Desalination and Water Treatment
www.deswater.com

doi: 10.5004/dwt.2021.26990

218 (2021) 260–269
April

Characterization of extracellular phosphorus in enhanced biological phosphorus removal granular sludge

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Received 20 July 2020; Accepted 19 December 2020

ABSTRACT

The effects of carbon sources and calcium on the phosphorus accumulation performance of extracellular polymers (EPS) were investigated. Results showed that polyphosphate (poly-P) with low and high molecular weight were distinguished in EPS. Total extracellular phosphorus accounted for 68.21%~71.05% and 26.97%~36.99% of the total phosphorus content of raw sludge in the acetic acid system and propionic acid system, respectively, suggesting that the ability of phosphorus absorption is poor in the EPS of the propionic acid system. It was found that when the influent Ca^{2+} concentration was elevated, the proportion of extracellular calcium phosphate (Ca-phosphate) precipitation in the two sludge systems increased remarkably. The variation tendency of $P_{release}/VFA_{uptake}$ and $glycogen_{degraded}/VFA_{uptake}$ ratios with the influent Ca^{2+} concentration increasing suggested that polyphosphate-accumulating organisms (PAO) were able to shift their metabolic pathways from polyphosphate-accumulating metabolism to glycogen-accumulating metabolism at high influent Ca^{2+}. The accumulation of calcium phosphate in EPS is a process that can affect the PAO abundance and metabolic patterns.

Keywords: Biological phosphorus removal granular sludge; Extracellular phosphorus; Carbon sources; Calcium; Microbial metabolism

1. Introduction

As an efficient and low-cost technology, enhanced biological phosphorus removal (EBPR) is widely applied in numerous full-scale wastewater treatment plants (WWTPs) [1–5]. In recent years, studies have shown that extracellular polymers (EPS) have an important effect on biological phosphorus removal and participate in the enhancement of biological phosphorus removal [6–10]. However, the extent of the effect of EPS on biological phosphorus removal was still unclear. The contribution of EPS to phosphorus uptake and the forms of accumulated extracellular phosphorus vary substantially in different studies, and the underlying mechanism of phosphorus transformation and transportation in EPS remains poorly understood. [11–15]. Therefore, it is difficult to study the phosphorus removal technology and application from the perspective of EPS.

Acetate and propionate are the typical volatile fatty acids (VFAs) in sewage or municipal wastewater [16,17], which are both key carbon sources for the EBPR process. The contents and species of phosphorus in EPS as well as their roles in biological phosphorus removal might also be influenced by the type of organic substrates. Therefore the changes of intracellular and EPS and total phosphorus contents in different carbon sources systems would be investigated, as well as the effects of carbon sources on the morphology and distribution of phosphorus in granular sludge to further clarify the role of EPS in biological phosphorus removal.

The Ca^{2+} concentration in municipal wastewater is usually in the range of 30~100 mg/L [18,19]. As an important

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component of sludge, EPS can absorb a large number of phosphate and calcium ions, leading to the formation of Ca-phosphate precipitation [20,21], which has a negative effect on the process performance [22,23]. Calcium (Ca) plays a limiting role in EBPR. At high Ca^{2+} concentration, the amount of polyphosphates available as an energy source decreases, thereby decreasing the amount of phosphate released per unit of VFA taken up. By studying the phosphorus removal mechanism of the EBPR system under different Ca^{2+} concentrations, the possible role of EPS in the formation of phosphorus precipitation in granular sludge was discussed, since direct evidence is quite insufficient now.

This work discussed the main influential factors for the phosphorus accumulation properties of EPS and studied the new mechanism of phosphorus removal in the EBPR system from two perspectives of biological phosphorus removal and chemical precipitation phosphorus removal in EPS. Thus the contribution of both to phosphorus removal was clearly understood. On the basis of these results, the role of EPS was better understood in the phosphorus removal mechanism of EBPR systems, which makes possible an improved design and optimization of the EBPR process to better adapt to complex and changeable sewage water quality.

2. Materials and methods

2.1. Operation of the EBPR reactor

The granular sludge used was taken from two laboratory-scale sequencing batch reactors (SBR) with a working volume of 12 L operated stably at 10°C for more than 6 months. SBRs were supplied with synthetic wastewater containing VFAs (400 mg/L as COD basis; one used acetic acid as carbon source (R1) and the other used propionic acid as carbon source (R2)), NH_4Cl (20 mg/L as NH_4^+–N basis), KH_2PO_4 (25 mg/L as PO_4^{3-}–P basis), and trace element solution. The trace element solution consisted of the following compounds per liter: 0.1 mg $ZnCl_2$, 0.5 mg $MgSO_4$, 0.5 mg $FeCl_3{\cdot}6H_2O$, 0.1 mg $MnSO_4{\cdot}H_2O$, 0.1 mg KI, 0.5 mg $CaCl_2$, 0.1 mg H_2BO_4, and 0.1 mg $NiCl_2$. The average PO_4^{3-}–P concentration in effluent of R1 and R2 in the stable period was 0.62 and 0.54 mg/L, and the average PO_4^{3-}–P removal rate was 97.52% and 97.84%, respectively. Both R1 and R2 showed good phosphorus removal performance by anaerobic/aerobic (A/O) cycling during a stable period.

2.2. Experimental design

The content of different forms of phosphorus in sludge and bacterial cells was determined by an improved STS method [24,25]. The phosphorus in the samples can be divided into orthophosphate (PO_4^{3-}–P), low molecular weight polyphosphate (LMW poly-P), phospholipid (lipid-P), DNA phosphorus (DNA-P), high molecular weight polyphosphate (HMW poly-P), protein phosphorus, and residue phosphorus (protein + residue-P). The TP content in the mixed centrifugal extract was determined by the closed reflux digestion method [26]. The TP and PO_4^{3-}–P contents were determined by the Mo-Sb-Vc-method [27]. The content of DNA-P was calculated by multiplying the content of DNA by the coefficient 9.2% (phosphorus is the characteristic element of DNA and the content is relatively constant), and the content of LMW poly-P and HMW poly-P was calculated by the subtraction method.

In order to investigate the effect of Ca^{2+} concentration on phosphorus content and morphological distribution in granular sludge, the two SBRs were both conducted in three stages (total of 108 d). The influent Ca^{2+} concentration was gradually increased from 28 mg/L (days 1–36) to 45 mg/L (days 37–72) and 80 mg/L (days 73–108) to examine the phosphorus removal performance and sludge characteristics. During the whole experimental period, other inlet matrix concentrations were kept constant.

2.3. Microscopy

The morphology of sludge before and after EPS extraction [28] was examined with a high-resolution scanning electron microscopy (SEM) (model JEM 7800F, Japan). Sludge samples were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer saline (PBS). The samples were subsequently washed and dehydrated in a series of ethanol solutions (50%, 70%, 80%, 90%, and 100%). Dewatered samples were dried by the Critical Point method [29]. The dried sludge samples were further sputter coated with gold for SEM observation.

2.4. Fluorescent in situ hybridization

An estimation of the biomass fractions of the populations of interest (PAO type I, PAO type II, and GAO) was based on fluorescent *in situ* hybridization (FISH) analyses, following the procedure described by Winkler et al. [30]. The oligonucleotide probes used in FISH were: EUBMIX, comprising EUB338, EUB338-II, and EUB338-III [31,32] to target all bacteria; PAOMIX (PAO651, 462, and 846) to target Accumulibacter [33]; ACC-I-444 and ACC-II-444 to target Accumulibacter type I and II, respectively [34]; GAOMIX (GAOQ989 and GAOQ431) to target Competibacter [35,36] and *Defluviicoccus vanus* related GAOs were targeted through probes TFO_DF218, TFO_DF618, and TFO_DF862 for cluster I [37]; DF988 and DF1020 for cluster II [38]. Image J software (NIH, USA) was used for quantitative analysis of micrographs. The biomass volume of Accumulibacter and Competibacter in relation to other bacteria was calculated as the area covered by the specific probes divided by the area covered by EUBMIX. The standard error of the mean was calculated as the standard deviation divided by the square root of the number of images.

3. Results

3.1. Influence of carbon sources and Ca^{2+} concentration on the phosphorus concentration in the liquid phase

Fig. 1 shows the typical profiles of PO_4^{3-}–P in the anaerobic–aerobic process at the influent Ca^{2+} of 28, 45, and 80 mg/L in R1 and R2. It exhibited a typical EBPR pattern, with the obvious release in the anaerobic phase and uptake in the aerobic phase. Carbon sources affected the amount of phosphorus release and absorption, and the amount of which in the acetic acid system was higher than that in the

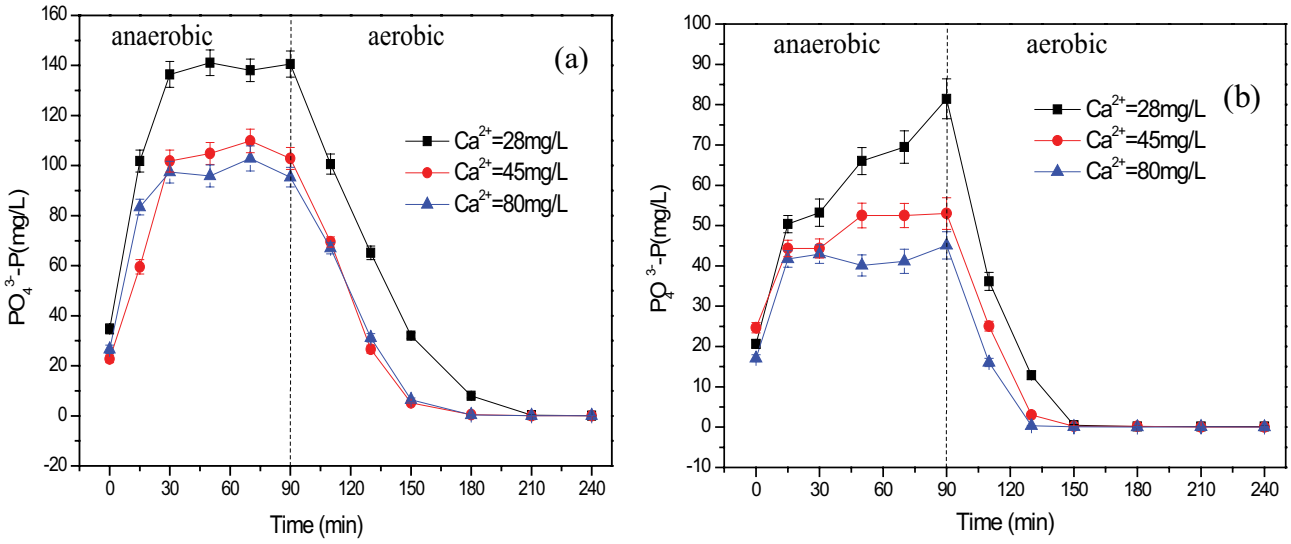

Fig. 1. Variation of phosphorus concentrations in the SBRs liquid during run cycle: (a) acetic acid system and (b) propionic acid system.

propionic acid system. In both systems, with the increase of Ca^{2+} concentration, the amount of phosphorus released and absorbed both showed a downward trend, but the final phosphorus removal rate all reached about 99%. In addition to biological phosphorus removal, phosphate precipitation also made a certain contribution to phosphorus removal with the increase of Ca^{2+} concentration, which is mentioned in section 3.3 (effects of Ca^{2+} concentration on phosphorus content and morphological distribution in EPS). Interestingly, whether the amount and rate of phosphate release in the anaerobic phase or the amount and rate of phosphate absorption in the aerobic phase, they were all higher for the acetate SBR than for the propionate SBR. However, the ratios of phosphate absorption and release were higher for the propionate SBR than for the acetate SBR. It achieved effluent quality in 210 min with acetic acid as a carbon source and achieved effluent quality in 150 min with propionic acid as a carbon source. In other words, R2 did it faster. It showed that a propionate carbon source was more favorable toward stable EBPR performance than an acetate carbon source. Furthermore, we can achieve treatment goals with a short cycle with propionic acid as a carbon source, the effects of higher influent flow on the cycle, and effluent quality in enhanced biological phosphorus removal system with propionic acid as carbon source will be investigated in later work.

3.2. Effects of carbon sources on phosphorus content and morphological distribution in EPS

Fig. 2 shows the granular sludge morphology in the two systems. In this study, the propionic acid system had a higher degree of granulation, and the average particle size was about 768 μm. By contrast, the average particle size of the acetic acid system was small, which was about 568 μm. The reason may be that the abundance of PAOs in propionic acid system was significantly higher than that in acetic acid system in this study, and more EPS would be produced to coagulate with other microorganisms thus forming larger particles.

Table 1 shows the polymer content in granular sludge during the anaerobic–aerobic process. Total polymers include intracellular polymers (glycogen, PHA, and polyphosphate) and EPS (proteins, polysaccharides, and nucleic acids). As seen from Table 1, the total extracellular polymer content in acetic acid system was about 255.5~278.9 mg/g, accounting for 48.76%~62.91% of the total polymer content. The total extracellular phosphorus content was 33.36~38.30 mg/g, accounting for 68.21%~71.05% of the total phosphorus content of raw sludge. The total extracellular polymer content in propionic acid system was about 254.1~332.80 mg/g, accounting for 59.53%~67.59% of the total polymer content. The total extracellular phosphorus content was 8.17~11.60 mg/g, accounting for 26.97%~36.99% of the total phosphorus content of raw sludge. The above results indicated that the polymers in the two systems are more distributed in the extracellular. Moreover, the content of extracellular polymer in propionic acid system was higher than that in acetic acid system, but the content of total extracellular phosphorus in propionic acid system was lower, while the content of total intracellular phosphorus was higher. It is speculated that there are two possible reasons. First, the propionic acid system contained more phosphorus accumulating bacteria (PAOs) than acetic acid system. FISH was used to quantify the bacteria abundance, the results showed that the abundance of Accumulibacter PAO were 34% ± 6% and 65% ± 8% in R1 and R2 (as seen in Table 2). The abundance of GAOs in two systems were relatively low, which was both about 1%~2%. The abundance of Accumulibacter PAO may be proportional to the content of intracellular phosphorus.

Second, polysaccharides play a major role in the adsorption of phosphorus by extracellular substances. When the production of EPS is relatively stable and the secretions are dominated by polysaccharides, the ability of phosphate adsorption is strong, which is reflected in the higher

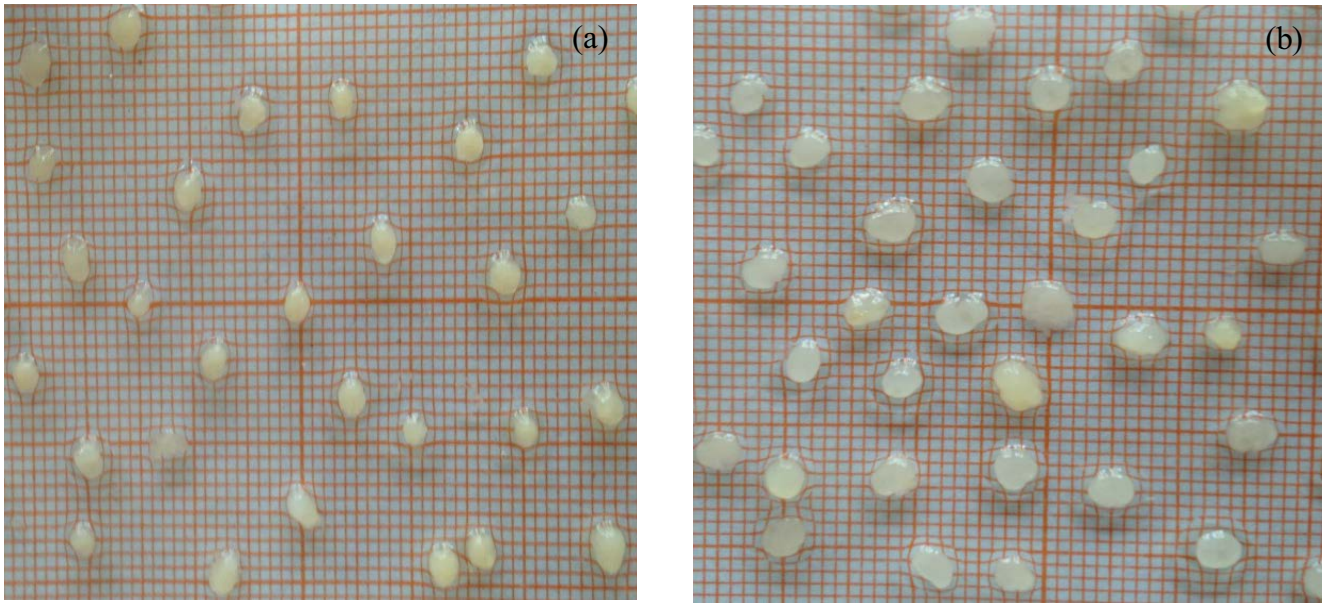

Fig. 2. Mature granular sludge: (a) acetic acid system and (b) propionic acid system.

Table 1
Variation of polymer and total phosphorus content in granular sludge during anaerobic-aerobic process

Sludge system		Intracellular polymer content (mg g^{-1})	TP_{cell} (mg g^{-1})	TP_{cell}/TP_{sludge} (%)	Extracellular polymer content (mg g^{-1})	TP_{EPS} (mg g^{-1})	TP_{EPS}/TP_{sludge} (%)
R1	A1	166.81 ± 4.73	13.59 ± 0.21	28.95 ± 0.44	278.90 ± 5.7	33.36 ± 0.48	71.05 ± 1.01
	A2	268.51 ± 5.28	15.78 ± 0.20	31.79 ± 0.59	255.50 ± 5.9	33.86 ± 0.40	68.21 ± 0.65
	O2	156.76 ± 4.50	15.84 ± 0.19	29.26 ± 0.41	265.94 ± 4.2	38.30 ± 0.50	70.74 ± 0.60
R2	A1	159.59 ± 3.40	22.53 ± 0.24	66.01 ± 0.87	332.80 ± 6.4	11.60 ± 0.19	33.99 ± 0.47
	A2	196.95 ± 5.94	22.14 ± 0.23	73.03 ± 0.72	289.70 ± 3.9	8.17 ± 0.05	26.97 ± 0.18
	O2	163.58 ± 3.30	23.26 ± 0.27	68.60 ± 0.61	254.10 ± 3.0	10.65 ± 0.10	31.40 ± 0.19

A1: anaerobic initial-stage of SBR systems; A2: anaerobic end-stage of SBR systems;
O2: aerobic end-stage of SBR systems under anaerobic-aerobic operation;
TP_{cell}: intracellular total phosphorus content; TP_{EPS}: extracellular total phosphorus content;
TP_{sludge}: total phosphorus content of raw sludge.

phosphorus removal efficiency of the system [39]. In this work, a low content of polysaccharides in the extracellular polymer secreted by microorganisms was found in the propionic acid system, which were only 64.0~71.2 mg/g (108.2~120.7 mg/g in acetic acid system), therefore a limited amount of phosphate can be adsorbed. This explains why the content of extracellular polymer in propionic acid system is high but the ability of phosphorus absorption is poor.

As shown in Fig. 3, the EBPR granular sludge before and after EPS extraction were studied by using SEM and energy dispersive spectrometry (EDS). Figs. 3a and b are the SEM images of EBPR granule before EPS extraction in acetic acid and propionic acid system. The organizational structure of bacterial cells exhibited sphericity or spheroidicity shape on a microscopic level. More EPS produced to cover the cell surface forming a kind of higher density outer layer in propionic acid system compared with acetic acid system. Figs. 3c and d are the SEM images of EBPR granule after EPS extraction in acetic acid and propionic acid system, the structural integrity was both significantly disrupted into smaller pieces without appreciable destruction of bacterial cells. The P scanning with EDS after EPS extraction in R1 and R2 (Figs. 3e and f) shows that the content of total intracellular phosphorus in propionic acid system was higher than acetic acid system. The content of total extracellular phosphorus in propionic acid system was lower. Phosphorus atomic percentage before and after EPS extraction were 3.79% and 1.45% in R1, and 3.93% and 2.91% in R2. The extracellular phosphorus content accounted for 61.74% and 25.95% of the total sludge phosphorus content in acetic acid system and propionic acid system respectively by calculation. The result was largely consistent with the data in Table 1.

As can be seen from Tables 3 and 4 (Ca^{2+} = 28 mg/L), phosphorus forms of EPS in the two systems were mainly PO_4^{3-}–P, LMW poly-P, and HMW poly-P, the three kinds of

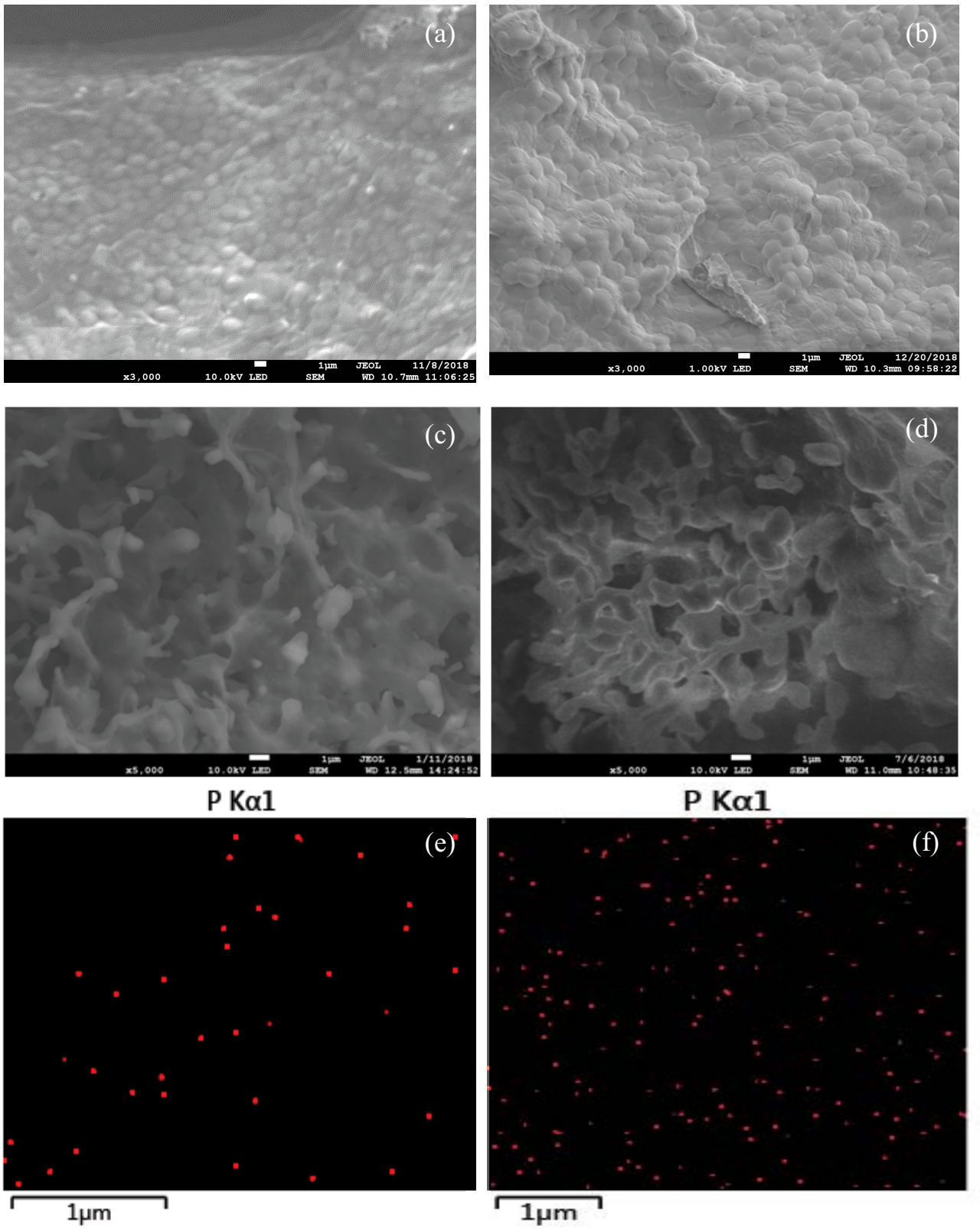

Fig. 3. SEM image of EBPR granule and P scanning with EDS in R1 and R2. SEM image of EBPR granule before EPS extraction in (a) acetic acid system, (b) propionic acid system, (c) acetic acid system, and (d) propionic acid system. P scanning with EDS after EPS extraction in (e) acetic acid system and (f) propionic acid system.

Table 2
Comparison of metabolic index and abundance of dominant bacteria in different systems

Sludge system	Ca^{2+} (mg/L)	$P_{release}$/VFA_{uptake}	$Glycogen_{degraded}$/VFA_{uptake}	PAOs (%)		GAOs (%)		$P_{precipitated}/TP_{EPS}$
				Acc I	Acc II	Competibacter	Defluviicocus	
R1	Ca^{2+} = 28 mg/L	0.93	0.45	11 ± 6	23 ± 13	1 ± 0	1 ± 0	13.33% ± 1.95%
	Ca^{2+} = 45 mg/L	0.61	0.65	10 ± 5	16 ± 5	5 ± 3	3 ± 1	41.87% ± 4.52%
	Ca^{2+} = 80 mg/L	0.52	1.35	13 ± 10	8 ± 5	6 ± 5	2 ± 1	52.91% ± 4.87%
R2	Ca^{2+} = 28 mg/L	0.72	1.88	43 ± 8	22 ± 13	1 ± 0	1 ± 0	10.52% ± 6.27%
	Ca^{2+} = 45 mg/L	0.70	4.12	37 ± 11	15 ± 4	1 ± 0	2 ± 1	27.15% ± 5.86%
	Ca^{2+} = 80 mg/L	0.28	6.28	6 ± 5	12 ± 3	1 ± 0	5 ± 3	43.55% ± 8.24%

Table 3
Variation of phosphorus morphological contents in extracellular polymers in R1

R1		PO_4^{3-}–P	LMW-poly-P	HMW-poly-P	DNA-P	Lipid-P	Protein + residue-P
Ca^{2+} = 28 mg/L	A1	3.10 ± 0.01	19.85 ± 0.12	4.28 ± 0.02	0.74 ± 0.03	0.25 ± 0.22	0.02 ± 0.01
	A2	4.52 ± 0.02	18.78 ± 0.16	6.30 ± 0.03	1.17 ± 0.04	0.42 ± 0.11	0.11 ± 0.04
	O2	3.69 ± 0.01	22.66 ± 0.20	1.12 ± 0.01	1.17 ± 0.04	0.49 ± 0.01	0.05 ± 0.01
Ca^{2+} = 45 mg/L	A1	13.17 ± 0.02	20.11 ± 0.42	0.92 ± 0.01	0.71 ± 0.01	0.22 ± 1.17	0.11 ± 0.06
	A2	15.88 ± 0.20	18.91 ± 0.20	0.67 ± 0.01	1.19 ± 0.01	0.23 ± 0.57	0.25 ± 0.02
	O2	14.46 ± 0.25	16.54 ± 0.17	0.71 ± 0.01	0.88 ± 0.02	0.15 ± 0.11	0.42 ± 0.14
Ca^{2+} = 80 mg/L	A1	17.87 ± 0.40	17.90 ± 0.16	1.16 ± 0.01	0.74 ± 0.02	0.27 ± 0.12	0.39 ± 0.04
	A2	19.27 ± 0.11	11.99 ± 0.11	1.17 ± 0.01	0.60 ± 0.01	0.36 ± 0.05	0.28 ± 0.02
	O2	21.86 ± 0.16	14.41 ± 0.13	0.84 ± 0.01	0.99 ± 0.01	0.53 ± 0.64	0.12 ± 0.08

Table 4
Variation of phosphorus morphological content in extracellular polymers in R2

R2		PO_4^{3-}–P	LMW-poly-P	HMW-poly-P	DNA-P	Lipid-P	Protein + residue-P
Ca^{2+} = 28 mg/L	A1	0.72 ± 0.01	10.82 ± 0.21	5.40 ± 0.03	0.86 ± 0.04	0.31 ± 0.07	0.09 ± 0.03
	A2	2.20 ± 0.03	5.94 ± 0.10	4.96 ± 0.18	0.69 ± 0.04	0.37 ± 0.08	0.08 ± 0.02
	O2	1.42 ± 0.01	9.19 ± 0.22	7.51 ± 0.21	0.80 ± 0.06	0.72 ± 0.15	0.10 ± 0.03
Ca^{2+} = 45 mg/L	A1	2.86 ± 0.10	7.46 ± 0.12	1.78 ± 0.03	1.16 ± 0.01	0.60 ± 0.09	0.73 ± 0.11
	A2	4.11 ± 0.06	6.22 ± 0.10	1.17 ± 0.02	0.88 ± 0.01	0.33 ± 0.05	0.50 ± 0.06
	O2	3.99 ± 0.03	7.76 ± 0.06	2.14 ± 0.02	0.86 ± 0.02	0.23 ± 0.01	0.14 ± 0.03
Ca^{2+} = 80 mg/L	A1	2.73 ± 0.08	6.07 ± 0.03	0.30 ± 0.01	0.76 ± 0.01	0.22 ± 0.04	0.14 ± 0.02
	A2	5.24 ± 0.12	4.07 ± 0.02	0.43 ± 0.01	0.65 ± 0.01	0.42 ± 0.08	0.20 ± 0.02
	O2	4.66 ± 0.21	8.16 ± 0.10	0.25 ± 0.01	0.60 ± 0.01	0.25 ± 0.02	0.11 ± 0.01

phosphorus in the acetic acid system were 11.38%~15.27%, 63.45%~82.49%, and 4.08%~21.28% of the total phosphorus content in EPS, respectively. While the sum of DNA-P, lipid-P, and protein + residue-P were only 0.93%~4.80%. The same three forms of phosphorus in the propionic acid system were 4.25%~16.78%, 45.31%~63.87%, and 31.88%~41.45% of total phosphorus content in EPS, respectively. The sum of DNA-P, lipid-P, and protein + residue-P were only 1.50%~7.80%.

By calculation, three forms of phosphorus (PO_4^{3-}–P, LMW poly-P, and HMW poly-P) in the cells of the acetic acid system were 57.58%~79.86%, 8.22%~28.30%, and 10.68%~28.30% of intracellular total phosphorus content respectively. Three forms of phosphorus in the cells of the propionic acid system were 82.78%~88.37%, 8.54%~12.88%, and 1.47%~12.88% of intracellular total phosphorus content, respectively. The above results showed that the phosphorus in the cells was dominated by orthophosphate and indicated that the microbial cell in EBPR sludge directly release or uptake ortho-P. Poly-P content in the microbial cells of the two reactors was very low, implying poly-P should be stored in EPS rather than microbial cell of EBPR sludge, and the poly-P of EPS should not come from the lysis of the microbial cell. Thereby, the poly-P of EPS might be synthesized and decomposed by the extracellular enzyme. This matches the conclusion of Long et al. [9].

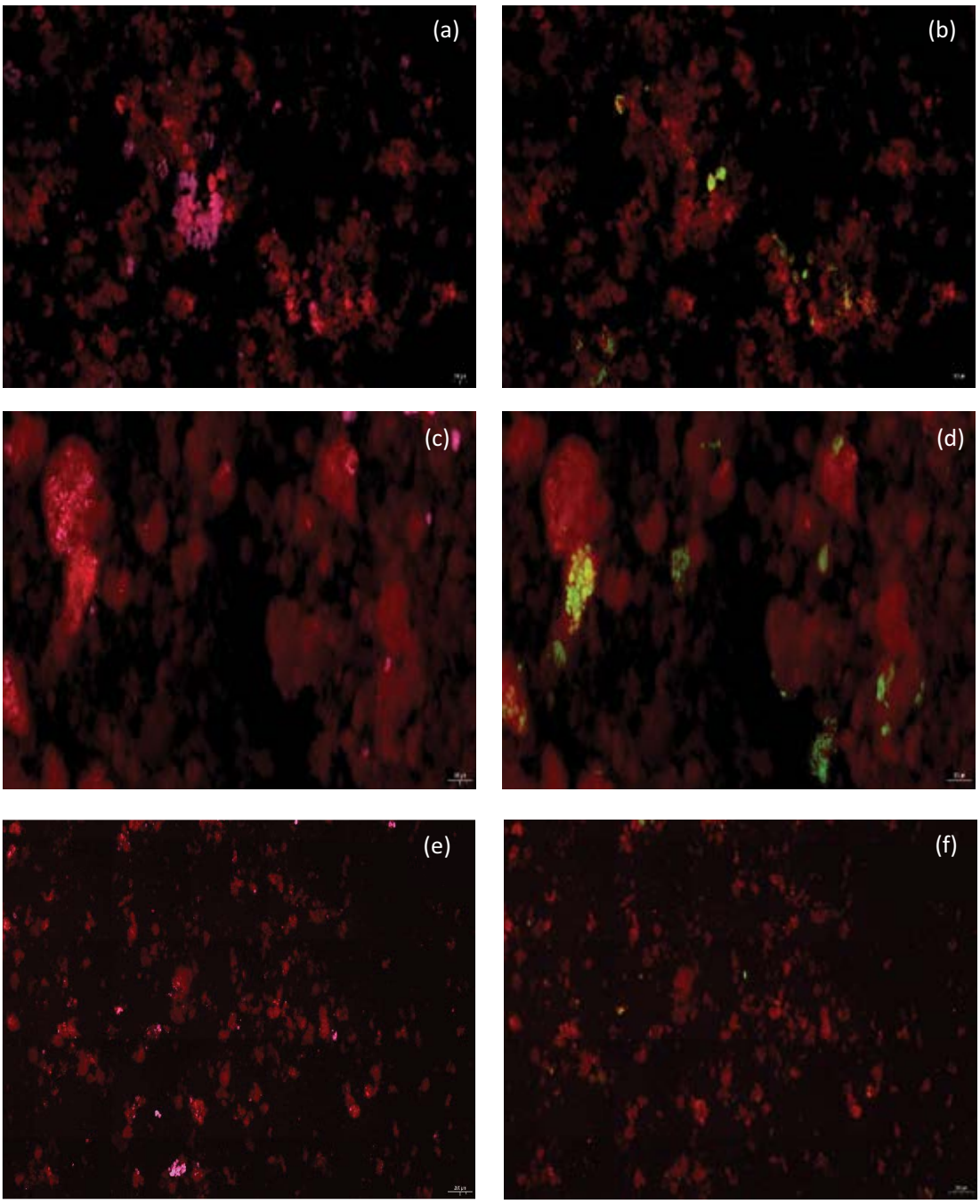

Fig. 4. FISH micrographs during the cycle at influent calcium concentrations of (a and b) 28 mg/L, (c and d) 45 mg/L, and (e and f) 80 mg/L in R1. Acc I appeared as pink fluorescence, Acc II appear as green fluorescence and all bacteria appear as red fluorescence. Bar = 200 μm.

3.3. Effects of Ca^{2+} concentration on phosphorus content and morphological distribution in EPS

In this work, the relationship between phosphorus content, morphological distribution in EPS and Ca^{2+} concentration was investigated. The cold perchloric acid (PCA) extraction steps in the improved STS method was used to determine the phosphorus precipitated (orthophosphate in the PCA extracts represents the chemical precipitated phosphorus) inside the EPS and the poly-P which participated in anaerobic degradation and aerobic synthesis (calculated from the difference between TP and orthophosphate) in this study.

The results showed that PO_4^{3-}–P in the EPS increased dramatically with increasing influent Ca^{2+} concentration in two reactors (as seen in Tables 3 and 4). The proportion of Ca-phosphate precipitation in the EPS of sludge was 11.29%~15.19%, 37.35%~46.38%, and 48.04%~57.78% in the

acetic acid system, and was 4.24%~17.17%, 21.29%~33.33%, and 35.31%~51.78% in the propionic acid system. This revealed that more phosphorus could be removed through precipitation at higher influent Ca^{2+} concentrations. The decline of LMW poly-P at higher Ca^{2+} concentration might be due to extracellular calcium phosphate precipitation, poly-P of EPS was decomposed and remained in EPS in the form of PO_4^{3-}–P. As the phosphorus source required by microbial growth, the content of HMW poly-P decreased in the meanwhile.

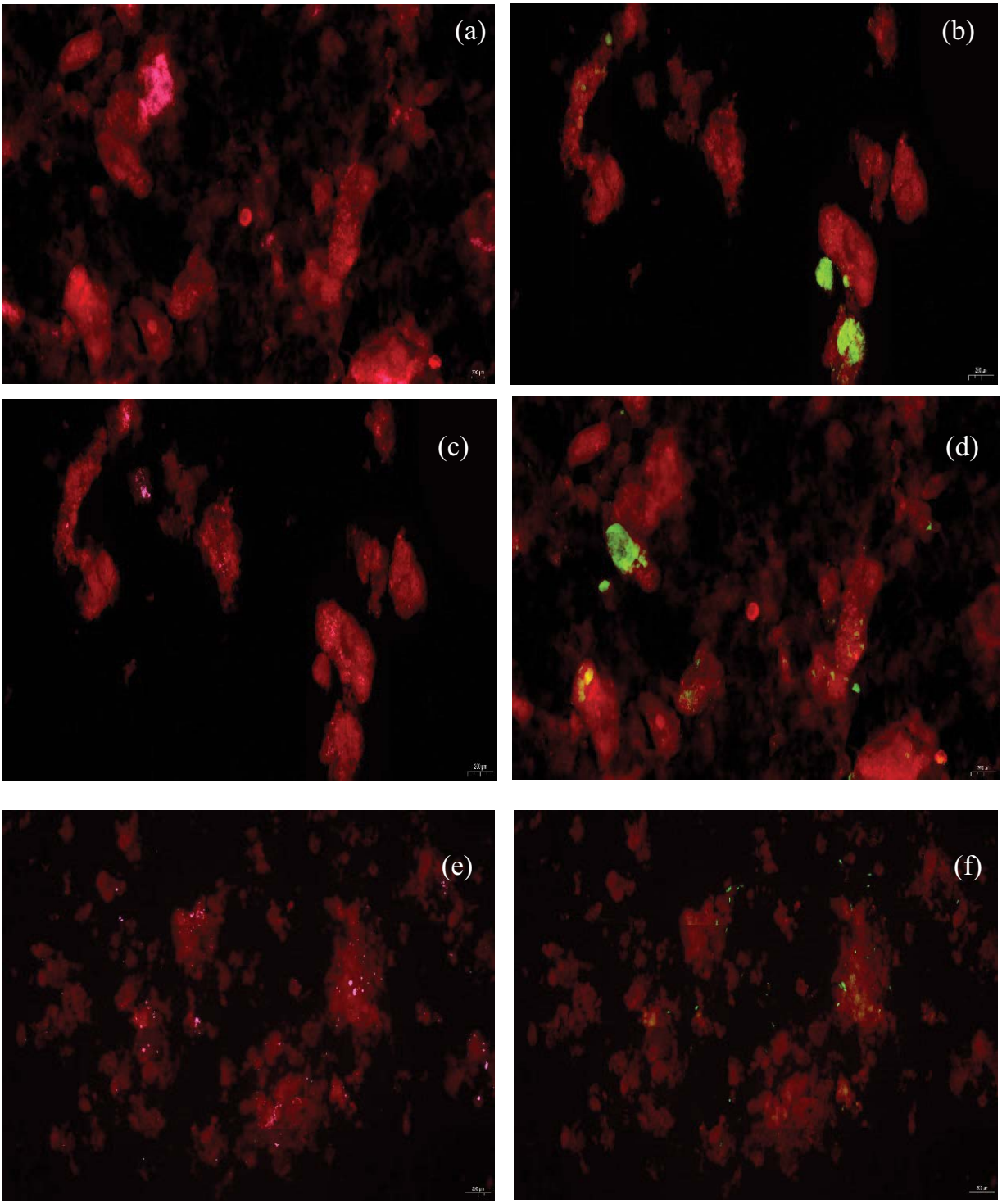

Fig. 5. FISH micrographs during the cycle at influent calcium concentrations of (a and b) 28 mg/L, (c and d) 45 mg/L, and (e and f) 80 mg/L in R2. Acc I appeared as pink fluorescence, Acc II appear as green fluorescence and all bacteria appear as red fluorescence. Bar = 200 μm.

Figs. 4 and 5 show the FISH images of sludge samples taken from the EBPR processes operated at different Ca^{2+} concentrations. The abundance of Accumulibacter PAO decreased from 34% ± 6% to 21% ± 6% in R1 and 65% ± 8% to 18% ± 3% in R2 (as seen in Table 2). We conclude from the results that the calcium accumulation in granules had a negative effect on their bioactivity. However, Ca-phosphate precipitation contributed to the removal of phosphorus in the EBPR process. Therefore, balanced consideration of both the biological and chemical factors will be required for operation of practical EBPR processes.

3.4. Effects of carbon sources and Ca^{2+} concentration on microbial metabolism in EBPR system

$P_{release}/VFA_{uptake}$ is an important metabolic index in the process of biological phosphorus removal. In general, $P_{release}/VFA_{uptake}$ in PAOs enrichment system has a large amplitude, ranging from 0.50 to 0.87 m mol P/m mol C, and $glycogen_{degradation}/VFA_{uptake}$ ranged from 0.31 to 0.61 m mol C/m mol C, which identified the system was carrying out phosphorus accumulation metabolism (PAM). In GAOs enrichment system, $P_{release}/VFA_{uptake}$ was usually between 0 and 0.02 m mol P/m mol C, and $glycogen_{degradation}/VFA_{uptake}$ was between 0.92 and 1.25 m mol C/m mol C, which identified the system was carrying out glycogen-accumulating metabolism (GAM) [19,40]. The $P_{release}/VFA_{uptake}$ in R1 and R2 systems in this study were 0.93 and 0.72 m mol P/m mol C, $glycogen_{degradation}/VFA_{uptake}$ were 0.45 and 1.88 m mol C/m mol C, respectively (as shown in Table 2). Thus, the metabolism mode of acetic acid system was PAM, while the metabolism mode of propionic acid system was PAM-GAM. Analysis showed that the propionic acid system has a significantly different utilization degree of glycogen than the acetic acid system, which may indicate that PAOs adopt different metabolic patterns for different carbon sources.

With the increase of Ca^{2+} concentration, the metabolic mode of acetic acid system changed from PAM to PAM-GAM, while the metabolic mode of propionic acid system changed from PAM-GAM to GAM. Both the system's PAOs had the ability to convert its metabolic mode to GAM, and the PAOs of the propionic acid system showed more significant GAM characteristics. There are two main reasons for the transformation of PAOs metabolism from PAM to GAM due to the increase of Ca^{2+} concentration, one is that Ca^{2+} enter the cell to synthesize Ca-polyP; the other is that Ca^{2+} form Ca-P precipitation outside the cell. It is almost impossible to quantify the amount of calcium entering the cell to synthesize Ca-polyP and forming Ca-P precipitation outside the cell. In further studies, the system can be simplified for research, such as controlling pH to inhibit the formation of phosphate precipitation and studying the influence of Ca^{2+} on the metabolic mechanism of PAOs. Investigating the effect of phosphate precipitation on PAOs metabolism may be achieved by fixing Ca^{2+} concentration.

4. Conclusion

Orthophosphate, LMW poly-P, and HMW poly-P were identified in granular sludge, among which LMW poly-P and HMW poly-P were distinguished in EPS. Orthophosphate was the dominant phosphorus in microbial cells. The content of total extracellular phosphorus in the propionic acid system was lower than that in the acetic acid system.

The dramatic increase of PO_4^{3-}–P and the decline of LMW poly-P and HMW poly-P in the EPS with the increase of Ca^{2+} concentration suggested that more phosphorus was removed through precipitation in the two systems compared to the condition with lower Ca^{2+} concentration. Intriguingly, both the system's PAOs had the ability to convert its metabolic mode to GAM, and the PAOs of the propionic acid system showed more significant GAM characteristics with the increase of Ca^{2+} concentration.

Acknowledgments

The study was financially supported by Tianjin Education Commission scientific research project (2016CJ09).

References

[1] A. Oehmen, P.C. Lemos, G. Carvalho, Z.G. Yuan, J. Keller, L.L. Blackall, M.A.M. Reis, Advances in enhanced biological phosphorus removal: from micro to macro scale, Water Res., 41 (2007) 2271–2300.
[2] X.L. Zheng, P.D. Sun, J.Y. Han, Y.Q. Song, Z.R. Hu, H.Q. Fan, S.Y. Lv, Inhibitory factors affecting the process of enhanced biological phosphorus removal (EBPR)-a mini-review, Process Biochem., 49 (2014) 2207–2213.
[3] A.B. Lanham, A. Oehmen, A.M. Saunders, G. Carvalho, P.H. Nielsen, M.A.M. Reis, Metabolic modelling of full-scale enhanced biological phosphorus removal sludge, Water Res., 66 (2014) 283–295.
[4] E.R. Coats, C.K. Brinkman, S. Lee, Characterizing and contrasting the microbial ecology of laboratory and full-scale EBPR systems cultured on synthetic and real wastewaters, Water Res., 108 (2017) 124–136.
[5] F. De Vleeschauwer, M. Caluwé, T. Dobbeleers, H. Stes, L. Dockx, F. Kiekens, J. D'aesa, C. Copotd, J. Dries, Performance and stability of a dynamically controlled EBPR anaerobic/aerobic granular sludge reactor, Bioresour. Technol., 280 (2019) 151–157.
[6] H.L. Zhang, W. Fang, Y.P. Wang, G.P. Sheng, R.J. Zeng, W.W. Li, H.Q. Yu, Phosphorus removal in an enhanced biological phosphorus removal process: roles of extracellular polymeric substances, Environ. Sci. Technol., 47 (2013) 11482–11489.
[7] R.D. Wang, Y.Z. Peng, Z.L. Cheng, N.Q. Ren, Understanding the role of extracellular polymeric substances in an enhanced biological phosphorus removal granular sludge system, Bioresour. Technol., 169 (2014) 307–312.
[8] W.W. Li, H.L. Zhang, G.P. Sheng, H.Q. Yu, Roles of extracellular polymeric substances in enhanced biological phosphorus removal process, Water Res., 86 (2015) 85–95.
[9] X.Y. Long, R. Tang, Z.D. Fang, C.X. Xie, Y.Q. Li, G. Xian, The roles of loosely-bound and tightly-bound extracellular polymer substances in enhanced biological phosphorus removal, Chemosphere, 189 (2017) 679–688.
[10] Y.H. Shi, J.H. Huang, G.M. Zeng, Y.L. Gu, Y.N. Chen, Y. Hu, B. Tang, J.X. Zhou, Y. Yang, L.X. Shi, Exploiting extracellular polymeric substances (EPS) controlling strategies for performance enhancement of biological wastewater treatments: an overview, Chemosphere, 180 (2017) 396–411.
[11] N. Li, N.Q. Ren, X.H. Wang, H. Kang, Effect of temperature on intracellular phosphorus absorption and extracellular phosphorus removal in EBPR process, Bioresour. Technol., 101 (2010) 6265–6268.
[12] H.L. Zhang, W. Fang, Y.P. Wang, G.P. Sheng, C.W. Xia, R.J. Zeng, H.Q. Yu, Species of phosphorus in the extracellular polymeric substances of EBPR sludge, Bioresour. Technol., 142 (2013) 714–718.

[13] A.G. Geyik, F. Çeçen, Variations in extracellular polymeric substances (EPS) during adaptation of activated sludges to new feeding conditions, Int. Biodeterior. Biodegrad., 105 (2015) 137–145.
[14] A.G. Geyik, B. Kılıç, F. Çeçen, Extracellular polymeric substances (EPS) and surface properties of activated sludges: effect of organic carbon sources, Environ. Sci. Pollut. Res. Int., 23 (2016) 1653–1663.
[15] S.S. Yang, J.W. Pang, W.Q. Guo, X.Y. Yang, Z.Y. Wu, N.Q. Ren, Z.Q. Zhao, Biological phosphorus removal in an extended ASM2 model: roles of extracellular polymeric substances and kinetic modeling, Bioresour. Technol., 232 (2017) 412–416.
[16] D.B. Wanga, X.M. Li, Q. Yang, W. Zheng, Y. Wua, T.J. Zeng, G.M. Zeng, Improved biological phosphorus removal performance driven by the aerobic/extended-idle regime with propionate as the sole carbon source, Water Res., 46 (2012) 3868–3878.
[17] A. Mielcarek, J. Rodziewicz, W. Janczukowicz, A. Thornton, The feasibility of citric acid as external carbon source for biological phosphorus removal in a sequencing batch biofilm reactor (SBBR), Biochem. Eng. J., 93 (2015) 102–107.
[18] H. Carlsson, H. Aspegren, N. Lee, A. Hilmer, Calcium phosphate precipitation in biological phosphorus removal systems, Water Res., 31 (1997) 1047–1055.
[19] H.L. Zhang, G.P. Sheng, W. Fang, Y.P. Wang, C.Y. Fang, L.M. Shao, H.Q. Yu, Calcium effect on the metabolic pathway of phosphorus accumulating organisms in enhanced biological phosphorus removal systems, Water Res., 84 (2015) 171–180.
[20] R. Barat, T. Montoya, L. Borrás, A. Seco, J. Ferrer, Calcium effect on enhanced biological phosphorus removal, Water Sci. Technol., 53 (2006) 29–37.
[21] A. Manas, B. Biscans, M. Spérandio, Biologically induced phosphorus precipitation in aerobic granular sludge process, Water Res., 45 (2011) 3776–3786.
[22] R. Barat, T. Montoya, L. Borrás, J. Ferrer, A. Seco, Interactions between calcium precipitation and the polyphosphate-accumulating bacteria metabolism, Water Res., 42 (2008) 3415–3424.
[23] H.L. Dai, Y.F. Wu, L.H. Peng, Z.Q. Dai, X. Li, X.W. Lu, Effects of calcium on the performance, bacterial population and microbial metabolism of a denitrifying phosphorus removal system, Bioresour. Technol., 243 (2017) 828–835.
[24] T. Mino, T. Kawakami, T. Matsuo, Behaviour of intracellular polyphosphate in the biological phosphate removal process, Water Sci. Technol., 17 (1985) 11–21.
[25] Z.D. Fang, G. Xian, X.Y. Long, R. Tang, H.B. Li, S. Lu, Effect of phosphate species in extracellular polymer substances on biological phosphorus removal process, Acta Sci. Circum., 34 (2014) 2205–2212.
[26] T.R. Long, J. Kang, X.Y. Long, S.S. Gao, Y.H. Zhang, R. Tang, Determination of fouling by ultrasonic-resin method/closed reflux digestion total phosphorus in mud, China Water Wastewater, 25 (2009) 81–85.
[27] S.E.P.A. Chinese, Water and Wastewater Monitoring Methods, 4th ed., Chinese Environmental Science Publishing House, China, 2002.
[28] Y.J. Li, S.P. Wang, Z. Li, L.P. Sun, Extraction method and structural and composition characteristics of extracellular polymeric substances in granular sludge from an enhanced biological phosphorus removal system, Desal. Water Treat., 185 (2020) 41–50.
[29] C. Laforsch, R. Tollrian, A new preparation technique of daphnids for scanning electronic microscopy using hexamethyldisilazane, Arch. Hydrobiol., 149 (2000) 587–596.
[30] M.K.H. Winkler, J.P. Bassin, R. Kleerebezem, L.M.M. De Bruin, T.P.H. Van den Brand, M.C.M. Van Loosdrecht, Selective sludge removal in a segregated aerobic granular biomass system as a strategy to control PAO-GAO competition at high temperature, Water Res., 45 (2011) 3291–3299.
[31] R.I. Amann, B.J. Binder, R.J. Olson, S.W. Chisholm, R. Devereux, D.A. Stahl, Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations, Appl. Environ. Microbiol., 56 (1990) 1919–1925.
[32] H. Daims, A. Bruhl, R. Amann, K.H. Schleifer, M. Wagner, The domain-specific probe EUB338 is insufficient for the detection of all bacteria: development and evaluation of a more comprehensive probe set, Syst. Appl. Microbiol., 22 (1999) 434–444.
[33] G.R. Crocetti, P. Hugenholtz, P.L. Bond, A. Schuler, J. Keller, D. Jenkins, L.L. Blackall, Identification of polyphosphate-accumulating organisms and design of 16S rRNA-directed probes for their detection and quantitation, Appl. Environ. Microbiol., 66 (2000) 1175–1182.
[34] J.J. Flowers, S. He, S. Yilmaz, D.R. Noguera, K.D. McMahon, Denitrification capabilities of two biological phosphorus removal sludges dominated by different "Candidatus Accumulibacter" clades, Environ. Microbiol. Rep., 1 (2009) 583–588.
[35] G.R. Crocetti, J.F. Banfield, J. Keller, P.L. Bond, L.L. Blackall, Glycogen-accumulating organisms in laboratory-scale and full-scale wastewater treatment processes, Microbiology, 148 (2002) 3353–3364.
[36] Y. Kong, S.L. Ong, W.J. Ng, W.T. Liu, Diversity and distribution of a deeply branched novel proteobacterial group found in anaerobic-aerobic activated sludge processes, Environ. Microbiol., 4 (2002) 753–757.
[37] M.T. Wong, F.M. Tan, W.J. Ng, W.T. Liu, Identification and occurrence of tetrad-forming Alphaproteobacteria in anaerobiceaerobic activated sludge processes, Microbiology, 150 (2004) 3741–3748.
[38] R.L. Meyer, A.M. Saunders, L.L. Blackall, Putative glycogen-accumulating organisms belonging to the Alphaproteobacteria identified through rRNA-based stable isotope probing, Microbiology, 152 (2006) 419–429.
[39] F. Ma, F.F. Yang, A. Li, Study on biological characteristics of a highly efficient denitrifying phosphorus accumulating bacteria, Environ. Sci., 32 (2011) 2710–2715.
[40] B. Acevedo, A. Oehmen, G. Carvalho, A. Seco, L. Borras, R. Barat, Metabolic shift of polyphosphate-accumulating organisms with different levels of polyphosphate storage, Water Res., 46 (2012) 1889–1900.