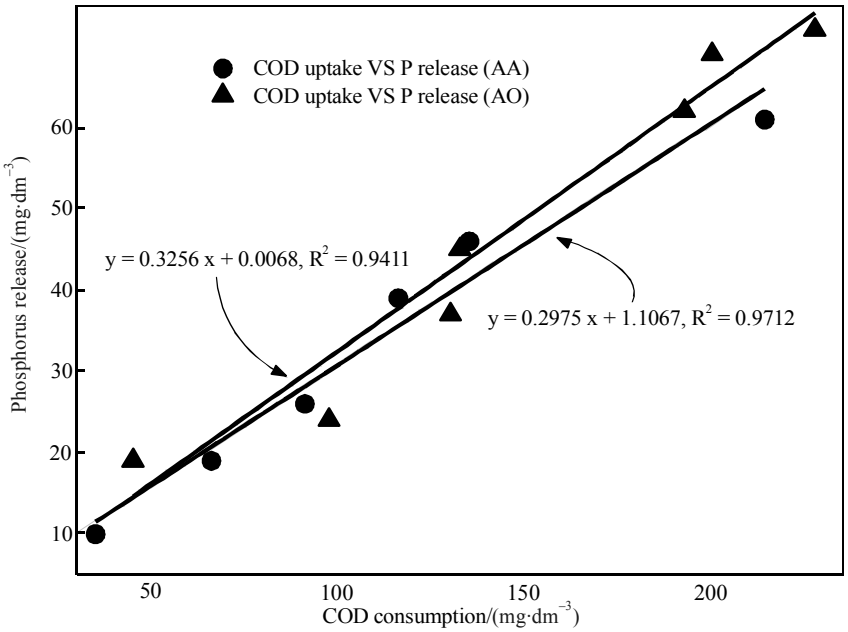

Fig. 3. Dependences of phosphorus release on COD uptake for AA and AO sludge in anaerobic conditions

3.2. ANAEROBIC-AEROBIC BATCH TEST WITH DPAO SLUDGE

The phosphorus release and uptake capacities of DPAO during two different cycles (anaerobic-aerobic and anaerobic-anoxic) at the end of acclimatization phase were investigated through two batch tests proposed here. Typical profiles (time dependence of carbon, nitrogen and phosphorus) monitored in these batch tests are shown in Fig. 4. Under the anaerobic conditions, sodium acetate was mostly taken up, which was accompanied by phosphorus release, additionally showing a good correlation between sodium acetate uptake and phosphorus release here (Fig. 3). The phosphorus anaerobic release rate of DPAO obtained here was 13.56 mg P/g MLSS lower than that of PAO (19.46 mg P/g MLSS), likely due to the less amount of *Accumulibacter* enriched in AA reactor compared to AO reactor (Fig. 6). After a two hour anaerobic phase, DPAO sludge exhibited a good phosphorus uptake performance both under anoxic and aerobic conditions. The phosphorus uptake rates obtained in these batch tests were 17.33 mg P/g MLSS in anoxic mode and 17.76 mg P/g MLSS under aerobic conditions, indicating that DPAO was able to immediately use oxygen as the electron acceptor when exposed to aerobic conditions, as evidenced by the rapidly phosphorus uptake rate (Fig. 4).

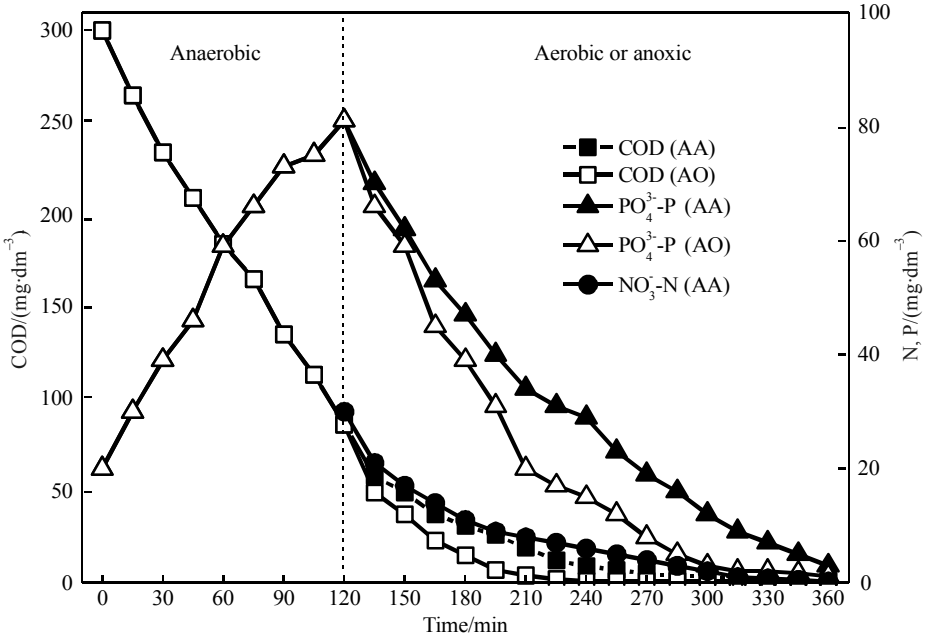

Fig. 4. Carbon, nitrogen and phosphorus profiles of DPAO sludge exposed to anaerobic-aerobic or anoxic conditions

Concurrently, residual sodium acetate from anaerobic phase was completely consumed by the denitrifying bacteria or by the heterotrophic bacteria. Obviously, nitrate added in the anoxic phase was removed from wastewater by the denitrifying phosphorus removing bacteria, namely *Accumulibacter*, with the function of simultaneous denitrificaiton and phosphorus removal.

3.3. ANAEROBIC-ANOXIC BATCH TEST WITH PAO SLUDGE

Two batch tests similar to those conducted in Sect. 3.2 were performed to compare the phosphorus uptake capacity of PAO from AO reactor in anaerobic-anoxic and anaerobic-aerobic modes at the end of acclimatization phase. This result obtained in these batch tests is shown in Fig. 5. During a 2 h anaerobic phase, a good performance of both phosphorus release and sodium acetate uptake was present for PAO sludge from the AO reactor, as also illustrated in Fig. 3, where the phosphorus release rate was 19.46 mg P/g MLSS and the sodium acetate uptake rate was 61.49 mg COD/g MLSS, both higher than that of DPAO (13.56 mg P/g MLSS, 47.56 mg COD/g MLSS, as shown in Fig. 4). In contrast with DPAO (see Fig. 4), however, a significant difference of phosphorus uptake performance of PAO between aerobic and anoxic was clearly present in Fig. 5. Here, the aerobic phosphorus uptake rate was 24.74 mg P/g MLSS, while that was 4.86 mg P/g MLSS in anoxic conditions, suggesting that the phosphorus uptake ability of PAO sludge was

inhibited when exposed to anoxic conditions, as also evidenced by the less nitrate reduction in this batch test.

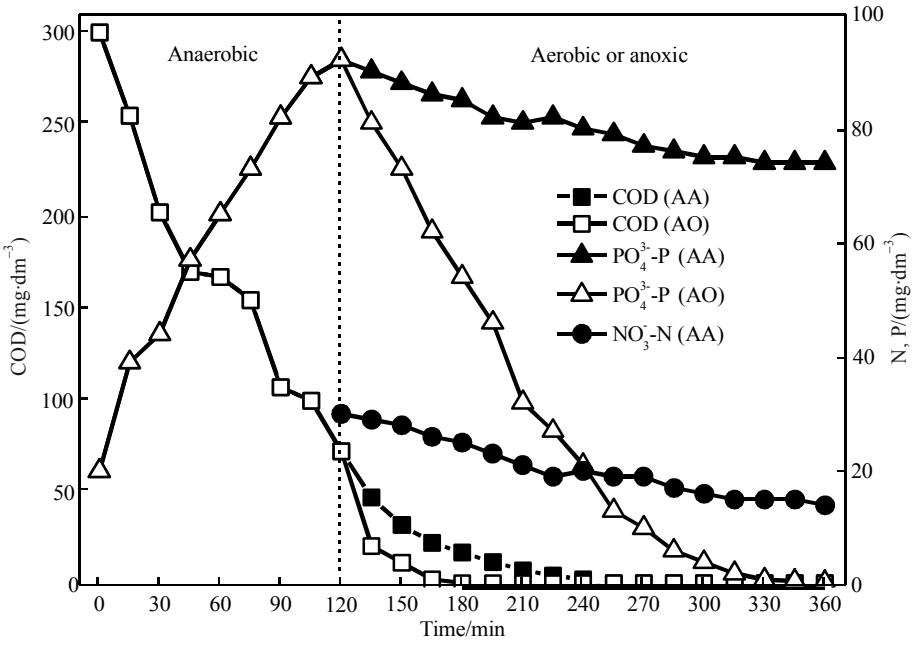

Fig. 5. Carbon, nitrogen and phosphorus profiles of PAO sludge exposed to anaerobic-aerobic or anoxic conditions

3.4. MICROBIAL CHARACTERISTICS ANALYSIS

Throughout the entire start-up period of AA and AO reactors, different phosphorus removal performance observed in the two reactors could be due to the variations in the microbial population. For this, the techniques of both fluorescence in situ hybridization (FISH) and scanning electron micrograph (SEM) were adopted to obtain a better understanding of the microbial community shift both in AA and AO reactors during the acclimatization phase. The results from AA and AO reactors are shown in Fig. 6.

FISH analysis showed the *Accumulibacter* population clearly increasingd with time, during the start-up period, from 9.3 % of all bacteria in seed sludge, to 61.6 % in AA sludge and to 79.3 % in AO sludge, indicating that *Accumulibacter* was substantially dominant at the end of acclimatization phase, which was consistent with phosphorus removal performance in respective reactor as described above. As shown in Fig. 6, the dominance was much higher in the AO reactor than in the AA reactor. From this FISH quantification, phosphorus removal microorganisms can be considerably enriched in EBPR systems at low temperature when a strategy suitable for the growth of *Accumulibacter* is provided, as the new strategy proposed in this study.

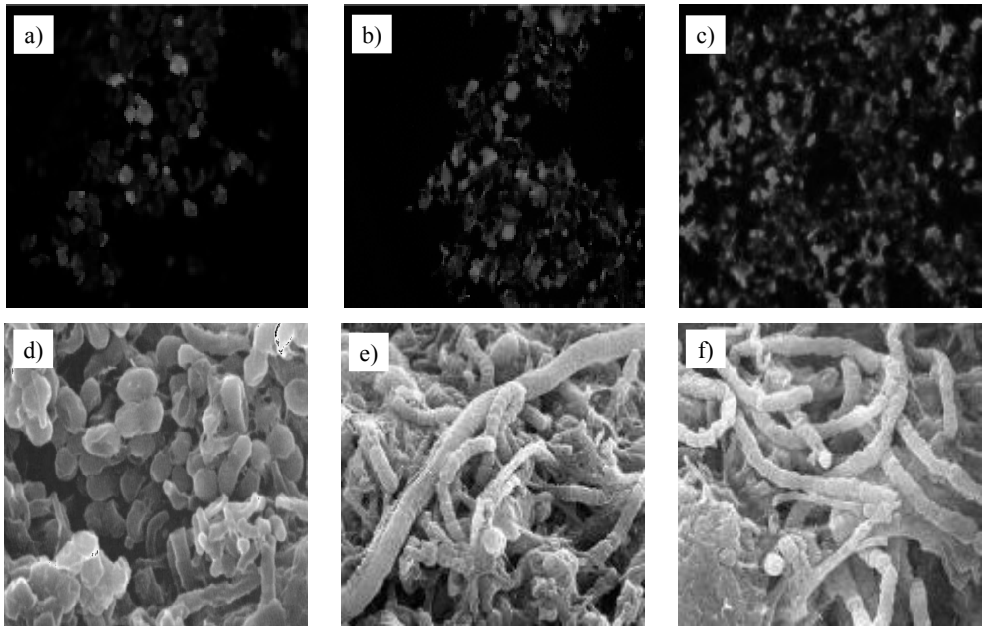

Fig. 6. FISH and SEM images of activated sludge in seed sludge and at the end of acclimatization period: a) seed sludge, PAO (9.3%) + DAPI, 50×, b) AA sludge, DPAO (61.6%) + DAPI, 50×, c) AO sludge, PAO (79.3%) + DAPI, 50×, d) seed sludge, 5000×, e) AA sludge, 5000×, f) AO sludge, 5000×

From SEM images (see Fig. 6), it is clear that significant differences in microbial morphologies were observed between the seed sludge from A_2O and the AA or AO sludge from EBPR systems studied here. Long-rod morphology microbes were abundantly enriched both in the AA and AO reactors, while seed sludge has a higher proportion of cocci or short-rod morphology microorganisms. Similar microbes were enriched in the two reactors during the acclimatization process suggested that the long-rod morphology of *Accumulibacter* responsible for phosphorus removal may preferably take up sodium acetate, as supplied in the influent in this study, regardless of the types of electron acceptors.

4. DISCUSSION

4.1. DEVELOPMENT OF THE OPERATIONAL STRATEGY TO PROMOTE THE GROWTH OF PAO AND DPAO

The strategy of enrichment PAO and DPAO under anaerobic-aerobic and anaerobic-anoxic mode respectively at lower temperature was developed based on the previous reports that *Accumulibacter*, a known PAO, contains two different types: one is

capable of not only aerobic phosphorus uptake by using oxygen as the electron acceptor, but also anoxic phosphorus uptake by using nitrate, namely DPAO, and the other only using oxygen instead of nitrate as the electron acceptor for phosphorus removal [16, 17], and that temperature seems to be one of the most important influence factors on wastewater systems containing EBPR in practical operation, particularly at low temperature [11].

It can be seen from Fig. 1 that both AO and AA reactors operated in anaerobic--aerobic and anaerobic-anoxic conditions confirmed the phenotypes of PAO and DPAO responsible for phosphorus removal and reached a similar stable state, as evidenced by the effluent phosphorus concentrations, MLSS, MLVSS, the ratio of MLVSS/MLSS, phosphorus release rate and uptake rate and their ratio. The AO and AA reactors reached the stable state after 40 and 80 days, respectively, suggesting the higher activities of PAO at low temperature than DPAO, probably due to the fact that the energy generated from the oxidative phosphorylation with NO_3^- is about 40% lower than that with O_2 [5]. The ratio of MLVSS to MLSS from 0.86 (3.6/4.2) of the start-up phase (namely, seed sludge collected from an aerobic basin within an A_2O process) decreased to 0.70 (2.6/3.7) of stable-state phase in the AO reactor and to 0.78 (3.5/4.5) in the AA reactor which are in agreement with the reports [18] indicating that the higher amount of phosphorus was stored in PAO or DPAO than in seed sludge, suggesting that this strategy studied here dramatically promoted the growth of PAO and DPAO in their respective reactor.

The specific phosphorus release and uptake rates for PAO were estimated to be 19.46 and 24.74 mg P/g MLSS both higher than that for DPAO, 13.56 and 17.33 mg P/g MLSS, respectively. This was likely due to the following two reasons: the energy produced by PAO with oxygen was higher than that of DPAO with nitrate (as discussed above) and the size of DPAO was higher than that of PAO, causing limited transfer of carbon, nitrogen and phosphorus to the active biomass [19]. Indeed, SEM conducted in this study showed that DPAO grow with the aggregation of biomass into similar granules, while PAO grow with flocs. Overall, these results obtained here demonstrated that the operational strategy at low temperature proposed in this study is rather effective in acclimatization of PAO and DPAO in EBPR systems, therefore providing a practical strategy for stable state operation of this process at low temperature such as in winter.

4.2. CHARACTERISTICS OF PAO AND DPAO DURING SWITCHING TESTS

Based on characteristics of *Accumulibacter*, it was expected that sludge enriched with phosphorus removing bacteria would show various phosphorus removal abilities according to the change in electron acceptors. Figures 4 and 5 show the correlation between the types of electron acceptor and the phosphorus removal performance. For DPAO, no considerable difference in the phosphorus uptake rate with nitrate and oxygen as electron acceptors was observed by switching the mode from normal anoxic to

aerobic, namely that when DPAO is exposed to aerobic conditions it can take up phosphorus immediately, which agrees well with the report [20]. However, the phosphorus uptake performance of PAO was obviously inhibited when it was exposed to anoxic rather than aerobic conditions. These results obtained through the switching batch tests suggested that DPAO can readily produce the quantity of enzymes for aerobic metabolisms similar to anoxic metabolisms, while PAO lacks the enzymes required for anoxic metabolisms using nitrate instead of oxygen as an electron acceptor [21]. The phosphorus uptake rate of PAO was very low in anoxic conditions as compared with the aerobic conditions (Fig. 5). Interestingly, some studies [19, 22] have demonstrated that when the anoxic phase was extended to 30 h rather than to 4 h, the phosphorus uptake rate of PAO can be obviously improved, suggesting that a several hour lag phase may exist in phosphorus uptake when PAO is exposed to anoxic conditions. From these studies, we hypothesize that PAO could gradually develop the required amount of enzymes for anoxic metabolisms during the lag time, which may be in agreement with the above explanation (PAO lacks the enzymes required for anoxic metabolisms using nitrate).

Through four batch tests, comparison of the phosphorus removal performance of PAO between aerobic and anoxic conditions, and similar comparison to DPAO were conducted, demonstrating that *Accumulibacter* has, at least, two different types, which supports the reports [16, 17] described above. Nevertheless, based on Carvalho et al. findings [1], a new type of *Accumulibacter* was found not capable of using nitrate or oxygen as electron acceptors but able to use nitrite for phosphorus removal under anoxic conditions, which may further promote the development of EBPR techniques in practice, especially in the wastewater treatment containing phosphorus. Similarly, the nitrite accumulation and then elimination under anoxic conditions was also observed both in the acclimatization phase and batch tests studied here, probably supporting the existence of three different types of *Accumulibacter* in EBPR systems according to the provided various electron acceptors.

4.3. IDENTIFICATION OF ACCUMULIBACTER IN AA AND AO REACTORS

A clear shift in bacterial populations towards *Accumulibacter* responsible for phosphorus removal was observed both in AA and AO reactors during the 80 day operation. Similar findings have been reported in other EPBR systems [13, 23]. Analysis by FISH showed that *Accumulibacter* enriched in AA and AA reactors accounted for 61.6%, 79.3% of all bacteria, respectively, lower than that of the report (greater than 90%) by Lu et al. [18] but significantly higher than those by Kang et al. (41.49%) [10] and Crocetti et al. (41%) [24]. It is likely due to the fact that different adopted operational conditions, such as temperature, pH, carbon sources, etc., result in the difference in enrichment capacities of phosphorus removal microbes. The relative abundance of *Accumulibacter* in AA and AO reactors linked well with their respective

EBPR performance observed in this study (as described above).These results demonstrated that the operational strategy proposed in this study may be highly effective in enrichment of *Accumulibacter*, and therefore may provide a new practical method for obtaining a good phosphorus removal performance at low temperature.

Analysis of SEM showed that identical microbial morphologies (long-rod microbes) were present in the two reactors at the end of acclimatization period, suggesting that this kind of *Accumulibacter* may display good affinity to sodium acetate as the carbon source. Indeed, two different types (rods or cocci) of *Accumulibacter* were found by Martin et al. [21] in two EBPR systems, where one was feed with sodium acetate and the other with propionate. Similarly, He et al. [25] also found the distribution of different types of *Accumulibacter* in one lab-scale reactor and six full-scale reactors both presenting good phosphorus performance. These studies may support the hypothesis that different carbon sources feed to the phosphorus removal microorganisms could promote the growth of different types of *Accumulibacter*, probably regardless of electron acceptors which is also partially supported by the results obtained in this study. Overall, the combination of chemical analysis with microbial analysis suggested that *Accumulibacter*, both PAO and DPAO, with a long-rod morphology was more preferably enriched with sodium acetate as compared with other carbon sources such as sodium propionate.

5. CONCLUSIONS

A strategy for obtaining microorganisms responsible for phosphorus removal (i.e., *Accumulibacter* including PAO and DPAO) at low temperature ranging from 8 °C to 11 °C has been demonstrated in this study. The approach is as follows:

- Switching the ratio of COD to P of two EBPR systems operated in anaerobic-aerobic (AO) and anaerobic-anoxic (AA) modes respectively, from 20:1 to 15:1; with the decrease of concentration COD, PO_4^{3-}-P and NO_3^--N in the influent from 800 mg/dm^3, 40 mg/dm^3 and 50 mg/dm^3 to 300 mg/dm^3, 20 mg/dm^3 and 30 mg/dm^3, respectively.
- Adopting different solids retention time (SRT) for enrichment of different types of *Accumulibacter*; no excess of activated sludge was wasted at the beginning of start-up, and then 10 days SRT for PAO and 20 days SRT for DPAO.
- Maintaining a high and different MLSS concentrations in AA and AO reactors, based on the metabolic characteristics of *Accumulibacter* supplied the different electron acceptors, 4.5 g/dm^3 MLSS for the AA reactor and 3.7 g/dm^3 MLSS for the AO reactor.

This proposed strategy here was shown to be effective in achieving a very high enrichment of *Accumulibacter* at low temperature by linking chemical analysis with microbial observation. From chemical analysis, similar stable state was achieved both

in AO and AA reactors when different enrichment time provided. Chemical analysis of batch tests indicated the existence of two types of *Accumulibacter*, one using oxygen as the electron acceptor and the other – nitrates. Through microbial observation, a high abundance of *Accumulibacter* was found in both AA and AO reactors. The identical microbial morphology observed both in the two reactors suggested the long-rod phosphorus removal microorganisms may display affinities to sodium acetate used as the carbon sources in this study. Although the strategy may not be the unique method for the enrichment of phosphorus removal microorganisms at low temperature, it is recommendable for the future studies in practical application.

ACKNOWLEDGEMENT

We wish to thank M.M.SU for assistance with activated sludge treatment and Dr. Wang for enlightening discussion. This research is supported by grant 2012ZX07101-005 from National Key Technology in Water Pollution Control and Treatment in 12th Five-year Plan of China and grant 51078074 from National Natural Science Foundation of China.

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