The Effect of Extracellular Polyphosphate in the Process of Enhanced Biological Phosphorus Removal (EBPR): from the Perspective of Transformations of Extracellular Polyphosphate and Intracellular Energy-storing Substances

Ran Tang1, Xiangyu Long2,*, Guangjian Tao2, Tao Wang2, Jiayue Wang2, Xiao Xiao2 and Haiwei Zhou2

1College of Chemistry and Chemical Engineering, Chongqing University of Science and Technology, University Town, Shapingba District, Chongqing 401331, China
2Department of Military installation, Army Logistics University of the People’s Liberation Army, University Town, Shapingba District, Chongqing 401311, China

*Corresponding author email: 2002longxy@sina.com

Abstract. The distributions and cycle changes of orthophosphate (ortho-P), polyphosphate (poly-P), glycogen and polyhydroxyalkanoates (PHAs) in the 2 reactors fed with acetate at 20°C or 35°C were investigated to understand the effect of extracellular polyphosphate (poly-P) in enhanced biological phosphorus removal (EBPR) process. The specific anaerobic-uptake acetate rate in the 20°C EBPR reactor was about 2.24 times that in the 35°C non-EBPR reactor. The anaerobic-hydrolysis and the aerobic-synthesis amounts of intracellular glycogen in the former were only half those in the latter, with the comparable corresponding-transformation amounts of intracellular PHAs between the 2 reactors. The anaerobic-hydrolysis / aerobic-synthesis of extracellular poly-P and the anaerobic-release / aerobic-uptake of intracellular ortho-P occurred in the 20°C EBPR sludge, corresponding to the lower transformation level of intracellular glycogen and the higher anaerobic-uptake rate of acetate. A hypothesis that the anaerobic-hydrolysis of extracellular poly-P could accelerate the acetate migration though anion-exchange was proposed. In addition, phosphorus accumulating organisms (PAOs) was the microbial population basis for the existence and transformation of extracellular poly-P.

Keywords: Enhanced biological phosphorus removal; Extracellular polyphosphate; Extracellular polymeric substances (EPS); Intracellular energy-storing substances; Phosphate accumulating organisms (PAOs).

1. Introduction
Enhanced biological phosphorus removal (EBPR) is applied in global to remove phosphorus (P) from wastewater [1, 2]. The success operation of EBPR system is mostly relied on the phosphorus accumulating organisms (PAOs), which excessively absorb P under the aerobic/anoxic condition and then store intracellular polyphosphate (poly-P) that is an important energy substance for PAOs to anaerobically absorb carbon source [3-6] and is beneficial for PAOs to gain advantage over glycogen accumulating organisms (GAOs) [7]. PAOs also can behave as GAOs under low poly-P condition [8, 9] or at high influent Ca concentration [10, 11], because intracellular glycogen is also used as an energy source for PAOs to absorb carbon source and synthesize polyhydroxyalkanoates (PHAs) [5, 6].

The bacterial cells in biological aggregate are enclosed by extracellular polymer substances (EPS), which contained 27% - 57% extracellular P of the sludge P content [12- 15], illustrating that the role of...
EPS in biological P removal could not be ignored. Furthermore, the \(^{31}\)P-NMR (nuclear magnetic resonance) spectra results illustrated that poly-P, pyrophosphate (pyro-P) and orthophosphate (ortho-P) were coexisted in the EPS of EBPR sludge \cite{16, 17}. Importantly, the anaerobic-decrease and aerobic-increase profile of the extracellular total P (TP) content was accompanied with the aerobic-hydrolysis and aerobic-synthesis of the extracellular poly-P \cite{17, 18}, indicating extracellular poly-P was involved in EBPR mechanism. EPS can be divided into outer loosely-bound EPS (LB-EPS) and inner tightly-bound EPS (TB-EPS) \cite{19-21}. Recently, the authors found that LB-EPS and TB-EPS played different roles in EBPR process \cite{19, 22}, in which the anaerobic-hydrolysis and aerobic-synthesis of the poly-P in TB-EPS occurred \cite{19}. In the EBPR process, intracellular glycogen and PHAs are important energy-storing substances of PAOs and GAOs, of which the transformation processes can reveal some microbial metabolism characteristics \cite{8 - 11}. However, the transformation relationships between extracellular poly-P and intracellular energy-storing substances are still unclear, thus it is essential to understand the effect of extracellular poly-P in EBPR process.

Temperature is an important factor on the EBPR performance, influencing the competition between PAOs and GAOs and the transformation of intracellular energy-storing substances \cite{1}. Temperatures higher than 25°C are generally unfavorable to EBPR process due to the domination of GAOs without accumulating poly-P \cite{2, 23}. The effect of extracellular poly-P in the EBPR process can be discovered, through comparing the structures of microbial population and the transformations of intracellular energy-storing substances in different temperature systems.

This aim of the study is to understand the effect of extracellular poly-P in EBPR process, taking 2 lab-scale anaerobic/oxic reactors respectively at 20°C and 35°C as the objects. The contents, distributions and cycle changes of the ortho-P, poly-P, glycogen and PHAs in the 2 reactors were firstly compared by fractionating sludge floc into LB-EPS, TB-EPS and bacterial cell. The effects of the microbial population structures on the transformations of the poly-P in TB-EPS and the intracellular energy-storing substances as well as the transformation relationships between extracellular poly-P and intracellular energy-storing substances were discussed, in order to understand the effect of extracellular poly-P in EBPR process.

2. Materials and Methods

2.1. Culture of Activated Sludge
The 2 A/O-sequencing batch reactors (SBR) at 20 ± 1 °C and 35 ± 1 °C were fed with sodium acetate, running for more than one year with 750 mg/L of the influent COD concentration and 100: 5: 5 of the COD: N: P ratio. The reactors were operated with 2 cycles per day with 15 L working volume, including instantaneous filling wastewater, 4-h anaerobic and 7-h aerobic conditions and 50-min settling, 5-min decanting and about 5-min idling stages. The approximately 20 d of solid retention time (SRT), the 7.4-8.8 of pH during running cycle and 2.5 - 4.5 mg/L of dissolved oxygen (DO) concentrations at the end of aeration phase were controlled. The concentrations of the influent phosphorus and nitrogen were detailed in the previous reports \cite{19, 22}. The volatile suspended solids (VSS), sludge volume index (SVI) and total phosphorus (TP) and orthophosphate (ortho-P) of influent and effluent were monitored every week. The tests were carried out after these parameter values kept relatively stable for more than 3 months.

2.2. Extraction of LB-EPS and TB-EPS
The LB-EPS and TB-EPS in sludge were extracted by ultrasonic (JY90-II; ScientzBioscience Co., Inc., Ningbo, China) at low frequency (21 kHz) and low power density (1 w/mL) and cation exchange resin (CER, 001×7 Type, 20-40 mesh, Suqing Co., Jiangsu, China), respectively, which were detailly described in the previous reports \cite{19, 22}. In addition, the LB-EPS and TB-EPS contents of sludge between the 2 reactors were respectively close (data not shown).

2.3. \(^{31}\)P NMR Analysis
These extractions were freeze-dried and then were kept at -20°C before \(^{31}\)P NMR analysis. The procedures of re-dissolution of the lyophilized LB-EPS and TB-EPS and lysis of the lyophilized
bacterial cell were described in the literature [19]. A 600 MHz NMR spectrometer (Agilent Technologies, Santa Clara, USA) was utilized to record the \(^3\)P NMR spectrum with the 0.56-\(\mu\)s 90° pulse width, 25 °C regulated temperature and 128 scan number, followed by the analysis with the NMR data processing software (MestReNova v6.1.0-6224) [16,19].

2.4. Measurement of Acetate, PHAs and Glycogen
The acetate concentration in bulk solution and the PHAs contents (including PHB and PHV) of activated sludge or bacterial cell were respectively determined by gas chromatograph (GC, 7890A, Agilent Technologies, Santa Clara, USA) equipped with a HP-FFAP [24] or DB-5 column [25]. The contents of glycogen or polysaccharide in activated sludge, LB-EPS and TB-EPS were determined with the anthranone sulfate method [25]. The glycogen content in bacterial cell (intracellular glycogen) was calculated by subtraction.

2.5. 16S rRNA Gene Amplicon Sequencing
The DNA of sludge sample collected at the end of aerobic phase was extracted with soil genomic DNA extraction kit (Tiangen Biochemical Technology Co., Ltd., Beijing, China) and then was stored at -20°C. The extract were amplified through PCR using primer set 338F (5/-ACTCTACGGGAGGCAGCAG-3/) and 806R (5/-GGACTACHVGGGTWTCTAAT-3/) for the V3-V4 region of 16S rRNA gene. The 20 \(\mu\)L mixture was employed to carry out PCR, which was detailed in the previous literature [22]. The PCR was performed in a Gene Amp_9700 (Applied Biosystems, U.S.) with the procedure reported in the previous literature [26], followed by sequencing on an Illumina MiSeq PE300 platform according to the standard protocols. The PCR amplification and sequencing were carried out by Meiji Biomedical Technology Co., LTD., (Shanghai, China) and the data were analyzed with Meiji Biocloud platform.

2.6. Other Analyses
The DO concentration was measured through a DO analyzer (Pro20, YSI, Ohio, USA). The SVI, the concentrations of suspended solids (SS) and volatile suspended solids (VSS), the ortho-P concentration (ortho-P\(_{\text{solution}}\)) and the TP concentration (TP\(_{\text{solution}}\)) in bulk solution as well the TP contents of LB-EPS (TP\(_{\text{LB-EPS}}\)) and TB-EPS (TP\(_{\text{TB-EPS}}\)) were determined by the standard methods [27]. The TP content of sludge (TP\(_{\text{sludge}}\)) was determined through the sonication dispersion, and the content of TP in bacterial cell (TP\(_{\text{cell}}\)) was calculated with subtraction. All measurements were conducted in triplicate.

3. Results and Discussion

3.1. TP and Acetate Concentrations in Bulk Solution

![Graph](image)  

**Figure 1.** Ortho-P (a) and acetate (b) concentrations in bulk solution of the 20°C EBPR and the 35°C non-EBPR reactors during steady cycle.

The 2 reactors at 20°C and 35°C had steadily runned more than 3 months before cycle experiments. From Figure 1a, the cycle profile of ortho-P\(_{\text{solution}}\) in the 20°C reactor presented a typical EBPR pattern, while the 35°C reactor had non-EBPR performance. In addition, the ortho-P\(_{\text{solution}}\) made up 98.5 - 99.6% of the TP\(_{\text{solution}}\) (The data not shown), illustrating that the 20°C EBPR sludge anaerobically released or aerobically took up ortho-P. From Figure 1b, the acetate was completely taken up in the 20°C and 35°C
reactors at 30 min and 45 min, respectively, when the VSS concentration (2450 ± 69 mg VSS/L) in the former was lower than that (3661 ± 75 mg VSS/L) in the latter, indicating that the specific anaerobic-uptake acetate rate in the former was about 2.24 times that in the latter.

Figure 2. TP content and distribution of sludge in the 20°C (a) and 35°C (b) reactors during steady cycle.

Figure 3. $^{31}$P NMR spectra of extracts from the 2 reactors during steady cycle. (a), (b) and (c): LB-EPS, TB-EPS and bacterial cell for the 20°C reactor, respectively; (d), (e) and (f): LB-EPS, TB-EPS and bacterial cell for the 35°C reactor, respectively.
3.2. P Content, Distribution and Species of Sludge

From Figure 2, the TP content of sludge (TP_{sludge}) in the 20°C EBPR reactor was greater than that in the 35°C non-EBPR reactor. The TP content of TB-EPS (TP_{TB-EPS}) of the 20°C sludge was slightly more than that of cell (TP_{cell}), while the TP_{TB-EPS} of the 35°C sludge was slightly less than the TP_{cell}. The steady-cycle profiles of the TP_{sludge}, TP_{cell} and TP_{TB-EPS} in the 20°C EBPR reactor all presented anaerobic-decrease and aerobic-increase, while these in the 35°C non-EBPR reactor kept relatively stable, indicating that the anaerobic P-release and aerobic P-uptake of the EBPR sludge was due to the combined actions of TB-EPS and bacterial cell [19].

According to the $^{31}$P NMR spectra (Figure 3), ortho-P was the main P species in the bacterial cell and LB-EPS, while poly-P, pyro-P and ortho-P co-existed in the TB-EPS of 20°C EBPR sludge, but only ortho-P in that of 35°C non-EBPR sludge. From Table 1, the contents of poly-P, pyro-P and ortho-P (poly-P_{TB-EPS}, pyro-P_{TB-EPS} and ortho-P_{TB-EPS}) in TB-EPS of the 20°C sludge respectively accounted for 2.09% - 60.18%, 0.09% - 2.86% and 39.40% - 96.42% of TP_{TB-EPS}, thus poly-P and ortho-P were the main P species. In addition, the apoptosis / death of bacterial cell was very weak induced by sonication extraction of LB-EPS and CER extraction of TB-EPS and there were no bacterial cells in the LB-EPS and TB-EPS extracts (data not shown). The cycle profiles of the TP_{cell} and the ortho-P content (ortho-P_{cell}) of bacterial cell in the 20°C reactor presented anaerobic-decrease and aerobic-increase, indicating that the bacterial cell directly released or took up ortho-P, when the cycle profiles of poly-P_{TB-EPS} and TP_{TB-EPS} also exhibited anaerobic-decrease and aerobic-increase. Furthermore, the average chain length of poly-P in TB-EPS for the 20°C reactor reduced from 286.4 ± 16.3 to 17.28 ± 3.46 at the early anaerobic stage (0 - 1 h) and increased from 6.44 ± 2.63 to 286.0 ± 23.9 at the early aerobic stage (4 - 6 h), illuminating that the extracellular poly-P played an important role in the EBPR process.

Table 1. P contents and species of microbial cell and TB-EPS in the 20°C reactor during steady cycle. (units: mg P/g VSS).

| Sampling time (h) | 0        | 1        | 4        | 6        | 11       |
|------------------|----------|----------|----------|----------|----------|
| TP_{cell}        | 24.91 ± 2.50 | 19.03 ± 2.67 | 14.41 ± 1.96 | 25.11 ± 2.16 | 26.08 ± 1.85 |
| Ortho-P_{cell}   | 24.47 ± 2.46 | 18.37 ± 2.58 | 13.57 ± 1.84 | 24.24 ± 2.08 | 25.62 ± 1.82 |
| Pyro-P_{cell}    | -        | -        | -        | -        | -        |
| Poly-P_{cell}    | -        | -        | 0.18 ±0.02 | 0.26 ± 0.02 | -        |
| TP_{TB-EPS}      | 27.29 ± 1.51 | 20.51 ± 2.13 | 17.29 ± 1.20 | 26.96 ± 1.49 | 27.85 ± 1.96 |
| Ortho-P_{TB-EPS} | 14.78 ± 1.94 | 16.13 ± 2.26 | 16.67 ± 2.27 | 17.19 ± 1.48 | 10.97 ± 0.78 |
| Pyro-P_{TB-EPS}  | 0.25 ± 0.03 | 0.59 ± 0.08 | 0.24 ± 0.03 | -         | 0.09 ± 0.01 |
| Poly-P_{TB-EPS}  | 12.20 ± 1.22 | 3.43 ± 0.48 | 0.36 ± 0.05 | 9.67 ± 0.83 | 16.76 ± 1.19 |
| Average chain length of poly-P | 286.4 ± 16.3 | 17.28 ± 3.46 | 6.44 ± 2.63 | 286.0 ± 23.9 | 220.0 ± 18.6 |

3.3. Glycogen Content and Distribution of Sludge

From Figure 4, the glycogen content of sludge (glycogen_{sludge}) in the 20°C EBPR reactor was obviously lower than that in the 35°C non-EBPR reactor. The glycogen of sludge mainly existed in bacterial cell, differently from the TP distribution. The intracellular glycogen contents (glycogen_{cell}) of the 20°C sludge and the 35°C sludge were 154.85 - 235.16 mg/g VSS and 329.28 - 434.74 mg/g VSS, respectively, which accounted for 89.79% - 91.93% and 89.95% - 92.15% of the glycogen_{sludge}, indicating that the extractions of LB-EPS with sonication and TB-EPS with CER were mild and reliable. In addition, the cycle profiles of the glycogen_{sludge} and the glycogen_{cell} in the 2 reactors were anaerobic-decrease and aerobic-increase, while the glycogen_{LB-EPS} and the glycogen_{TB-EPS} kept relatively stable.
3.4. PHAs Content, Distribution and Component in Sludge

From Figure 5, the PHAs content of sludge (PHA\textsubscript{sludge}) in the 20°C EBPR reactor was obviously lower than that in the 35°C non-EBPR reactor. In addition, the PHAs also mainly existed in bacterial cell, similarly to the glycogen distributions. The intracellular PHAs contents (PHA\textsubscript{cell}) of the 20°C sludge and 35°C sludge were 23.67 - 67.80 mg/g VSS and 112.9 - 149.2 mg/g VSS, respectively, which accounted for 90.02% - 96.12% and 83.28% - 96.11% of the PHA\textsubscript{sludge}. The extracellular PHAs existed in LB-EPS and TB-EPS was the difference between the PHA\textsubscript{sludge} and the PHA\textsubscript{cell}. In general, the PHAs and the glycogen of sludge in the 2 reactors were mainly intracellular, while the poly-P of the 20°C sludge was almost extracellular. The cycle profiles of the PHA\textsubscript{sludge} and the PHA\textsubscript{cell} in the 2 reactors exhibited anaerobic-increase and aerobic-decrease, which were opposite to those of the glycogen\textsubscript{sludge} and the glycogen\textsubscript{cell}. In addition, the cycle changes of the PHA\textsubscript{sludge} and the glycogen\textsubscript{sludge} were respectively attributed to those of the PHA\textsubscript{cell} and the glycogen\textsubscript{cell}.

There was obvious difference in the component of intracellular PHAs between the 2 reactors (Figure 5). The intracellular PHB of the 20°C sludge was the dominant component of intracellular PHAs, of which the content (PHB\textsubscript{cell}) was 14.59 - 49.47 mg/g VSS, while the intracellular PHV content (PHV\textsubscript{cell}) was 9.07 - 18.33 mg/g VSS. On the contrary, the intracellular PHV of the 35°C sludge was the main component of intracellular PHAs. Specifically, The PHV\textsubscript{cell} and the PHB\textsubscript{cell} were 86.10 - 97.69 mg/g VSS and 15.26 - 62.48 mg/g VSS, respectively. The PHB\textsubscript{cell} of the 20°C EBPR sludge was close to that of the 35°C non-EBPR sludge, while the PHV\textsubscript{cell} of the former was much lower than that of the latter. The cycle profiles of the PHB\textsubscript{cell} and the PHV\textsubscript{cell} in the 20°C reactor presented anaerobic-increase and aerobic-decrease. But, the change of the PHV\textsubscript{cell} in the 35°C reactor was anaerobic-decrease and aerobic-increase, which was opposite to that of the PHB\textsubscript{cell}.
3.5. Transformations of Poly-P in TB-EPS and Intracellular Energy-storing Substances

Table 2. Variation amounts of poly-P in TB-EPS and intracellular energy-storing substances in the 2 reactors during steady cycle. (Units: mg/g VSS).

| Reactor | Variation | 0-1 h     | 1-4 h     | 4-6 h     | 6-11 h    |
|---------|-----------|-----------|-----------|-----------|-----------|
|         | Poly-P_{TB-EPS} | -8.77 ± 1.19 | -3.07 ± 0.49 | 9.31 ± 0.85 | 7.09 ± 1.34 |
|         | glycogen_{cell}  | -48.74 ± 4.94 | -15.07 ± 2.53 | 69.75 ± 5.86 | 10.56 ± 3.42 |
| 20 °C   | PHA_{cell}       | 38.00 ± 4.24 | 6.14 ± 1.74 | -33.74 ± 3.42 | -12.80 ± 2.68 |
|         | PHB_{cell}       | 29.96 ± 3.56 | 4.92 ± 1.51 | -27.46 ± 2.73 | -10.02 ± 2.31 |
|         | PHV_{cell}       | 8.04 ± 0.68 | 1.21 ± 0.23 | -6.28 ± 0.69 | -2.78 ± 0.37 |
|         | Poly-P_{TB-EPS} | -61.99 ± 4.37 | -12.40 ± 2.84 | 72.55 ± 3.56 | 32.92 ± 5.15 |
| 35 °C   | glycogen_{cell}  | 27.92 ± 3.61 | -0.66 ± 0.90 | -26.67 ± 3.95 | -8.96 ± 3.10 |
|         | PHA_{cell}       | 36.66 ± 2.75 | 1.12 ± 0.47 | -35.48 ± 3.01 | -11.74 ± 2.39 |
|         | PHV_{cell}       | 8.74 ± 0.86 | -1.79 ± 0.43 | 8.81 ± 0.94 | 2.78 ± 0.71 |

From Table 2, the poly-P_{TB-EPS} and the glycogen_{cell} in the 20°C EBPR reactor at the early anaerobic stage (0 - 1 h) decreased by 8.77 ± 1.19 mg/g VSS and 48.74 ± 4.94 mg/g VSS, respectively, while the PHB_{cell} and the PHV_{cell} increased by 29.96 ± 3.56 mg/g VSS and 8.04 ± 0.68 mg/g VSS. At this stage, the PHV_{cell} and the glycogen_{cell} in the 35°C non-EBPR reactor decreased by 8.74 ± 0.86 mg/g VSS and 61.99 ± 4.37 mg/g VSS, respectively, while the PHB_{cell} increased by 36.66 ± 2.75 mg/g VSS. At the middle and later anaerobic stage (1 - 4 h), the change values of these intracellular energy-storing substances and /or extracellular poly-P in the 2 reactors were less. In addition, the VSS concentrations in the 20°C EBPR reactor and 35°C non-EBPR reactor were 2450 ± 69 mg VSS/L and 3661 ± 75 mg VSS/L, respectively. During the anaerobic phase, the anaerobic-synthesis amounts of intracellular PHAs for the 20°C and 35°C reactors were about 108.1 mg/L and 101.1 mg/L, respectively, while the anaerobic-hydrolysis amounts of intracellular glycogen were about 156.3 mg /L and 272.3 mg /L. At the early aerobic stage (4 - 6 h), the PHB_{cell} and the PHV_{cell} in the 20°C EBPR reactor decreased by 27.46 ± 2.73 mg VSS and 6.28 ± 0.69 mg VSS, respectively, while the poly-P_{TB-EPS} and the glycogen_{cell} increased by 9.31 ± 0.85 mg/g VSS and 69.75 ± 5.86 mg/g VSS. At the stage, the PHB_{cell} of the 35°C non-EBPR reactor decreased by 35.48 ± 3.01 mg/g VSS, while the glycogen_{cell} increased by 72.55 ± 3.56 mg/g VSS and 8.81 ± 0.94 mg/g VSS, respectively. At the middle and later aerobic stage (6 - 11h), the change values of these intracellular energy-storing substances and /or extracellular poly-P in the 2 reactors were less. During the aerobic phase, the aerobic-hydrolysis amounts of the intracellular PHAs in the 20°C and 35°C reactors were about 114.0 mg /L and 130.4 mg /L, respectively, while the corresponding aerobic-synthesis amounts of the intracellular glycogen were about 196.8 mg/L and 386.1 mg/L. In brief, the transformation amounts of intracellular glycogen in the 35°C non-EBPR reactor were almost twice as much as those in 20°C EBPR reactor, but those of the intracellular PHAs between the 2 reactors were close.

3.6. Microbial Population Structure of Sludge

Temperature is an important factor to influence EBPR performance and microbial population structure [2, 23]. From Figure 6, there were obvious differences in the microbial population structure between the 2 reactors. In the 20°C EBPR sludge, Candidatus _Competibacter_ and Candidatus _Accumulibacter_ were abundantly co-existed, of which the abundances were 33.77% and 30.48%, respectively, while the abundances of _Anaerolineaceae_ and _Defluviicoccus_ all were very low. In the 35°C EBPR sludge, the abundances of _Anaerolineaceae_, _Defluviicoccus_ and Candidatus _Competibacter_ were 28.20%, 27.13% and 15.21%, respectively, but Candidatus _Accumulibacter_ was not observed. It is well known that...
Candidatus *Accumulibacter* has been recognized as PAOs, and *Defluviococcus* and Candidatus *Competibacter* has been considered as GAOs [1, 5]. Thus, the Candidatus *Accumulibacter* was the key PAOs in the 20°C EBPR sludge and the *Defluviococcus* was the dominant GAOs in the 35°C non-EBPR sludge. The differences of transformation characteristics of the extracellular poly-P and the intracellular glycogen and PHAs between the 2 reactors were related to the microbial population structures. Firstly, the anaerobic-hydrolysis and aerobic-synthesis of extracellular poly-P in the 20°C EBPR sludge occurred, while the extracellular poly-P in the 35°C non-EBPR sludge was not detected. In addition, Candidatus *Accumulibacter* was abundant in the 20°C sludge and not existed in the 35°C sludge (Figure 6b), which was the microbial population basis for the existence and the transformation of the poly-P in TB-EPS of the 20°C EBPR sludge. Secondly, the anaerobic-hydrolysis and aerobic-synthesis amounts of intracellular glycogen in the 35°C reactor were almost twice those in the 20°C reactor, respectively. Although the VSS concentration (3661 ± 75 mg/L) in the 35°C non-EBPR reactor was more than that (2450 ± 69 mg/L) in the 20°C EBPR reactor, the glycogen-accumulating metabolism (GAM) level of microorganism in the former was 1.16-1.31 times that in the latter (Figure 4 and Table 2), corresponding to that the total abundance of GAOs in the former was greater than that in the later. Specifically, the total abundance of *Defluviococcus* (27.13%) and Candidatus *Competibacter* (15.21%) in the 35°C sludge was about 1.25 times the abundance of Candidatus *Competibacter* (33.77%) in the 20°C sludge. Thirdly, the anaerobic-hydrolysis and aerobic-synthesis of intracellular PHV in the 35°C non-EBPR sludge occurred, which was contrary to the change of intracellular PHV in the 20°C EBPR sludge. It had been reported that PAOs anaerobically produced mainly PHB with little PHV production (< 10 %) when fed with acetate [28, 29], while GAOs anaerobically produced about 75 % PHB and 25 % PHV when fed with acetate [30, 31]. The formation amounts of PHBcell and PHVcell in the 20°C EBPR sludge at the anaerobic phase were respectively about 79 % and 21 % of the produced PHAcell, because the Candidatus *Accumulibacter* and Candidatus *Competibacter* abundantly co-existed (Figure 5 and Table 2). The different transformation characteristics of intracellular PHV in the 35°C non-EBPR sludge might be related to a great deal of *Defluviococcus* and *Anaerolineaceae* as well as some different Candidatus *Competibacter* at species level.
3.7. Effect of Extracellular Poly-P in EBPR Process

Figure 6. Phylogenetic tree on species level. R1: in 20°C reactor; R2: in 35°C reactor.

Figure 7. Anion exchange hypothesis to explain the effect of extracellular poly-P in EBPR process.
In the EBPR theory, intracellular poly-P is assumed as a main energy source for PAOs to anaerobically absorb carbon source [5, 6] and a key substance for PAOs to gain advantage over GAOs [7], which can promote PAOs to take up carbon sources, such as acetate and propionate [6]. In this study, the specific anaerobic-uptake acetate rate in the 20°C EBPR reactor was about 2.24 times that in the 35°C non-EBPR reactor (Figure 1). However, the $^{31}$P NMR resonance signals of intracellular poly-P (i.e., the end and the middle groups of poly-P) of the 20°C sludge were also weak (Figure 3c), which could not explain the difference of the specific anaerobic-uptake acetate rate between the 2 reactors. The proton motive force (PMF) by efflux of proton and ortho-P is also related to promoting carbon source uptake [5, 6]. From Figure 3 and Table 1, the bacterial cell in the 20°C EBPR sludge directly anaerobically released, indicating that the release of intracellular ortho-P into the cell outer surface produced PMF to promote acetate pass through cell membrane. There was a great deal of extracellular poly-P in the 20°C EBPR sludge, which produced ortho-P by anaerobic hydrolysis. These ortho-P all passed through TB-EPS and LB-EPS before entering bulk solution. In addition, the anion-exchange performance of ortho-P is superior to that of acetate, thus the ortho-P both released from the intracellular ortho-P and came from the hydrolyzed extracellular poly-P could accelerate acetate pass through LB-EPS and TB-EPS by the anion-exchange between ortho-P and acetate (Figure 7). From Table 2, the decrease amount (-8.77 ± 1.19 mg/g VSS) of the poly-P$_{TB-EPS}$ was almost 1.44 times that (-6.10 ± 1.32 mg/g VSS) of the ortho-P$_{cell}$ in the 20°C EBPR sludge at the early anaerobic stage (0 - 1 h), suggesting that the hydrolysis of extracellular poly-P played an important role in the acetate migration from the bulk solution to the cell surface (passing through LB-EPS and TB-EPS). During the aerobic phase, the aerobic-hydrolysis amount of intracellular PHAs in the 20°C EBPR reactor was comparable to that in the 35°C non-EBPR reactor, but the aerobic-synthesis amount of intracellular glycogen in the former was about half that in the latter. Meanwhile, the aerobic-synthesis amount of extracellular poly-P and the aerobic-uptake amount of intracellular ortho-P in the former were far greater than that in the latter, of which the energy should came from the hydrolysis of intracellular PHAs. In the future, it is very necessary to verify the anion exchange hypothesis and understand the different routes of P migration and transformation in EBPR process.

**4. Conclusions**

The anaerobic-hydrolysis and aerobic-synthesis amounts of intracellular glycogen in the 20°C EBPR reactor were only half those in the 35°C non-EBPR reactor, while the corresponding-transformation amounts of intracellular PHAs between them were comparable. Notably, the aerobic-synthesis amount of extracellular poly-P (in tightly-bound EPS (TB-EPS)) and the anaerobic-release / aerobic-uptake of intracellular ortho-P occurred in the 20°C EBPR sludge. An anion-exchange hypothesis that the ortho-P both released from the intracellular ortho-P and came from the hydrolyzed extracellular poly-P could accelerate acetate pass through LB-EPS and TB-EPS was proposed. PAOs were the microbial population basis for the existence and transformation of extracellular poly-P.

**Acknowledgements**

This work was supported by the National Natural Science Foundation of China under Grant [number 21577174]; and the Chongqing Natural Science Foundation under Grant [number cstc2015jcyjBX0012, number cstc2020jcyj-msxmX0406].

**References**

[1] Oehmen A Lemoe P C Carvalho G Yuan Z Jürg K Blackall L L Reis M A M 2007 Advances in enhanced biological phosphorus removal: from micro to macro scale Water Research 41 (11) pp 2271 - 2300

[2] Qiu G Zuniga-Montanez R Law Y Thi S S Nguyen T Q Nguyen T Eganathan K Liu X Nielsen P H Williams R B H Wuertz S 2019 Polyphosphate-accumulating organisms in full-scale tropical wastewater treatment plants use diverse carbon sources Water Research 149 (1) pp 496 - 510

[3] Lanham A B Oehmen A Saunders A M Carvalho G Nielsen P H Reis M A M 2014 Metabolic modelling of full-scale enhanced biological phosphorus removal sludge Water Research 66 pp 283 - 295
[4] Zheng X Sun P Han J Song Y Hu Z Fan H 2014 Inhibitory factors affecting the process of enhanced biological phosphorus removal (EBPR) - a mini-review *Process Biochemistry* 49 (12) pp 2207 - 2213

[5] Nielsen P H Saunders A M Hansen A A Larsen P Nielsen J L 2012 Microbial communities involved in enhanced biological phosphorus removal from wastewater - a model system in environmental biotechnology *Current Opinion in Biotechnology* 23 (3) pp 452 - 459

[6] Oehmen A Carvalho G Lopez-Vazquez C M Van-Loosdecht M C M Reis M A M 2010 Incorporating microbial ecology into the metabolic modelling of polyphosphate accumulating organisms and glycogen accumulating organisms *Water Research* 44 (17) pp 4992 - 5004

[7] Seviour R J McIlroy S 2008 The microbiology of phosphorus removal in activated sludge processes-the current state of play *Journal of Microbiology* 46 (2) pp 115 - 124

[8] Acevedo B Oehmen A Carvalho G Seco A Borrás L Barat R 2012 Metabolic shift of polyphosphate-accumulating organisms with different levels of polyphosphate storage *Water Research* 46 (6) pp 1889 - 1900

[9] Acevedo B Murgui M Borrás L Barat R 2017 New insights in the metabolic behaviour of PAO under negligible poly-P reserves *Chemical Engineering Journal* 311 pp 82 - 90

[10] Lv J H Yuan L J Chen X Liu L Luo D C 2014 Phosphorus metabolism and population dynamics in a biological phosphate-removal system with simultaneous anaerobic phosphate stripping *Chemosphere* 117 pp 715 - 721

[11] Zhang H L Sheng G P Fang W Wang Y P Fang C Y Shao L M 2015 Calcium effect on the metabolic pathway of phosphorus accumulating organisms in enhanced biological phosphorus removal systems *Water Research* 84 pp 171 - 180

[12] Sheng G Yu H Li X 2010 Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment systems: a review *Biotechnology Advances* 28 (6) pp 882 - 894

[13] Borovec J Sirová P Mošnerová E Rejmánková E Vrba J 2010 Spatial and temporal changes in phosphorus partitioning within a freshwater cyanobacterial mat community *Biogeochemistry (Dordrecht)* 101 pp 323 - 333

[14] Cloete T E Oosthuizen D J 2001 The role of extracellular exopolymers in the removal of phosphorus from activated sludge *Water Research* 35 (15) pp 3595 - 3598

[15] Wang R Peng Y Cheng Z Ren N 2014 Understanding the role of extracellular polymeric substances in an enhanced biological phosphorus removal granular sludge system *Bioresource Technology* 169 pp 307 - 312

[16] Zhang H L Fang W Wang Y P Sheng G P Xia C W Zeng R J Yu H J 2013 Species of phosphorus in the extracellular polymeric substances of EBPR sludge *Bioresource Technology* 142 pp 714 - 718

[17] Zhang H L Fang W Wang Y P Sheng G P Zeng R J Li W W Yu H J 2013 Phosphorus removal in an enhanced biological phosphorus removal process: roles of extracellular polymeric substances *Environmental Science & Technology* 47 (20) pp 11482 - 11489

[18] Li W W Zhang H L Sheng G P Yu H Q 2015 Roles of extracellular polymeric substances in enhanced biological phosphorus removal process *Water Research* 86 pp 85 - 95

[19] Long X Y Tang R Fang Z D Xie C X Li Y Q Xian G 2017 The roles of loosely-bound and tightly-bound extracellular polymer substances in enhanced biological phosphorus removal *Chemosphere* 189 pp 679 - 688

[20] Jia F Yang Q Han J Liu X Li X Peng Y 2016 Modeling optimization and evaluation of tightly bound extracellular polymeric substances extraction by sonication *Appl. Microbiol. Biot.* 100 pp 8485 - 8494

[21] Li X Y Yang S F 2007 Influence of loosely bound extracellular polymeric substances (EPS) on the flocculation, sedimentation and dewaterability of activated sludge *Water Research* 41 (5) pp 1022 - 1030

[22] Long X Y Tang R Xie C X Fang Z D Li Y Q 2019 Content and species of extracellular phosphorus in activated sludge of biological phosphorus removal systems *Polish Journal of Environmental Studies* 28 (5) pp 3779 - 3790

[23] Ong Y H Chua A S M Fukushima T Ngoh G C Shoji T Michinaka A 2014 High-temperature
EBPR process: the performance, analysis of PAOs and GAOs and the fine-scale population study of Candidatus “Accumulibacter phosphatis” *Water Research* 64, pp 102 - 110

[24] Ullah M A Kim K H Szulejko J E Cho J 2014 The gas chromatographic determination of volatile fatty acids in wastewater samples: evaluation of experimental biases in direct injection method against thermal desorption method *Analytica Chimica Acta* 820 pp 159 - 167

[25] Li D Fang Z Long X Tang R Di S 2016 Effects of matrix types on formation and transformation of energy-accumulating substances in enhanced biological phosphorus removal (EBPR) *Cellular and Molecular Biology* 62 (14) pp 34 - 37

[26] Wei Z Zhang L Fan P Guo J Peng Y 2018 Community structures and population dynamics of “candidatus accumulibacter” in activated sludges of wastewater treatment plants using ppx1 as phylogenetic marker *Journal of Environmental Sciences* 67 (5) pp 237 - 248

[27] Clesceri L S Greenberg A E E Eaton A D 1999 Standard methods for the examination of water and wastewater 20th ed APHA Washington D.C.

[28] Smolders G J F Smolders, J. Vandermeij J Van - Loosdrecht M C M Heijnen J J 1994 Model of the anaerobic metabolism of the biological phosphorus removal process: Stoichiometry and pH influence *Biotechnology and bioengineering* 43 (6) pp 461 - 470

[29] Mino T Van - Loosdrecht M C M Heijnen J J 1998 Microbiology and biochemistry of the enhanced biological phosphate removal process *Water Research* 32 (11) pp 3193 - 3207

[30] Filipe C D M Daigger G T Grady C P L 2001 Effects of pH on the rates of aerobic metabolism of phosphate-accumulating and glycogen-accumulating organisms *Water Environment Research* 73 (2) p 213 - 222

[31] Zeng R J Van - Loosdrecht M C M Yuan Z Keller J 2010 Metabolic model for glycogen-accumulating organisms in anaerobic/aerobic activated sludge systems *Biotechnology & Bioengineering* 81 (1) pp 92 - 105