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Performance of biological phosphorus removal and characteristics of microbial community in the oxic-settling-anaerobic process by FISH analysis*

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Abstract: Performance of biological phosphorus removal in the oxic-settling-anaerobic (OSA) process was investigated. Cell staining and fluorescent in situ hybridization (FISH) were used to analyze characteristics and microbial community of sludge. Experimental results showed that phosphorus removal efficiency was near 60% and the amount of biological phosphorus accumulation in aerobic sludge of the OSA system was up to 26.9 mg/g. Biological phosphorus removal efficiency was partially inhibited by carbon sources in the continuous OSA system. Contrasted to the OSA system, biological phosphorus removal efficiency was enhanced by 14% and the average total phosphorus (TP) contents of aerobic sludge were increased by 0.36 mg/g when sufficient carbon sources were supplied in batch experiments. Staining methods indicated that about 35% of microorganisms had typical characteristics of phosphorus accumulating organisms (PAOs). FISH analysis demonstrated that PAOMIX-binding bacteria were predominant microbial communities in the OSA system, which accounted for around 28% of total bacteria.

Key words: Excess sludge reduction, Biological phosphorus removal, Phosphate accumulating organisms (PAOs), DAPI (4′,6′-diamidino-2-phenyl indol dihydrochloride), Fluorescent in situ hybridization (FISH)

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INTRODUCTION

Enormous excess sludge has been a rising challenge for conventional activated sludge (CAS) processes due to economic, environmental and legislative constraints (Liu *et al.*, 2001; Wei *et al.*, 2003). The oxic-settling-anaerobic (OSA) process is a modified CAS process by adding an anaerobic reactor in sludge return line. The OSA process reduces excess sludge by 20%~65% and improves the sludge settleability (Chudoba *et al.*, 1991; Saby *et al*., 2003; Wang *et al*., 2007) without any chemicals addition. The OSA process is an attractive approach to solve sludge-associated problems by reducing sludge production in the wastewater treatment rather than the post-treatment of the sludge.

The anaerobic reactor inserted in the recycling bypass of sludge is the key unit of the OSA process. Microorganisms are subjected to alternative aerobic food-enriched and anaerobic starvation environment, which stimulates energy uncoupling (Chudoba *et al.*, 1991). In addition, sludge decay is the main cause that results in minimization of excess sludge in the OSA process. Contrasting to the anaerobic zone of the conventional biological nutrient removal process, the anaerobic reactor has hardly any external organic substrate and high sludge concentration in the OSA process (Chen *et al.*, 2003). However, performance of biological phosphorus removal in the OSA process is still a controversial issue. Chudoba *et al.*(1992) reported that 50%~60% of the bacteria in the OSA

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system were found to be polyphosphate (poly-P) bacteria with a low growth yield. But Chen *et al*.(2003) concluded that dominant bacteria in the OSA system were not found to be poly-P bacteria. Because no significant intake of phosphate was observed under aerobic conditions with sufficient food available, although phosphate release was observed under the anaerobic condition; moreover, there is no report on microbial community characteristics of the OSA process affected by the inserted anaerobic sludge reactor; therefore, it is evident that investigation of phosphorus removal efficiency and phosphorus accumulating organisms (PAOs) in the OSA process is necessary. It is now well recognized that microbial diversity is greatly underestimated by cultivation studies because most microorganisms observable in nature typically cannot be cultivated by standard techniques. Fluorescent in situ hybridization (FISH) by specific targeted oligonucleotide probes allows us to analyze the microbial composition and diversity in a given system without cultivation of individual species (Sanz and Köchling, 2007). In this study, biological phosphorus removal efficiency was investigated and microbial community structure was analyzed by cell staining and FISH, in order to explore the effect of the inserted anaerobic tank on the biological phosphorus removal performance in the OSA process.

MATERIALS AND METHODS

Experimental designs

1. Continuous experiments

Two identical reactors with an aerobic tank available volume of 12 L and a settling tank volume of 4.0 L were used in two continuous systems. Contrasting to the CAS system, an anaerobic tank with a volume of 4.5 L was inserted in sludge recycling line in the OSA system, as shown in Fig.1. The oxidation reduction potential (ORP) was maintained below –250 mV in anaerobic sludge tank by occasional injection of pure nitrogen gas during the start-up period. Sludge retention time (SRT) in the anaerobic tank was kept at 8~10 h. The sludge tank was mixed by magnetic stirrer at 150 r/min and covered with a gas release outlet for keeping gas pressure balance. Influent flow rate was controlled at 40.8 L/d. The mixed liquor suspended solid (MLSS) was maintained at 3000 mg/L in aerobic tank, and the volumetric loadings (measured in chemical oxygen demand (COD)) were 1.24 kg/(m^3·d). Dissolved oxygen (DO) concentration in aerobic tanks was kept at 2~3 mg/L. Flow rates of influent and returning sludge were controlled by peristaltic pumps.

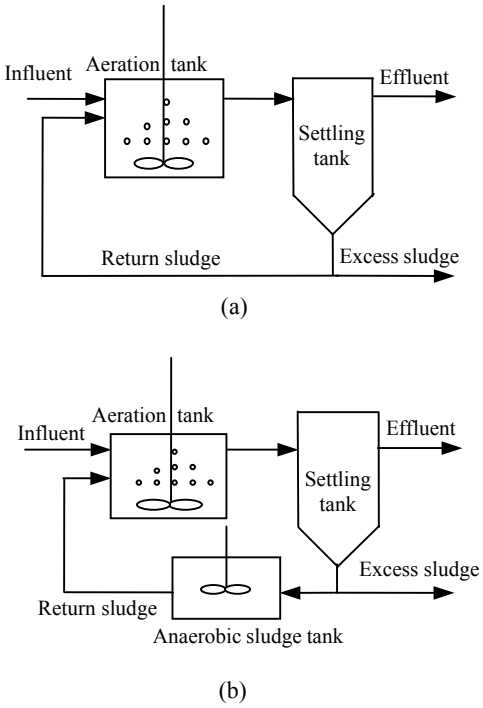

Fig.1 Schematic diagrams of the CAS (a) and OSA (b) experimental setups

2. Batch experiments

The main purposes in the batch experiments are to evaluate the biological phosphorus removal performance of sludge from the OSA system supplied with enough carbon substrate. 300 ml seed sludge taken from the anaerobic reactor in the OSA process was inoculated to a cylindrical vessel with a 1 L working volume. The sludge was washed with sterile deionized water twice and MLSS was maintained at approximate 2650 mg/L by adding synthetic wastewater. The synthetic wastewater for batch experiments consisted of sodium acetate (245 mg/L as COD basis), NH_4Cl (52 mg/L as NH_4^+-N basis), KH_2PO_4 (10 mg/L as PO_4^{3-}-P basis) and mineral solution. The sequencing batch reactor (SBR) was operated in a fill-and-draw mode as follows: anaerobic stirring 3 h, then aerating 3 h, and settling 0.5 h.

Synthetic wastewater

Synthetic wastewater contained starch (157.25 mg/L), tryptone (81.80 mg/L), beef extract (37.75 mg/L), glucose (113.25 mg/L), $Co(NH_2)_2$ (58 mg/L), $(NH_4)_2SO_4$ (112 mg/L), KH_2PO_4 (44 mg/L), $CaCl_2{\cdot}2H_2O$ (15 mg/L), $MgSO_4{\cdot}7H_2O$ (66 mg/L), $NaHCO_3$ (44 mg/L) and nutrient solution (1.0 ml/L). The nutrient solution consisted of the following compounds per liter: 0.05 g H_3BO_3, 0.05 g $ZnCl_2$, 0.03 g $CuSO_4{\cdot}5H_2O$, 0.05 g $MnSO_4{\cdot}H_2O$, 0.05 g $(NH_4)_6Mo_7O_{24}$, 0.05 g $AlCl_3$ and 0.05 g $CoCl_2{\cdot}6H_2O$.

Analytical methods

COD was measured using HACH COD kits and meter (DR890, HACH, USA). NH_4^+-N, total nitrogen (TN), total Phosphorus (TP) and MLSS were analyzed according to the Standard Methods (APHA, 1995). Phosphorus (P) contents of sludge were measured (Liu *et al.*, 2005a). Sludge samples taken from the reactors were digested first by potassium persulfate, and the supernatant recovered by high-speed centrifugation was used to determine the P content in sludge by the ascorbic acid method. In order to differentiate chemical precipitation related P accumulation from biological P accumulation, sludge samples taken from the reactors were put into contact with 0.5 mol/L HCl for 5 min, under gently mixing condition. At the end of 5 min, phosphate released into the supernatant was determined. Thus, the biological P accumulation can be calculated as the difference between the TP content in sludge and that released into solution after the contact with acid. The measurements were conducted periodically during the stable operation of both systems.

Staining and fluorescent in situ hybridization

Staining for lipophilic granules was carried out with the Sudan black stain in (Murray *et al.*, 1994). In this paper, the lipophilic granules are referred to as PHB (poly-*β*-hydroxybutyrate) granules. A black-blue granule in a clear or light pink background indicates the presence of this storage polymer (Serafim *et al.*, 2002).

DAPI (4′,6′-diamidino-2-phenyl indol dihydrochloride) staining was carried out in (Liu *et al.*, 2005b). DAPI stained intracellular poly-P particles and microbial cells. The stained poly-P particles emit yellow light under UV (ultraviolet) excitation while the stained cell emits dim blue light (Liu *et al.*, 2005b). The samples were observed with epifluorescence microscope using an excitation wavelength 330~385 nm.

Table 1 summarizes the oligonucleotide probes used in this study and various formamide concentrations. The probes were purchased as derivatives labeled at the 5′-end with Cy3 or N-(3-Fluoranthyl) maleimide (FAM) (Sangon, Shanghai, China). Sludge samples were fixed in 4% paraformaldehyde phosphate-buffered saline (PBS) solution for 2 h at 4 °C. The fixed sludge samples were washed twice in PBS solution, resuspended in PBS-ethanol solution (1:1, v/v) and then stored at –20 °C for further processing. FAM-labeled EUB338 was specific for most bacteria and Cy3-labeled PAOMIX probes comprising equal amounts of probes PAO 462, PAO 651 and PAO 846 were specific for *Rhodocyclus* associated PAO cluster (Crocetti *et al.*, 2000). Hybridization was performed according to the protocol described by Bond *et al.*(1999). After the hybridized slides were rinsed and dried, an anti-fading mounting solution was amounted. A fluorescent microscope (DM RA 2, LEICA, Germany) fitted with a digital camera was used for microscopic observation and image acquisition. These images were analyzed with LEICA CW 4000 image analysis software. Quantitative FISH analysis was enumerated by the software (Q500 IW, LEICA, Germany). For quantitative FISH analysis, at least 12 microscopic fields (magnification, 1000×) were analyzed. Within each field, cells hybridized to a given probe were expressed as a percentage of the total area of bacteria hybridized by the EUB probe (Kong *et al.*, 2005). Because this quantification method can underestimate colony formers, the sample was homogenized and just one layer of sludge in each well of the slide was applied when FISH was performed (Pijuan *et al.*, 2006).

Table 1 Information relevant to FISH oligonucleotides

Probe	Sequence (5′-3′)	16S rRNA target site	Specificity	Formamide (%)	Label
EUB338	GCTGCCTCCCGTAGGAGT	338-355	Most bacteria	20	FAM
PAO462	CCGTCATCTACWCAGGGTATTAAC	462-485	PAO cluster	35	Cy3
PAO651	CCCTCTGCCAAACTCCAG	651-668	PAO cluster	35	Cy3
PAO846	GTTAGCTACGGCACTAAAAGG	846-866	PAO cluster	35	Cy3

RESULTS AND DISCUSSION

Performance of nutrient removal in the OSA process

Fig.2 showed the removal efficiency of phosphorus when the OSA process and the CAS process were operated stably. The removal efficiency of TP in the OSA system exceeded that in the CAS process by near 50%. Phosphorus removal efficiency in the CAS process was merely up to 11%, which may be consumed by microbial growth since 2.3 g phosphorus is needed for per 100 g of cell biomass (Tchobanoglous *et al.*, 2003). However, about 61% of phosphorus was removed in the OSA process, which was far more than the need of microbial growth. The results showed that enhanced biological phosphorus removal was occurred remarkably in the OSA process. Comparing with the anaerobic zones of conventional biological phosphorus removal process, there were few external substrates in the anaerobic reactor of the OSA system. The release of soluble COD and TP was observed in the anaerobic tank of the OSA system (Chen *et al.*, 2003; Wang *et al.*, 2007). Soluble COD was released from sludge decay in the anaerobic tank, which could be used as carbon sources for anaerobic phosphorus release (Tchobanoglous *et al.*, 2003).

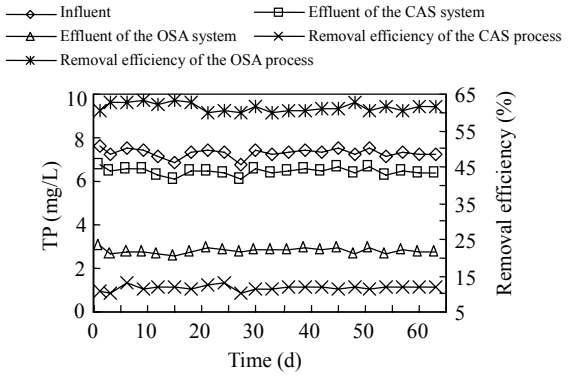

Fig.2 Change of phosphorus concentrations and removal efficiencies in the CAS and OSA processes

Precipitate-bound P and biological P accumulation in the aerobic sludge from the both processes were shown in Table 2. TP contents of aerobic sludge in the OSA process were twice more than those in the CAS process while the precipitate-bound P contents in both sludge were nearly similar. The results indicated that biological phosphorus accumulation led to higher phosphorus removal efficiency in the OSA system. There existed some bacteria, which had capability to take up excess phosphate in the aerobic OSA sludge. Therefore, performance of biological phosphorus removal in the OSA system should be further studied.

Table 2 TP, precipitate-bounded P and biological P accumulation in aerobic sludge from the CAS and OSA processes

Process	TP (mg/g)	Precipitate-bounded P (mg/g)	Biological P accumulation (mg/g)
CAS	14.9	3.8	11.1
OSA	30.5	3.6	26.9

Note: the ratio of P/COD of influent is 1.72/100

Phosphorus uptake and release capability of PAOs in the OSA system

The rates of phosphorus uptake and phosphorus release of the OSA sludge were investigated by batch experiments when enough carbon substrates were supplied. Fig.3 showed the change of TP and COD fed with CH_3COONa as the sole carbon substrate in the batch experiments. In the anaerobic phase, phosphorus was released rapidly while COD was utilized by PAOs. The phosphorus release rate (PRR) of sludge (MLSS) was 6.3 mg/(g·h) in the anaerobic treatment. Then a large amount of poly-P is synthesized by PAOs in the aerobic zone. The aerobic phosphate uptake rate (PUR) was 10.5 mg/(g·h). However, the rate of phosphorus uptake in our experiment was lower than that of Lee *et al*.(2001), who studied performance of enhanced biological phosphorus removal (EBPR) process in a sequencing batch reactor and reported the rates of aerobic phosphorus uptake were 14.9 mg/(g·h) to 26.8 mg/(g·h) in different operating conditions. The activity of PAOs was usually reflected by PUR (Tsuneda *et al.*, 2006). Thus, the amount of PAOs in the OSA system was lower than that in EBPR system, which was also reflected by the phosphorus removal performance. Phosphorus removal efficiency was about 60% in the OSA system while that was up to nearly 100% in EBPR system (Lee *et al.*, 2001; Tsuneda *et al.*, 2006).

As shown in Table 3, efficiency of biological phosphorus removal was enhanced by 14% when the sufficient external substrate was supplied. Meanwhile, average TP content in aerobic sludge was increased

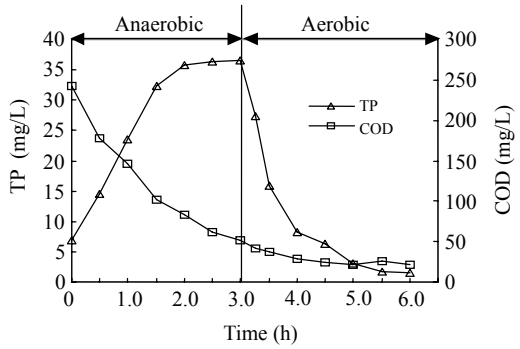

Fig.3 Change of the concentrations of TP and COD in the batch experiments (fed with CH_3COONa as the sole carbon source)

Table 3 TP removal efficiency and TP content of aerobic sludge in OAS system and the batch experiments

No.	TP removal efficiency (%)		TP content of aerobic sludge (mg/g)	
	OSA	Batch	OSA	Batch
1	59.45	76.76	30.45	31.22
2	63.64	72.94	30.99	31.03
3	62.10	75.54	30.86	31.10
Mean	61.73	75.08	30.75	31.12

by 0.36 mg/g in batch experiments. The results showed readily that biological organic substrate had been a restrictive factor for biological phosphorus removal in the continuous OSA system. Some of organic substrates could not be utilized for microorganism although soluble COD were released from hydrolysis and acid fermentation in anaerobic tank of the OSA system.

Characteristics of PAO in the OSA process

DAPI and Sudan black staining were employed to confirm the storage and release of poly-P granules and PHB granules, respectively. PHB-staining microscopic results in Figs.4a and 4b showed that percentages of black-blue granules in sludge obtained from various operation periods (85 d and 135 d) were relative stable. Bacteria with positive reactions were large coccoid cells with a diameter of 2 μm to 4 μm, accounting for about 30% to 35% of bacteria in anaerobic sludge of the OSA system.

DAPI staining revealed that poly-Ps were accumulated by PAOs in the OSA process. Approximately, 35% of bacteria were capable of accumulating poly-P (Figs.4c and 4d). The finding was consistent with the relative percentage of bacteria with positive PHB staining. Yellow spots of sludge slightly increased from 85 d to 135 d. The staining results demonstrated that there were 35% of bacteria with characteristics of PAOs in the OSA process. Contrasting to aerobic sludge of the OSA system, there were scarcely any yellow spots in that of the CAS system.

In EBPR process, PAOs store volatile fatty acids (VFA) as PHB and release phosphate under anaerobic conditions, followed by accumulation of excess amounts of phosphate in the form of poly-P under aerobic conditions. Therefore, the amount of cellular poly-P granule and PHB granule represented the number of PAOs in a certain extent. But the amount of poly-P accumulating bacteria in our experiments was less than that of Chudoba *et al.*(1992)'s conclusion that the OSA biomass contained about 60% of poly-P accumulating bacteria by Neisser staining, contrary to 10% in the activated sludge from the CAS system. DO concentration of aerobic tank was maintained at 8 mg/L in Chudoba *et al.*(1992)'s OSA system while DO of aerobic tank was controlled at 2~3 mg/L in our OSA system. High oxygen concentration might stimulate poly-P accumulation in the OSA system. In higher oxygen concentration, sufficient oxidization in aerobic tank may accumulate more energy in the cells. More energy was thus stored by means of the intracellular adenosine triphosphatase (ATP) pool and the surplus was transferred in poly-Ps accumulated by poly-P bacteria (Chudoba *et al*., 1992).

In situ detection of PAOs using FISH analysis

An initial characterization of the sludge in the OSA system was performed using FISH to assess the amount of PAOs. The micrographs in Fig.5 visually showed the percentage of PAOMIX-binding cells and active bacteria. The numbers of PAO probe-positive cells were counted and expressed as a proportion of EUB338-binding cells. The ratio of area of EUB3338 to area of DAPI staining represents the percentage of active bacteria. Active bacteria in sludge were around 80% to 90% of total microorganisms via image analysis while the ratio of active bacteria in anaerobic sludge was slightly lowed by about 4% (Fig.5). The results accorded to Chen *et al.*(2003)'s conclusion that the amount of active microorganisms was scarcely affected by sludge decay in anaerobic reactor.

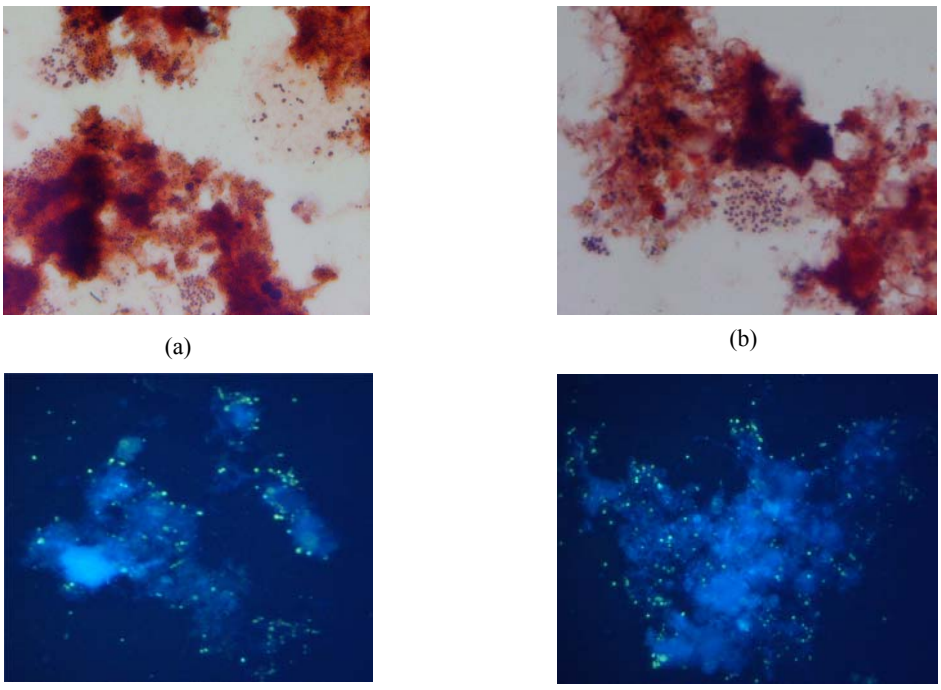

Fig.4 Images of Sudan black and DAPI staining for sludge in the OSA process. (a) Anaerobic sludge, 85 d; (b) Anaerobic sludge, 135 d; (c) Aerobic sludge, 85 d; (d) Aerobic sludge, 135 d

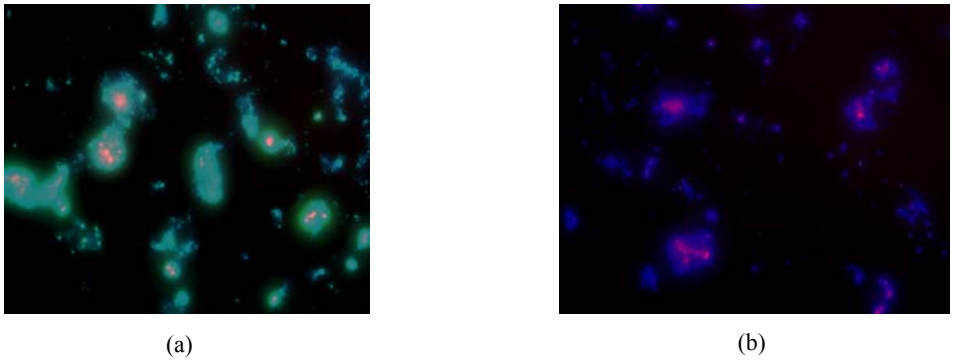

Fig.5 FISH images of activated sludge from the OSA process. (a) Aerobic sludge, 135 d; (b) Anaerobic sludge, 135 d
Blue represents DAPI staining; light green represents domain bacteria; pink represents PAO

PAOs were quantified as around 28% of total active microorganisms represented by the ratio of PAO/EUB338, and around 31% of total biomass represented by the ratio of PAO/DAPI. The findings showed that PAOMIX-binding bacteria were one of important communities in the OSA sludge.

The PAOs were identified by staining and FISH assay in the OSA system. The amount of PAOs identified by FISH was slightly lower than that identified by special staining (poly-P staining and PHB staining).

CONCLUSION

Based on the investigations of biological phosphorus removal in the OSA system and characterization of microbial community analyzed by staining and FISH technology, the main conclusions could be drawn as follows.

(1) Phosphorus removal efficiency in the OSA system was up to near 60%, exceeding that in the CAS process by about 50%. Biological phosphate accumulations of aerobic sludge in the OSA process

were 26.9 mg/g, which was twice more than that in the CAS sludge.

(2) The batch experiments results showed that the aerobic phosphate uptake rate of OSA sludge was 10.5 mg/(g·h) when sufficient carbon sources were supplied. Contrasted to the continuous OSA system, biological phosphorus removal efficiency was enhanced by 14% and average TP contents of aerobic sludge were increased by 0.36 mg/g in batch experiments. Biological phosphorus removal efficiency was partially inhibited by carbon sources in the OSA system.

(3) Approximately 35% of total microorganisms were capable of accumulating poly-P and synthesizing cellular PHB granules. FISH analysis demonstrated that PAOMIX-binding bacteria were predominant microbial communities in the OSA system, which accounted for around 28% of total bacteria.

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