Acetate favors more phosphorus accumulation into aerobic granular sludge than propionate during the treatment of synthetic fermentation liquor

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Acetate favors more phosphorus accumulation into aerobic granular sludge than propionate during the treatment of synthetic fermentation liquor

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Abstract

Anaerobic digestion (AD) is an efficient biotechnology widely applied for energy and resource recovery from organic waste and wastewater treatment. The effluent from AD or fermentation liquor containing organic substances like volatile fatty acids (VFAs) and mineral nutrients (such as N and P), however, will trigger serious environmental issues if not properly dealt with. In this study two identical sequencing batch reactors (SBRs), namely Ra and Rp were used to cultivate aerobic granules for P recovery from synthetic fermentation liquor respectively using acetate and propionate as additional carbon source. Larger and more stable granules were achieved in Ra with higher P removal capability (9.4 mgP/g-VSS·d) and higher anaerobic P release (6.9 mgP/g-VSS·h). In addition to much higher P content (78 mgP/g-SS), bioavailable P in Ra-granules increased to 45 mgP/g-SS, approximately 2-times those of seed sludge and Rp-granules. Microbial community analysis indicated that more GAOs were accumulated in Rp-granules.

Key words: Fermentation liquor; Aerobic granular sludge; Phosphorus recovery; Phosphorus bioavailability; Acetate; Propionate

1. Introduction

Anaerobic digestion (AD) is regarded as one of the most important and effective stabilization, disposal and energy recovery processes for wasted activated sludge (WAS), livestock manure and other high-strength organic wastewaters or solid wastes. Through AD, organic carbonaceous substances can be converted into biogas to a greater extent, leaving all the other components such as mineral materials, non- or slowly biodegradable organics, and some intermediate products like volatile fatty acids (VFAs) in the digestate or fermentation liquor (Barker et al., 1999; Ji and Chen, 2010). This huge amount of fermentation liquor, if not properly treated or discharged directly to the environment, will trigger serious environmental problems. Traditionally, fermentation liquor is either directly spread as liquid fertilizer or treated by solid-liquid separation, drying, filtration, etc., before land application (Möller and Müller, 2012). These practices sometimes encounter problems relating to land availability, long-distance transportation, cost-effectiveness, etc. Since fermentation liquor contains high levels of nutrients, especially nitrogen (N) and phosphorus (P), it is more applicable to firstly recover these resources and then treat it to meet the standards for final usage or disposal. Magnesium ammonium phosphate (MAP) precipitation can realize simultaneous recovery of NH_4-N and ortho-P from WAS alkaline fermentation liquor, and after MAP precipitation the liquor can serve as additional carbon source for enhanced N and P removal from wastewater (Tong and Chen, 2009) . However, due to the complex nature of fermentation liquor and the requirement for MAP formation ideally at

Mg^{2+}:NH_4-N:PO_4^{3-} of 1:1:1 (molar ratio), it is challenging to achieve stable nutrients recovery and high purity target product by using MAP method without adding other chemicals (like Mg^{2+}) and proper pretreatment processes. Other alternative and prospective processes are necessary for a better management of the fermentation liquor. Ammonia stripping has been proven to be effective for NH_4-N recovery from digestate, and about 80-95% of total ammonia can be recovered after wet stripping at 35°C and pH 10-11 for 3 hours, leaving high levels of PO_4^{3+} and VFAs in the liquid phase (Huang et al., 2016). Further work is still demanding for VFAs utilization and P recovery from fermentation liquor.

Aerobic granule sludge (AGS) is considered to be one of the most promising biotechnologies for wastewater treatment. Up to the present, AGS with high treatment efficiency and much lower investment and operation costs has been successfully applied in large-scale domestic and industrial wastewater treatment plants (WWTPs) (Pronk et al., 2015; Nereda, 2016). Still, little information is available for AGS application in the treatment of fermentation liquor.

As reported, AGS can be used for effective P accumulation from wastewater treatment, achieving stable N and P removal (Schönborn et al., 2001; Wu et al., 2010). Solovchenko et al. (2016) claimed that P is always present in a form that does not meet the specifications for agriculture use when recovered chemically or biologically from wastewater. A previous work (Huang et al., 2015a) achieved P-rich granules with 93-95% of P bioavailability (i.e. the proportion of organic P and non-apatite inorganic

P (NAIP) to the total P stored in granules) through enhanced P removal AGS process. Specifically, if stable P-rich granules with high P bioavailability could be cultivated during the treatment of fermentation liquor, P recovery from this huge amount of wastewater would not only greatly ameliorate environmental contamination but also ease the burden of rapid consumption of phosphate rock which is estimated to be depleted in 50-100 years (Cordell et al., 2009). Up to now, little information is available with respect to this aspect.

Restated, acetate and propionate are the two dominant VFA products which generally amount to 60-80% of the total VFAs, about 350-1330 mg/L in fermentation liquor regardless of solids retention time (SRT) varied from 5 to 10 days during anaerobic digestion of WAS (Yuan et al., 2009). According to previous studies, acetate- or propionate- dominant VFAs fermentation liquor can be achieved after adjustment or optimization of the AD operation conditions. In addition, how to get acetate- or propionate-dominant fermentation liquor for multi-functional utilizations is one of the research focuses of AD during recent years. Research works show that these two VFA species have different impact on phosphate accumulating organisms (PAOs) responsible for P uptake and accumulation from wastewater by using conventional enhanced biological P removal (EBPR) process (Chen et al., 2004) and on the structure of AGS (Lin et al., 2003; Wu et al., 2010). Currently no information can be found on the impact of these two major VFAs on P bioavailability of P-rich granules, the most important aspect of P recovery from fermentation liquor by using AGS process for

agricultural purpose.

This study aimed to investigate the feasibility of cultivation of P-rich AGS through 6 months' operation of sequencing batch reactors (SBRs) to treat synthetic fermentation liquor after ammonia being recovered by stripping process (Huang et al., 2016). In addition to glucose, the two VFAs, namely acetate and propionate were added as additional carbon source in the synthetic fermentation liquor. P species and its bioavailability in seed sludge and AGS were evaluated and compared. Finally, changes in microbial biodiversity in the granules cultivated with acetate and propionate were analyzed to shed light on the mechanisms involved in this complex granulation process.

2. Materials and methods

2.1. Experimental setup and operation conditions

Two identical laboratory scale SBRs made of acrylic plastic were used in this study. Their individual dimension was 6cm×6cm×60cm (L×W×H) with working volume of 1.40 L. Seed sludge was sampled from the secondary sedimentation tank of the Shimodate Sewage Treatment Plant, Ibaraki Prefecture, Japan. Conventional activated sludge process is applied in this plant to treat domestic wastewater, mainly including primary sedimentation tank, aeration tank and secondary sedimentation tank. The initial concentrations of mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) were 4830 mg/L and 3570 mg/L

(MLVSS/MLSS=0.74), respectively in both reactors. Based on our previous works (Lin et al., 2013; Huang et al., 2015; Huang et al., 2016), the synthetic wastewater used in these experiments consisted of 500 mg COD/L (glucose), 50 mg PO_4^{3-}-P/L (KH_2PO_4), 100 mg NH_4^+-N/L (NH_4Cl), 10 mg Ca^{2+}/L ($CaCl_2$), 5 mg Mg^{2+}/L ($MgSO_4 \cdot 7H_2O$), 5 mg Fe^{2+}/L ($FeSO_4 \cdot 7H_2O$), and 1 ml/L of trace element solution to mimic the fermentation liquor after ammonia recovery by stripping. The composition of the trace elements solution was the same as Huang et al. (2015b). In addition to 500mg COD/L of glucose, sodium acetate (~500mg COD/L) and sodium propionate (~500mg COD/L) were also used as carbon source and added into the influents of the two reactors, i.e. Ra and Rp, to simulate the acetate- and propionate- dominant fermentation liquor after mesophilic anaerobic digestion of WAS and livestock manure (Yuan et al., 2009; Huang et al., 2015).

The two SBRs were operated automatically at room temperature (25±2℃) under alternative anaerobic and aerobic conditions with each cycle of 6 h. The initial one cycle consisted of 2 min feeding, 60 min non-aeration, 270 min aeration, 15 min settling, 2 min decanting and 11 min idling. After 10 days operation, due to improved settleability of the sludge the settling time was reduced to 2 min to wash out the sludge with poor settleability and to accelerate the granulation process, and the residual 13 min was used for idling. During aeration, air was provided by an air pump (AK-30, KOSHIN, Japan) from the bottom of SBRs through air bubble diffusers at an air flow rate of 0.6 cm/s. For each cycle, 0.76 L of supernatant was discharged right after the

settling period (HRT~11 h), and sludge retention time (SRT) of the two SBRs was controlled around 22 days. Dissolved oxygen (DO) concentration was 5-8 mg/L during aeration period. The granules in Ra and Rp were labelled as Ra-granules and Rp-granules. On day 130, the previous Rp- granules were evacuated and replaced by half of the Ra- granules in order to further clarify the influence of propionate on mature AGS functioned as P removal and P accumulation. The two SBRs were operated as same as day 130 before, and thereafter the granules in Ra and Rp were referred as Ra'-granules and Rp'-granules, respectively.

2.2. Analytical methods

2.2.1. Physicochemical characteristics of granules and wastewater

MLSS and MLVSS were used to quantify biomass growth in accordance with standard methods (APHA, 2012). DO concentration in bulk liquor was measured with a DO meter (HQ40d, HACH, USA). pH was monitored using a compact pH-meter (Horiba, Japan).

Granular size was measured by a stereo microscope (STZ-40TBa, SHIMADZU, Japan) with a program Motic Images Plus 2.3S (Version 2.3.0). The strength of granules was estimated by using the increased turbidity in sludge sample after shaking at 200 rpm for 10 min (Teo et al., 2000). Granular morphology was observed using a scanning electron microscope (SEM, JSM6330F, Japan) after the granules being pretreated with the method described by Wu et al. (2010).

Extracellular polymeric substances (EPS) of granules were extracted with ethylene diamine tetraacetic acid (EDTA) and analyzed according to Sun et al. (2012). Glycogen stored in granules was measured by the phenol-sulfuric method with glucose as standard (Herbert et al., 1971) after two drops of 1M HCl being immediately added to the granular samples in order to stop bacterial activity (Wu et al., 2010). For the analysis of metal ions in granules, the sludge samples were pretreated according to the method described by Huang et al. (2015b) and then measured by ICP-OES (Perkin-Elmer Optima 7300DV, USA). C, H and N contents were determined by a CHN Elemental Analyzer (Perkin-Elmer 2400 Ⅱ, USA). Phosphorus (P) in granules, namely total phosphorus (TP), organic phosphorus (OP), inorganic phosphorus (IP), non-apatite phosphorus (NAIP) and apatite phosphorus (AP), was fractioned and quantified using the Standards, Measurements and Testing (SMT) Programme extraction protocol (Medeiros et al., 2005). Before analysis of metal ions, C, H and N contents and phosphorus fractionation, the granules were washed with deionized water for three times after being sampled, and then lyophilized. P species in granular sludge and in EPS were further analyzed by ^{31}P NMR using a Bruker Avance-600MHz NMR Spectrometer at 242.94 MHz after being extracted by cold perchloric acid ($HClO_4$, 0.5M) and NaOH (1M) as described by Huang et al. (2015a).

Water samples were collected at the end of operation cycle and the filtrates were used for analysis after filtration through 0.45 μm membrane. Total nitrogen (TN) and total phosphorus (TP) were analyzed according to the standard methods (APHA, 2012).

Dissolved organic carbon (DOC) was measured by TOC detector (TOC-V_{CSN}, SIMADZU, Japan) equipped with an auto-sampler (ASI-V, SIMADZU, Japan).

2.2.2. Microbial diversity analysis

The total DNA of granular sludge samples harvested on day 120 from Ra and Rp were extracted by using Mo-Bio PowerMax® Soil DNA Isolation Kit (MoBio Laboratories, Inc., USA) according to the manufacturer's protocol. After DNA extraction, polymerase chain reaction (PCR) and high-throughput sequencing were performed as described by Huang et al. (2014). Briefly, the rough full-length 16S rDNA gene was amplified by PCR with a forward primer V4F, 5-AYTGGGYDTAAAGNG-3 and an equimolar mixture of four reverse primers, i.e. V4R1 5-TACCRGGGTHTCTAATCC-3, V4R2 5-TACCAGAGTATCTAATTC-3, V4R3 5-CTACDSRGGTMTCTAATC-3, and V4R4 5-TACNVGGGTATCTAATCC-3 based on the RDP pyrosequencing pipeline (http://pyro.cme.msu.edu/pyro/help.jsp). The PCR conditions were as follows: 95℃ for 7 min, followed by 32 cycles at 95℃ for 1 min, 55℃ for 1 min, 72℃ for 1 min and a final extension step at 72℃ for 10 min. The PCR products were purified with the QIAquick PCR Purification Kit (Qiagen, Germany). After quantification using Qubit® 2.0 Fluorometer (Invitrogen, USA), the PCR products of all samples were taken for high-throughput sequencing on Ion Torrent PGM System (Life Technology, USA). Mothur (version: 1.31.2) was used for analysis of microbial biodiversity in the granules.

2.3. Calculations

DOC, TN and TP removal efficiencies were calculated according to Eq. (1).

$$\text{Removal (\%)} = 100 \times (\rho_{inf} - \rho_{eff})/\rho_{inf} \quad (1)$$

in which ρ_{inf} (mg/L) and ρ_{eff} (mg/L) are influent DOC, TN or TP concentration and effluent DOC, TN or TP concentration, respectively.

TP removal capacity was calculated according to Eq. (2), which can be used to compare the influence of acetate and propionate on P removal and storage.

$$\text{TP removal capacity (mg/g-VSS·d)} = 4 \times (TP_{inf} - TP_{eff}) \times 0.76/(\text{MLVSS} \times 1.40) \quad (2)$$

where TP_{inf} (mg P/L) and TP_{eff} (mg P/L) are average influent and effluent TP concentrations during the designated operation period, respectively. MLVSS (g/L) is the average MLVSS concentration in SBR during this period, and 4 and 0.76 are the number of cycles per day and exchange ratio, respectively, in this study.

In addition, anaerobic DOC uptake rate, anaerobic TP release rate and aerobic TP uptake rate in SBRs were estimated according to the following three equations (Eqs. (3) to (5)), respectively.

$$\text{DOC uptake rate (mg DOC/g-VSS·h)} = (DOC_0 - DOC_1)/(\text{MLVSS} \times 1) \quad (3)$$

$$\text{TP release rate (mg P/g-VSS·h)} = (TP_1 - TP_0)/(\text{MLVSS} \times 1)$$

(4)

TP uptake rate (mg P/g-VSS h) = $(TP_1 - TP_{eff}) / (MLVSS \times 4.5)$

(5)

where DOC_0 (mg/L) and TP_0 (mg P/L) are the initial DOC and TP concentrations respectively during one cycle, while DOC_1 (mg/L) and TP_1 (mg P/L) are their concentrations at the end of non-aeration period, respectively. TP_{eff} (mg P/L) is the effluent TP concentration, and '1' (h) and '4.5' (h) are the duration of non-aeration and aeration periods, respectively.

2.4. Statistics

For data analysis, one-way analysis of variance (ANOVA) was applied to compare the difference in P removal capacity between the two kinds of granules before and after the formation of mature granules by using SPSS 19.0 (IBM, US). Statistical difference was assumed significant at $p < 0.05$.

3. Results and discussion

3.1. Formation and characteristics of granules

From the startup of the two SBRs, the sludge particle size was recorded along with the operation as shown in Fig. 1a. Granules appeared on day 15 in both reactors and grew up gradually during the operation. From day 0 to day 60, the particle size in both reactors almost exhibited a similar increase trend, averagely from initial 0.14 mm

to 0.98 mm (Ra) and 0.94 mm (Rp), respectively. After day 60, the granular size in the two reactors continued to increase and stabilized at around 1.85 mm (Ra) and 1.53 mm (Rp) after operation for 110 days. Smaller granules were observed in Rp after day 60, most probably attributable to the following two reasons. Firstly, granules in Rp were not as stable as those in Ra, which can be clearly seen from Fig. 1b. After shaking for 20 min, the ΔTurbidity (increase in turbidity of the sludge suspension) was respectively 3.59 NTU (Ra) and 6.74 NTU (Rp), indicating more small fragmentations or particles generated in the suspension of Rp-granules under the same operation condition. Namely, Ra- granules were more stable than Rp-granules, and the latter were easily to disintegrate to smaller particles. Secondly, propionate is more complex than acetate and difficult to be assimilated by microorganisms; high concentration of propionate might inhibit the activities of microorganisms (Garrity et al., 2007), possibly resulting in the smaller particle size of Rp-granules.

The digital images show that the granules from both reactors had compact and dense structure, especially after 100 days' operation (Fig. S1). All granules exhibited three layers in their interior structure, i.e. inner core, intermediate layer, and yellowish edge layer. However, different surface morphologies were observed on Ra- and Rp-granules (from their SEM images, Fig. S1). In Ra-granules, most of the bacteria were distributed on the edge and few was observed in the core where was most probably occupied by inorganic precipitate (Huang et al., 2015b; Li et al., 2015). Still, many bacteria were observed in the core of Rp-granules, and Bacilli and Brevibacterium

dominated the edge while filamentous bacteria were found in the core, implying that different microbial communities existed in the two kinds of granules.

3.2. Performance of the two reactors during 130 days' operation

3.2.1. Overall performance for pollutants removal

Both reactors exhibited almost similar organics and N removals during the 6 months operation, achieving 96-97% of DOC removal and 78-79% of TN removal under the designed operation strategy (Table S1). During the whole operation period, Ra showed more stable TP removal efficiency (61-63%) than Rp, even after day 130 when half of its granules were taken out and used as the seed granules in Rp (Table S1). As for Rp, interestingly, the TP removal was averagely 44% (29.4-63.3%) before day 130, while increased to about 65% during the subsequent operation (from day 130 to day 170) after the original Rp-granules being discarded and replaced by half of the Ra-granules in Ra (on day 130). It is worthy to note that the Ra-granules could also use propionate and effectively remove TP from the synthetic wastewater during the short-term operation (40 days). Further research work is still ongoing to confirm the stability of P removal efficiency for Rp'-granules after long-term operation.

3.2.2. Profiles of ML(V)SS and P removal capacity in the two reactors together with changes in granular P content

The biomass growth indicated by MLSS and MLVSS increased in a similar trend

in the two reactors under the same operation strategy (Fig. 2a). A faster increase in MLVSS detected in Ra clearly reflected that acetate can be easily uptaken by the microorganisms in the sludge. Besides, the MLSS and MLVSS concentrations were observed to continuously increase from ~ 4.8 g MLSS/L and ~3.6 g MLVSS/L (MLVSS/MLSS =74%, day 0) to about 14.0 g MLSS/L and 9.5g MLVSS/L in Ra (MLVSS/MLSS = 68%), and 8.4 g MLSS/L and 6.6 g MLVSS/L in Rp (MLVSS/MLSS=79%) on day 90, respectively. From day 90 on, their biomass concentrations slightly decreased and fluctuated at 12.3-13.6 g MLSS/L and 7.9-9.5 g MLVSS/L in Ra, and 7.5-8.1 g MLSS/L and 6.0-6.4 g MLVSS/L in Rp, respectively. Lower MLVSS/MLSS ratios were obtained for Ra- granules, implying that more mineral substances were accumulated into the granules of Ra (Oehmen et al., 2005). Similar phenomenon was also observed during the extended operation trial (from day 130 to day 170) by using half of granules in Ra to replace all the original granules in Rp. MLVSS/MLSS ratio was observed to slightly decrease from 0.63 (day 130) to 0.61 (day 170) in Ra, while slightly increased to 0.66 (day 170) in Rp.

As for MLVSS-based P removal capacity (Fig. 2b), another indicator of P removal efficiency, the two reactors exhibited almost similar variation trend after granules formed on day 15, ranging between 6.9-12.2 mg P/g-VSS d (Ra) and 3.6-10.8 mg P/g-VSS d (Rp), respectively. More specifically, according to one-way ANOVA results, before mature P-rich granules formed (from day 15 to day 90), the average P removal capacity (10.0 mg P/g-VSS d) of Ra-granules was significantly higher than Rp (8.2 mg

P/g-VSS d) (p= 0.0274 < 0.05); while after day 90, their P removal capacities were averagely comparable at ~8.6-8.7 mg P/g-VSS d) (p= 0.9004 > 0.05).

Granular TP content was also recorded during the whole operation shown in Fig. 2c. Before granules appeared (day 15), TP content in the sludge particles decreased to some extent possibly due to loss in bioactivity of PAOs because of their adaptation to the laboratory operation conditions which were much different from where the seed sludge was sampled. After granules formed, the granular TP contents increased in a similar trend and reached to 45 mg P/g-SS on day 75. After day 75, the granular TP content in Rp decreased slightly and fluctuated at 36 - 43 mg P/g-SS. However, the TP content in Ra-granules kept its increase trend, achieving 77 mg P/g-SS on day 90, thereafter maintaining at 72 - 78 mg P/g-SS till the end of experiments. It should be noted that after day 90, averagely 78 mg P/g-SS and 39 mg P/g-SS were respectively achieved in Ra and Rp (Fig. 2c), about 2.7- and 1.3- times that of the seed sludge (29 mg P/g-SS) used in this study. The TP content in Ra-granules (78 mg P/g-SS) after reaching stable is in agreement with the result (about 80 mgP/g-SS) obtained by Lin et al. (2003) under the same influent P/COD (5/100) ratio, although different operation conditions including different cycle time (anaerobic/aerobic), HRT, SRT, and seed sludge were applied in this work. Obviously, the TP content of Ra-granules was significantly higher than that of Rp-granules, signaling more PAOs might be accumulated in the granules of Ra.

3.2.3. DOC and TP changes during a typical operation cycle

The variation of DOC and TP concentrations in the bulk liquor was also monitored in the two reactors during a typical operation cycle after reaching steady operation (like on day 120). Results (Fig. S2a) showed that more than 90% of influent DOC could be removed within 120 min in both SBRs. More specifically, during the initial 60 min (non-aeration period), about 70% of influent DOC was uptaken by the microorganisms in both reactors. However, TP concentration in the bulk liquor showed completely different variation trends in the two reactors (Fig. S2a). In Ra, TP was detected to significantly release during the non-aeration period (from 32.8 to 98.4 mg/L) and was taken up again during the subsequent aeration period (decreased to 22.0 mg/L). In contrast, only 4.0 mg/L of P was released in Rp during the same non-aeration period.

Table 1 compares the P release and uptake rates during one typical operation cycle on day 60, day 120 and day 170, respectively. The anaerobic P release rate and aerobic P uptake rate of Ra-granules on day 60 were lower than those on day 120, indicating that PAOs might have been enriched after feeding Ra with acetate for 120 days. Probably due to the reactors were operated at a fixed influent organics concentration (~ 1000 mg COD/L) while much higher biomass concentration in Ra, the mature Ra-granules uptook much less DOC to realize its effective P release, about 15 mg C/g-VSS ħ on day 120 in comparison to 26 mg C/g-VSS ħ on day 60, respectively (Table 1). Future research work is still necessary for detailed information about the

influence of organic loading rate on the relationship between granular P release/uptake and DOC consumption in the granules.

Compared to Ra, Rp-granules exhibited much lower anaerobic P release and aerobic P uptake rates, especially its P release rate which decreased by 15% (from 0.74 to 0.63 mg P/g-VSS ħ) during the 120 days' operation. This observation is possibly associated with the metabolic shift from PAOs to glycogen-accumulating organisms (GAOs) occurred when using propionate as carbon source (Acevedo et al., 2012; Zhou et al., 2008). In the typical cycle test, glycogen variation was also recorded in both reactors (Fig. S2b). Higher glycogen (7.1 mmol C/g-VSS) was detected to accumulate in Rp-granules during the non-aeration period, implying that more GAOs existed in these granules. Interestingly, these GAOs did not exert much influence on the average P removal capacity of Rp-granules, which was averagely 8.4 mg P/g-VSS ɗ (9.4 mg P/g-VSS ɗ for Ra-granules). This observation suggests that some other P removal pathways like precipitation/adsorption might co-exist with PAOs uptake in this kind of wastewater treatment systems (Huang et al., 2015b; Li et al., 2015).

Previous studies suggest that propionate may be a more favorable substrate than acetate for PAOs to compete with GAOs (Chen et al., 2004; Oehmen et al., 2007; Wu et al., 2010), which is different from the finding of this work. This difference is most probably attributable to the different characteristics of seed sludge (activated sludge without PAOs enrichment), different characteristics of influent (such as lower COD/P ratio (20) and mixed carbon sources (acetate/propionate + glucose)), no addition of

nitrification inhibitor, and different operation strategy (1 h of non-aeration and 4.5 h of aeration) applied in this study. The followed-up experiments are designed to further clarify the above phenomenon.

After day 130, the Rp-granules were discarded and replaced by half of the Ra-granules and both reactors were operated under the same strategy as day 130 before. During the next 40 days' operation, propionate was clearly observed to have inhibition effect on PAOs due to lower P release and uptake rates detected in Rp' than those in Ra' under the same tested conditions (Table 1).

3.3. Difference in P fractionation and bioavailability in both granules

3.3.1. Granular P fractionation by SMT protocol

The P fractionation of Ra- and Rp- granules on day 120 and day 170 was performed by using SMT method (Fig. 3). In the seed sludge, Ra- and Rp- granules, inorganic P (IP) was the dominant P fraction occupying 83%, 78% and 67% of TP, of which apatite P (AP) was about 33%, 55% and 48%, respectively. Non-apatite inorganic P (NAIP) was 57%, 35% and 40% of TP in the seed sludge, Ra- and Rp-granules. Compared to the seed sludge, OP contents in Ra- and Rp-granules increased from initial 17% to 22% and 36% of TP on day 120, respectively. In Ra-granules with the highest TP content (78 mg P/g-SS on day 120), the bioavailability of P ((NAIP+OP)/TP) significantly decreased from 74% to 58%. More accumulation of AP into the Ra-granules might reduce the amount of synthesized poly-P available as

energy source for PAO metabolism (Li et al., 2015; Schönborn et al., 2001). In Rp-granules, the bioavailability of P ((NAIP+OP)/TP) was 76%, almost similar to the seed sludge (74%). Compared to the seed sludge, the bioavailable P content doubled in Ra-granules, which increased from initial 21 mg P/g-SS (seed sludge) to 45 mg P/g-SS after 120 days' operation. The bioavailable P content in Rp-granules, however, slightly increased to 28 mg P/g-SS under the same operation strategy.

Table 2 lists the average contents of dominant elements and metals in the seed sludge and granules on day 120 and day 170, respectively. As it can be seen, more cations were accumulated in Ra-granules, especially Ca, Mg and Fe which were almost 2-3 times those of Rp-granules. Rp-granules, however, contained higher contents of organic- C, N and H. Compared with Ra'-granules, Rp'-granules had decreased contents of metals except Fe. In this study, the bulk liquor pH value varied from 7.1 to 8.2 during the whole operation cycle. Under alkaline conditions, Ca, Mg and Fe ions can precipitate with PO_4^{3-} and become more stable ultimately, while other metals may bind with poly-P to form metal-P complexes (Li et al., 2015). In Ra, PO_4^{3-} concentration in the bulk liquor was about 300 mg/L at the end of non-aeration period, which was significantly higher than that in Rp (120 mg/L). In this study, Visual MINTEQ 3.0 was also used to calculate the saturation indices (SI) of possible precipitates formed at the end of non-aeration period. Results showed that $Ca_3(PO_4)_2$, $Ca_4H(PO_4)_3$ $3H_2O$, vivianite and hydroxyapatite were most probably formed in Ra due to their positive SI values (3.51, 1.699, 8.696 and 10.094, respectively). On the other

hand, only SI values of hydroxyapatite and vivianite were positive (about 3.966 and 5.969, respectively) in Rp, which were significantly lower than those of Ra. This observation indicates that these precipitates were possibly formed in the two reactors, especially at the end of non-aeration period, and more PO_4^{3-} was likely precipitated as NAIP or AP in Ra to form the core of granules (Huang et al., 2015b; Li et al., 2015).

3.3.2. Analysis of granular P species by ^{31}P NMR

In order to further determine the existing forms of P in granules, ^{31}P NMR was applied to identify and quantify P species with the results presented in Table 3 (in addition to Fig. S3). In the seed sludge, Ra- granules and Rp-granules, poly-P is the dominant P species, about 59%, 76% and 37% of TP, respectively, indicating acetate is more favorable for poly-P accumulation by PAOs than propionate under the tested conditions. In addition, about 1.3% and 4.4% poly-P were detected in the EPS extracts of Ra-granules and Rp-granules, respectively. As for ortho-P content, about 13% of TP was detected in Ra-granules, much lower than those in Rp-granules and the seed sludge (about 24% for both). In the EPS extracts, ortho-P was the dominant P species taking about 78% and 47% of TP in Ra- and Rp-granules, respectively.

From Table 3, monoester-P is the major form of organic P (OP) in all the sludge samples, approximately 15%, 6% and 34% of TP in the seed sludge, Ra-granules and Rp-granules, respectively. That is, Rp-granules had the highest monoester-P content among all the tested sludge samples. As it is known, monoester-P is mainly associated

with the production of nucleotides such as glycerol-6-phoshate (found in cell membrane), which could be used as a direct microbial signal. In this work, some amount of monoester-P was also detected in the EPS samples, about 2.0 mg P/g-SS in Ra-granule and 3.4 mgP/g-SS in Rp-granule extracts, which might come from the membrane of dead cells. Being closely related to deoxyribonucleic acid (DNA-P) and teichoic acid (Teichoic-P), diester-P was also detected in the EPS samples (about 0.4 mg P/g-SS in Ra-granule and 0.8 mg P/g-SS in Rp-granule extracts, respectively).

The TP_{EPS}/TP_{sludge} ratios for Ra- and Rp-granules were respectively 15% and 20%, higher than that of conventional EBPR sludge (6-7%) obtained by Zhang et al. (2013) using the same EDTA extraction method. This observation indicates that EPS plays an important role in P removal from wastewater by using AGS process. Based on previous works (Huang et al., 2015b; Li et al., 2015), phosphate adsorption by EPS of granular sludge and other mechanisms to some extent might also contribute to the stable and efficient P removal achieved in this study.

3.4. Changes in microbial community in granules from the two reactors

As seen from above experiments, the noticeable difference in P removal efficiency between Ra and Rp might be brought about by the different microbial communities gradually established in the two reactors under the designed operation conditions. Fig. 4 shows the results of main classes in these two kinds of granules sampled on day 120 based on 16S rDNA clone library analysis (Table S2). Results

indicate that the predominant bacteria in the granules mainly covered 10 phylums revealing higher bacterial diversity than those from sole carbon reactors (Guo et al., 2011; Zengin et al., 2010). That is, supplementary carbon source or mixed carbon source does favor and better the development of microbial diversity in wastewater treatment systems. In the Ra- and Rp- granules, the Bacteroidetes (34% and 27%), Proteobacteria (31% and 36%) and Firmicutes (14% and 15%) were the dominant three phylums that amounted to almost 80% species. Among them, the Bacteroidetes and Proteobacteria are responsible for high COD and NH_4-N removal capability and the β-proteobacteria (like Rhodocyclus) is regarded as an important group of PAOs (Crocetti et al., 2000; Gonzalez-Gil and Holliger, 2011; Huang et al., 2014). Although very low anaerobic phosphorus release was detected, more Rhodocyclus were found in Rp-granules (7.4%) than Ra-granules (5.9%), which might also contribute to some extent to the relatively high P removal capacity of Rp under the designed operation conditions. Previous research works reported that PAOs were able to behave as GAOs under some operation conditions (Acevedo et al., 2012; Zhou et al., 2008). The α- and γ- proteobacteria groups in Rp-granules (11.4%) were more than those in Ra-granules (8.6%). These proteobacteria have been reported to be more related with GAOs species (Crocetti et al., 2000; Zengin et al., 2010) which can accumulate glycogen without P release under anaerobic conditions. Restated, more Bacteroidetes (34%) and β-proteobacteria (16.3%) or other unclassified species in Ra-granules might contribute a lot to the stably high organics removal and P accumulation capacity of Ra fed with

acetate.

4. Conclusions

In this study acetate was found to favor P accumulation into aerobic granules during the treatment of synthetic fermentation liquor, achieving 78 mg P/g-SS in Ra-granules in comparison to 37 mg P/g-SS in Rp-granules after 120 days' operation. Besides, bioavailable P content in Ra-granules was detected to double that of seed sludge while slightly increased in Rp-granules under the same operation strategy. Ra and Rp possessed average P removal capability of 9.4 and 8.4 mg P/g-VSS·d, respectively. P release/uptake tests in addition to microbial biodiversity analysis revealed that more GAOs were existed in Rp-granules under the tested operation conditions.

Acknowledgements

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References

1. Acevedo, B., Oehmen, A., Carvalho, G., Seco, A., Borras, L., Barat, R., 2012. Metabolic shift of polyphosphate-accumulating organisms with different levels of polyphosphate storage. Water Res. 46, 1889-1900.
2. APHA, 2012. Standard Methods for the Examination of Water and Wastewater, American Public Health Association/American Water Work Association/Water Environment Federation, Washington D.C., USA.

3. Barker, D. J., Mannucchi, G. A., Salvi, S. M. L., Stuckey, D. C., 1999. Characterisation of soluble residual chemical oxygen demand (COD) in anaerobic wastewater treatment effluents. Water Res. 33, 2499-2510.
4. Chen, Y., Randall, A.A., McCue, T., 2004. The efficiency of enhanced biological phosphorus removal from real wastewater affected by different ratios of acetic to propionic acid. Water Res. 38, 27-36.
5. Cordell, D., Drangert, J.-O., White, S., 2009. The story of phosphorus: Global food security and food for thought. Global Environ. Chang. 19, 292-305.
6. Crocetti, G.R., Hugenholtz, P., Bond, P.L., Schuler, A., Keller, J., Jenkins, D., Blackall, L.L., 2000. Identification of polyphosphate-accumulating organisms and design of 16S rRNA-directed probes for their detection and quantitation. Appl. Environ. Microb. 66, 1175-1182.
7. Garrity, J., Gardner, J.G., Hawse, W., Wolberger, C., Escalante-Semerena, J.C., 2007. N-Lysine propionylation controls the activity of propionyl-CoA synthetase. J. Biol. Chem. 282, 30239-30245.
8. Gonzalez-Gil, G., Holliger, C., 2011. Dynamics of microbial community structure of and enhanced biological phosphorus removal by aerobic granules cultivated on propionate or acetate. Appl. Environ. Microb. 77, 8041-8051.
9. Guo, F., Zhang, S.H., Yu, X., Wei, B., 2011. Variations of both bacterial community and extracellular polymers: The inducements of increase of cell hydrophobicity from biofloc to aerobic granule sludge. Bioresour. Technol. 102, 6421-6428.
10. Herbert, D., Phipps, P.J., Strange, R.E., 1971. Chapter III Chemical Analysis of Microbial Cells. in: Methods in Microbiology, (Eds.) J.R. Norris, D.W. Ribbons, Volume 5, Part B, Academic Press, pp. 209-344.
11. Huang, H., He, L., Lei, Z., Zhang, Z., 2015. Contribution of precipitates formed in fermentation liquor to the enhanced biogasification of ammonia-rich swine manure by wheat-rice-stone addition. Bioresour. Technol. 175, 486-493.
12. Huang, W., Huang, W., Li, H., Lei, Z., Zhang, Z., Tay, J.H., Lee, D.-J., 2015a. Species and distribution of inorganic and organic phosphorus in enhanced phosphorus removal aerobic granular sludge. Bioresour. Technol. 193, 549-552.
13. Huang, W., Cai, W., Huang, H., Lei, Z., Zhang, Z., Tay, J.H., Lee, D.-J.. 2015b. Identification of inorganic and organic species of phosphorus and its bio-availability in nitrifying aerobic granular sludge. Water Res. 68, 423-431.

14. Huang, W., Wang, W., Shi, W., Lei, Z., Zhang, Z., Chen, R., Zhou, B., 2014. Use low direct current electric field to augment nitrification and structural stability of aerobic granular sludge when treating low COD/NH_4-N wastewater. Bioresour. Technol. 171, 139-144.
15. Huang, W., Huang, W., Yuan, T., Zhao, Z., Cai, W., Zhang, Z., Lei, Z., Feng, C., 2016. Volatile fatty acids (VFAs) production from swine manure through short-term dry anaerobic digestion and its separation from nitrogen and phosphorus resources in the digestate. Water Res. 90, 344-353.
16. Ji, Z., Chen, Y., 2010. Using sludge fermentation liquid to improve wastewater short-cut nitrification-denitrification and denitrifying phosphorus removal via nitrite. Environ. Sci. Technol. 44, 8957-8963.
17. Lin, L., Wan, C., Liu, X., Lei, Z., Lee, D.-J., Zhang, Y., Tay, J.H., Zhang, Z., 2013. Anaerobic digestion of swine manure under natural zeolite addition: VFA evolution, cation variation, and related microbial diversity. Appl. Microbiol. Biot., 97(24), 10575-10583.
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 Res. 86, 85-95.
19. Lin, Y.M., Liu, Y., Tay, J.H., 2003. Development and characteristics of phosphorus-accumulating microbial granules in sequencing batch reactors. Appl. Microbiol. Biot. 62, 430-435.
20. Medeiros, J.G., Cid, B.P., Gómez, E.F., 2005. Analytical phosphorus fractionation in sewage sludge and sediment samples. Anal. Bioanal. Chem. 381, 873-878.
21. Möller, K., Müller, T., 2012. Effect of anaerobic digestion on digestate nutrient availability and crop growth: A review. Eng. Life Sci. 12, 242-257.
22. Nereda, 2016 http://www.royalhaskoningdhv.com/en-gb/nereda/about-nereda/downloads-and-pr (accessed 8th March 2016)
23. Oehmen, A., Lemos, P.C., Carvalho, G., Yuan, Z.G., Keller, J., Blackall, L.L., Reis, M.A.M., 2007. Advances in enhanced biological phosphorus removal: From micro to macro scale. Water Res. 41, 2271-2300.
24. Oehmen, A., Teresa Vives, M., Lu, H., Yuan, Z., Keller, J., 2005. The effect of pH on the competition between polyphosphate-accumulating organisms and glycogen-accumulating organisms. Water Res. 39, 3727-3737.

25. Pronk, M., de Kreuk, M.K., de Bruin, B., Kamminga, P., Kleerebezem, R., van Loosdrecht, M.C.M., 2015. Full scale performance of the aerobic granular sludge process for sewage treatment. Water Res. 84, 207-217.
26. Schönborn, C., Bauer, H.-D., Röske, I., 2001. Stability of enhanced biological phosphorus removal and composition of polyphosphate granules. Water Res. 35, 3190-3196.
27. Solovchenko, A., Verschoor, A. M., Jablonowski, N. D., Nedbal, L., 2016. Phosphorus from wastewater to crops: An alternative path involving microalgae. Biotechnol. Adv., http://dx.doi.org/10.1016/j.biotechadv.2016.01.002.
28. Sun, M., Li, W.-W., Yu, H.-Q., Harada, H., 2012. A novel integrated approach to quantitatively evaluate the efficiency of extracellular polymeric substances (EPS) extraction process. Appl. Microbiol. Biot. 96, 1577-1585.
29. Teo, K.-C., Xu, H.-L., Tay, J.-H., 2000. Molecular mechanism of granulation. II: proton translocating activity. J. Environ. Eng. 126, 411-418.
30. Tong, J., Chen, Y., 2009. Recovery of nitrogen and phosphorus from alkaline fermentation liquid of waste activated sludge and application of the fermentation liquid to promote biological municipal wastewater treatment. Water Res. 43, 2969-76.
31. Wu, C.-Y., Peng, Y.-Z., Wang, S.-Y., Ma, Y., 2010. Enhanced biological phosphorus removal by granular sludge: From macro- to micro-scale. Water Res. 44, 807-814.
32. Yuan, Q., Sparling R., Oleszkiewicz, J.A., 2009. Waste activated sludge fermentation: Effect of solids retention time and biomass concentration. Water Res. 43, 5180-5186.
33. Zengin, G.E., Artan, N., Orhon, D., Chua, A.S.M., Satoh, H., Mino, T., 2010. Population dynamics in a sequencing batch reactor fed with glucose and operated for enhanced biological phosphorus removal. Bioresour. Technol. 101, 4000-4005.
34. Zhang, H.-L., Fang, W., Wang, Y.-P., Sheng, G.-P., Xia, C.W., Zeng, R.-J., Yu, H.-Q., 2013. Species of phosphorus in the extracellular polymeric substances of EBPR sludge. Bioresour. Technol. 142, 714-718.
35. Zhou, Y., Pijuan, M., Zeng, R.J., Lu, H., Yuan, Z., 2008. Could polyphosphate-accumulating organisms (PAOS) be glycogen-accumulating organisms (GAOs)? Water Res. 42, 2361-2368.

Tables

Table 1

Changes in anaerobic P release and aerobic P uptake rates in the two SBRs during 6 months' operation (unit: mg/g-VSS ħ).

Reactor	Day 60			Day 120			Day 170		
	Anaerobic P release rate	Aerobic P uptake rate	Anaerobic DOC uptake rate	Anaerobic P release rate	Aerobic P uptake rate	Anaerobic DOC uptake rate	Anaerobic P release rate	Aerobic P uptake rate	Anaerobic DOC uptake rate
Ra	2.02	0.75	26.12	6.88	1.78	15.32	7.41	1.89	25.0
Rp	0.74	0.42	27.26	0.63	0.40	23.73	5.74	1.41	26.53

All data are the average values of three tests.

Table 2

Changes in average element contents in the seed sludge, Ra- , Rp-, Ra'- and Rp'-granules on day 120 and day 170 (unit: mg/g-SS).

Sample	Organic-C	Organic-N	Organic-H	Na	K	Mg	Ca	Fe	Mn	Al
Seed sludge	375.6	74.0	64.4	4.53	3.21	4.19	13.27	3.59	0.17	0.33
Ra-granule	289.0	64.2	55.2	8.04	14.39	20.67	52.32	11.69	0.76	2.28
Rp-granule	379.5	78.4	66.3	5.92	7.73	7.40	22.45	7.35	0.43	1.29
Ra'-granule	285.4	65.4	56.0	7.27	11.41	14.36	81.12	8.73	0.38	1.19
Rp'-granule	321.7	73.6	60.5	5.89	6.72	12.02	67.29	13.47	0.33	1.37

Table 3

Contents of different P fractions extracted from the granules on day 120 by using PCA + NaOH method followed by ^{31}P NMR spectroscopy.

Sample	TP	IP			OP	
	(mg/g-SS)	Ortho-P (%TP)	Pyro-P (%TP)	Poly-P (%TP)	Monoester-P (%TP)	Diester-P (%TP)
Seed sludge	29.0	24.6	N.D.	59.3	14.7	1.4
Ra-granules	77.5	12.8	0.5	76.4	6.4	3.8
Rp-granules	43.4	24.1	0.3	37.1	34.1	4.0
Ra-granule EPS	11.4	77.7	N.D.	1.3	17.4	3.7
Rp-granule EPS	8.8	47.3	N.D.	4.4	39.1	9.2

N.D., not detectable.

Figures

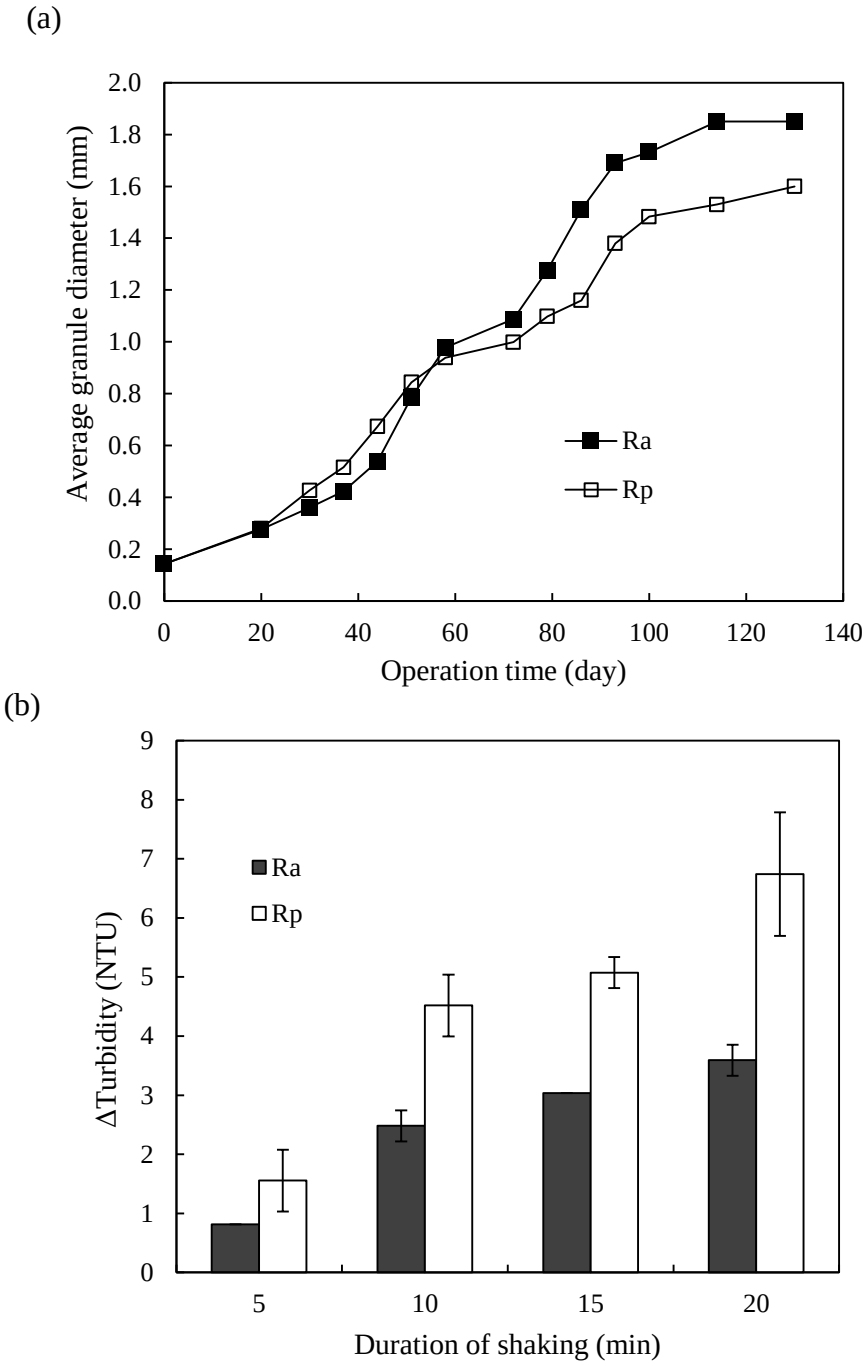
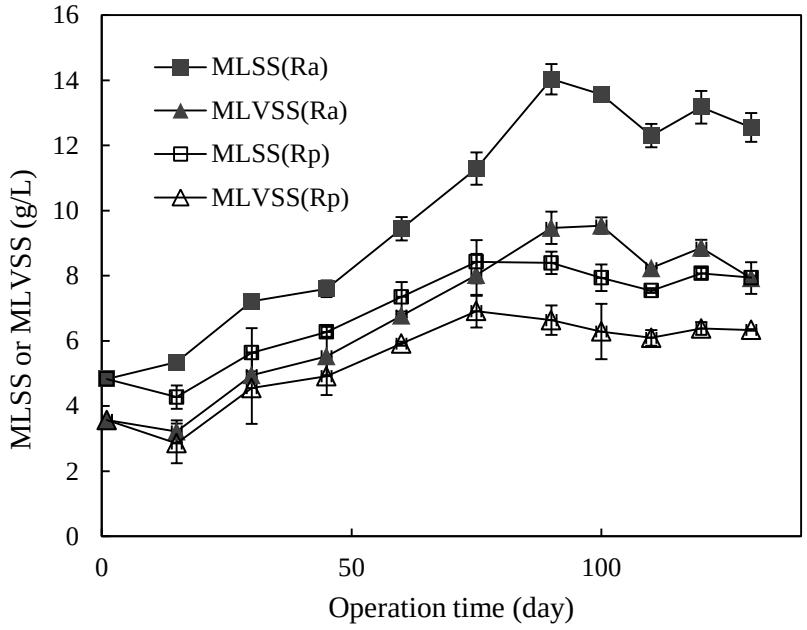
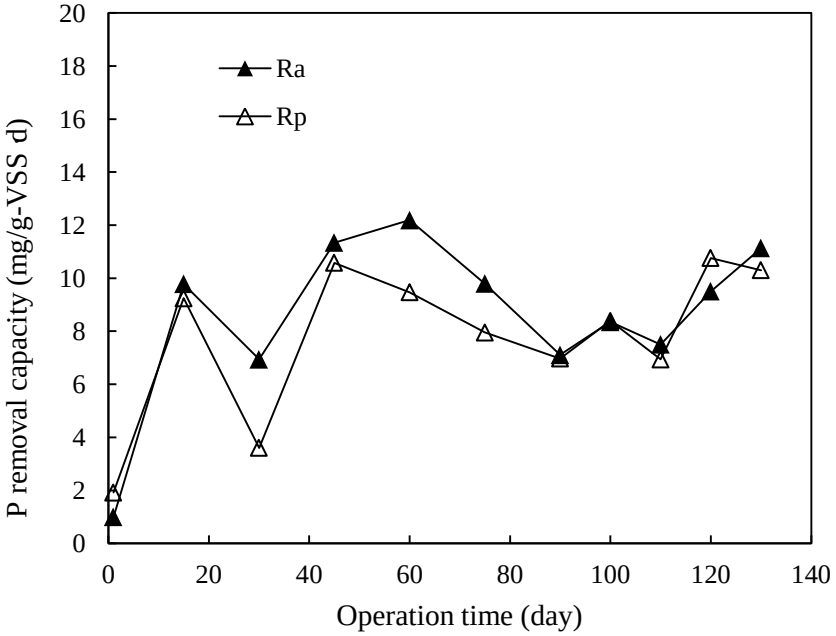


Fig. 1. Variation in granular size during 130 days' operation (a) and granular strength on day 120 (b). Ra, SBR fed with glucose and acetate; Rp, SBR fed with glucose and propionate.

(a)


MLSS(Ra)
MLVSS(Ra)
MLSS(Rp)
MLVSS(Rp)
MLSS or MLVSS (g/L)
Operation time (day)
0
2
4
6
8
10
12
14
16
0
50
100

(b)


Ra
Rp
P removal capacity (mg/g-VSS d)
Operation time (day)
0
2
4
6
8
10
12
14
16
18
20
0
20
40
60
80
100
120
140

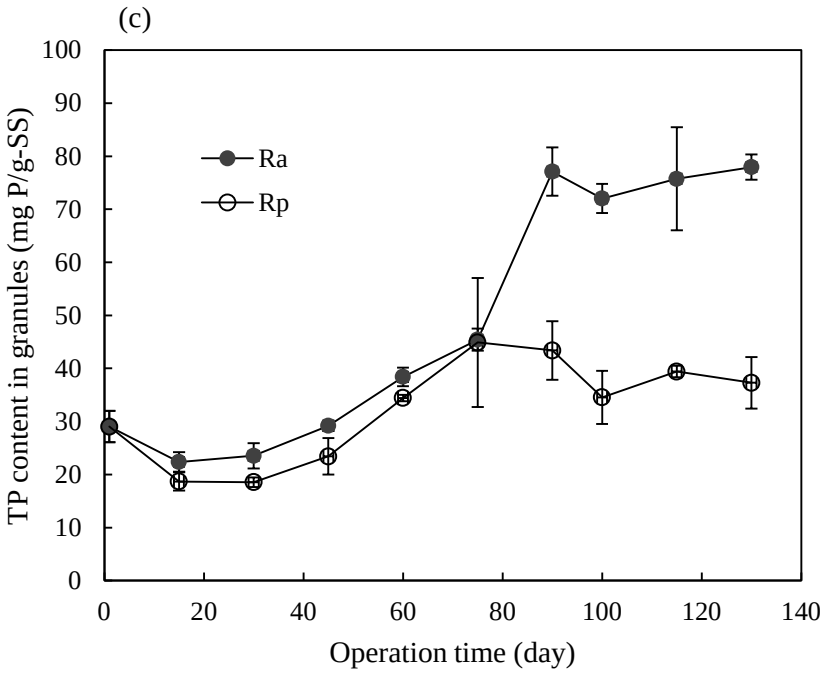


Fig. 2. Changes in MLSS, MLVSS (a), P removal capacity (b) and granular P content (c). Filled symbols refer to Ra (the SBR fed with glucose and acetate), and open symbols refer to Rp (the SBR fed with glucose and propionate).

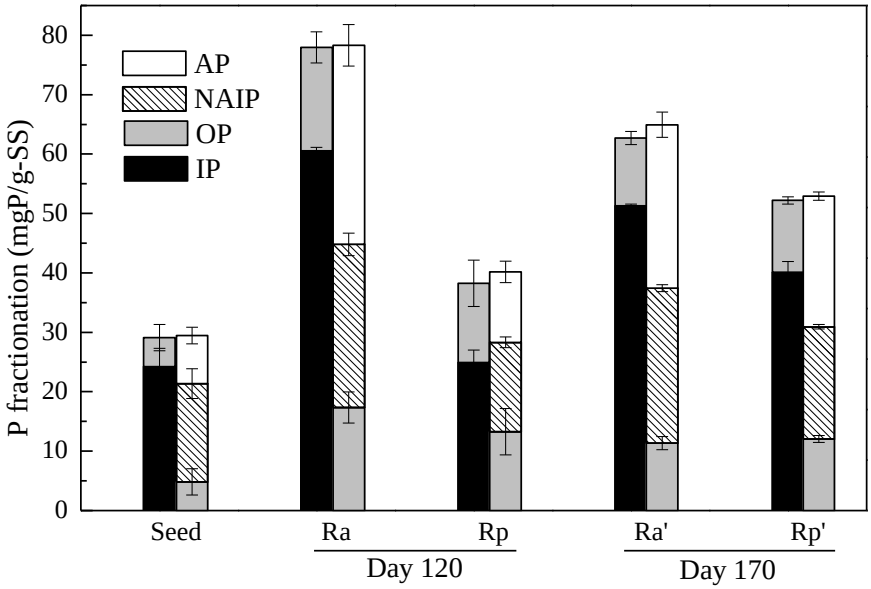


Fig. 3. Phosphorus species in different sludge samples measured by SMT method on day 120 (Ra and Rp) and day 170 (Ra’ and Rp’), respectively.

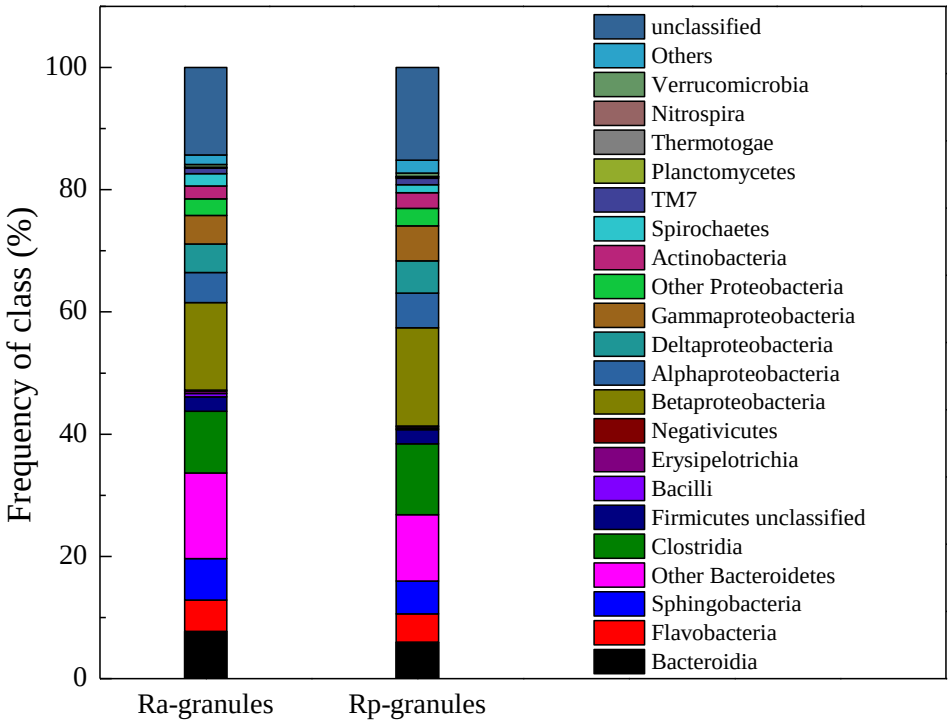


Fig. 4. Abundance of main classes in Ra- and Rp-granules on day 120.

Supporting Information

Acetate favors more phosphorus accumulation into aerobic granular sludge than propionate during the treatment of synthetic fermentation liquor

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Table S1

DOC, TN, and TP removal efficiencies during the operation period.

Items	Ra						Rp					
	Before day 130			After day 130			Before day 130			After day 130		
	Max	Min	**Avg**	Max	Min	**Avg**	Max	Min	**Avg**	Max	Min	**Avg**
DOC removal (%)	99.8	92.5	**97.0**	99.8	93.2	**96.9**	97.8	93.1	**95.8**	98.3	94.9	**97.1**
TN removal (%)	95.5	55.5	**78.2**	98.3	60.7	**78.0**	84.8	72.1	**79.1**	85.8	63.2	**77.5**
TP removal (%)*	81.3	37.5	**63.0**	71.7	49.0	**61.4**	63.3	29.4	**44.3**	81.0	48.7	**64.6**

* Data after day 40.

Table S2

Composition of the microbial community in the SBR biomass samples on day 120 obtained from 16S rDNA clone library.

Phylum	Class	Ra-granules (% of total clones)	Rp-granules (% of total clones)
Bacteroidetes	Bacteroidia	7.7	6.0
	Flavobacteria	5.1	4.6
	Sphingobacteria	6.8	5.4
	Others	14.0	10.8
Firmicutes	Clostridia	10.1	11.6
	Bacilli	0.6	0.2
	Erysipelotrichia	0.4	0.3
	Negativicutes	0.2	0.2
	Others	2.3	2.3
Proteobacteria	Betaproteobacteria	16.3	16.1
	Alphaproteobacteria	4.4	5.7
	Deltaproteobacteria	4.2	5.3
	Gammaproteobacteria	4.2	5.7
	Others	2.2	2.9
Actinobacteria	Actinobacteria	2.1	2.6
Spirochaetes	Spirochaetes	2.0	1.3
TM7	TM7	0.9	1.1
Verrucomicrobia	Opitutae	0.5	0.5
Planctomycetes	Phycisphaerae	0.0	0.2
Thermotogae	Thermotogae	0.1	0.1
Nitrospira	Nitrospira	0.1	0.1
Unclassified		15.8	17.3

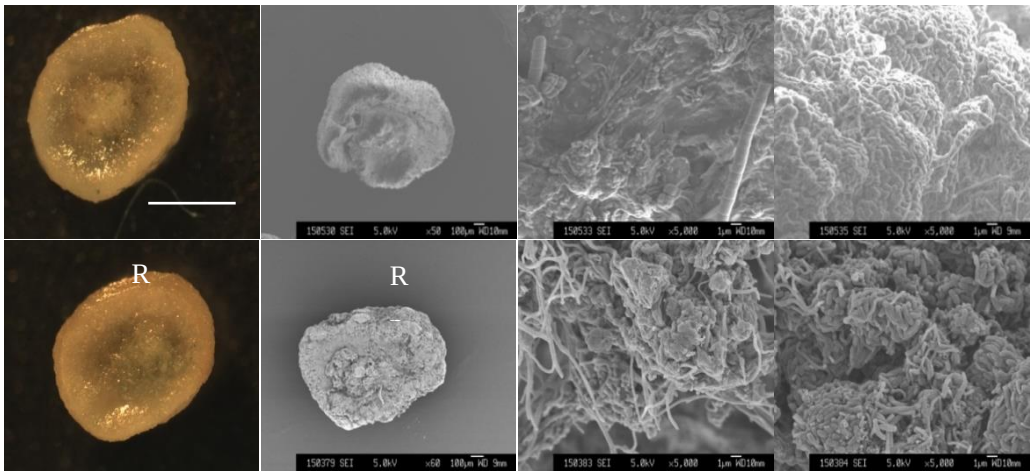

Fig. S1 Digital and SEM images of Ra-, Rp- granules on day 120. Ra and Rp, the cross section of the granule. Ba and Bp, the core; and Ca and Cp, the edge of the granule.

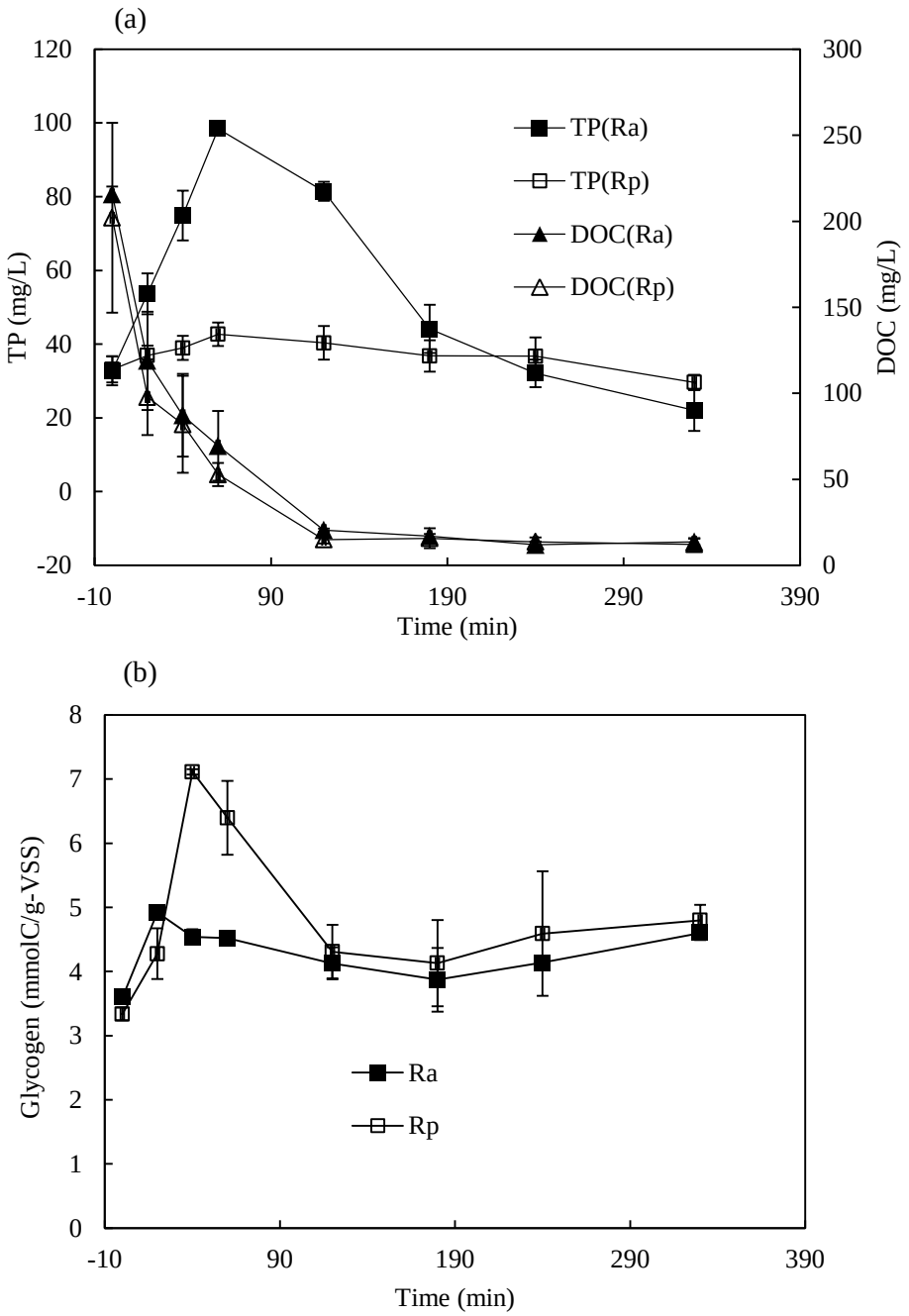

Fig. S2. Variations of TP, DOC and glycogen during a typical operation cycle on day 120.

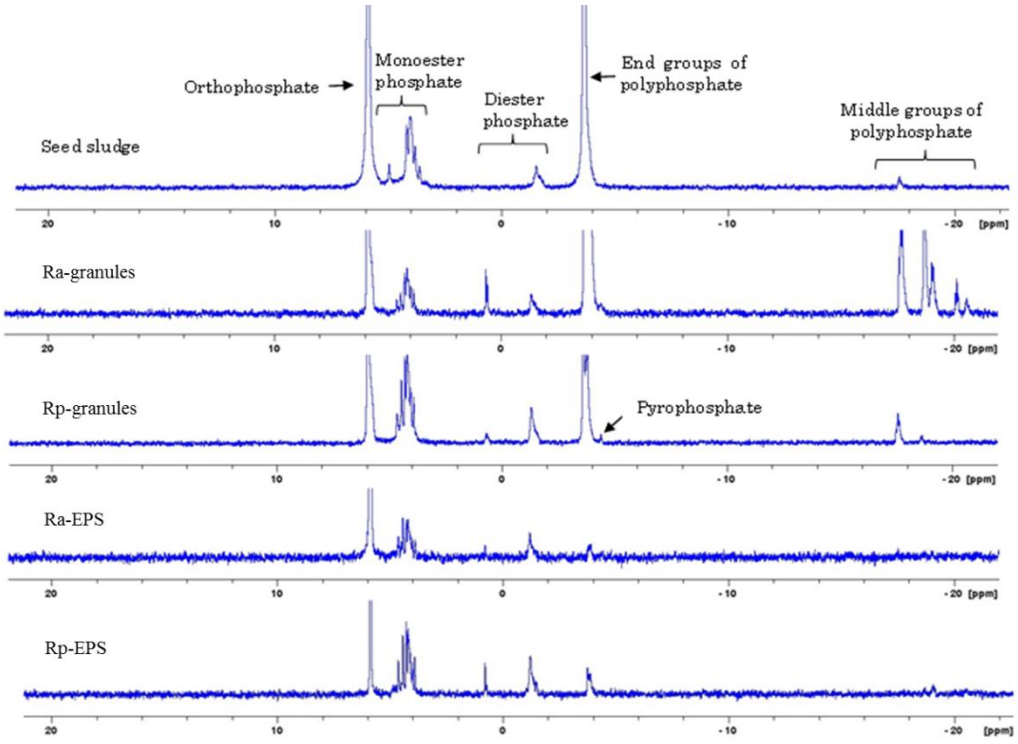

Fig. S3. Typical ^{31}P NMR spectra of PCA + NaOH extracts from the seed sludge and granular sludges on day 120