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RESEARCH ARTICLE

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# Effect of hydrodynamic conditions on the formation and structure of aerobic granular sludge performing enhanced biological phosphorus removal

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## Abstract

The study investigated the effect of hydrodynamic conditions on the formation of enhanced biological phosphorus removal (EBPR) granular sludge without losing the EBPR capacity, and its structure. The effect of hydrodynamic conditions on the formation of EBPR granular sludge was evaluated by using the minimum settling velocity, $(V_s)_{min}$, and superficial air velocity. The application of $(V_s)_{min}$ strategy was proved to be beneficial in maintaining granular biomass without losing the EBPR capacity. Microscale structure, the chemical composition and chemically stored phosphorus of the matured EBPR granular sludge were determined by using ESEM-EDX and cold perchloric acid (PCA) analyses. The results of both the ESEM-EDX and PCA analyses proved that the poly-P granules were stored extracellular in EPS structure of EBPR granular sludge. Therefore, the EBPR granular sludge has a high potential for both removal and recovery of phosphorus from wastewater, which needs to be investigated in future studies.

**KEYWORDS**
aggregation, batch processing, biokinetics, EBPR granular sludge, settling time, waste-water treatment

## 1 | INTRODUCTION

Aerobic granular sludge technology has recently been developed as an efficient alternative for conventional activated sludge process (de Bruin et al., 2004). Numerous factors affecting aerobic granular sludge formation were investigated extensively such as settling time, hydrodynamic shear force, feast–famine regime, organic loading rate, reactor configuration, and substrate composition (Beun et al., 2002; Liu & Tay, 2002; Qin et al., 2004; Tay et al., 2001a, 2001b, 2004; Wang et al., 2006). Liu, Wang et al. (2005) pointed out that the settling time as the main hydraulic selection pressures can significantly lead to aerobic granulation, and the other factors mentioned above have an effect on the characters of aerobic granular sludge including stability, compactness, and microbial population but may not individually trigger aerobic granulation. Normally, the short settling time was recommended to obtain fully aerobic granular in aerated systems (Qin et al., 2004). However, the nutrients removal capability of the fully granular sludge system was probably influenced because of excessive flocculent sludge washout. For example, both experimental evidences and theoretical model analysis showed the nitrification rate of pure aerobic granules were less than that of aerobic granules mixed with flocs (Filali et al., 2012). Furthermore, Winkler et al. (2012) explained that aerobic granules coexisted with flocs in a single reactor have various microbial population distribution and thus improved nutrients removal.

The enhanced biological phosphorus removal (EBPR) process is adapted as an effective biological method for phosphorus removal from wastewater (Mino et al., 1998). Previous studies reported that EBPR granular sludge could be formed at different settling times of 3, 5, 15, 30, 35, and 40 min (de Kreuk & van Loosdrecht, 2004; Dulekgurgen et al., 2003; Lang et al., 2015; Lin et al., 2003; Wu et al., 2012). Although the settling time was defined as the key

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parameter for aerobic granular formation in fully aerobic systems, it seemed that the settling time applied in aerobic EBPR granular sludge system was not a decisive parameter for granulation. In other words, the effect of the settling time on EBPR granular sludge system was not obvious because phosphate-accumulating organism (PAOs) as the dominant bacteria in EBPR are proved to be beneficial for the granules formation (de Kreuk & van Loosdrecht, 2004). Moreover, previous studies showed that the positive charged particles formed during the anaerobic period with the release of phosphorus in EBPR process could stimulate the formation of subgranules by attracting negative charged bacteria or clusters (Wu et al., 2010, 2012). This indicated that the formation mechanism of EBPR granular sludge may differ from that of other aerobic granular sludge. Additionally, during the EBPR process, it was observed that phosphorus precipitated with a range of cations presented in wastewater, that is, multivalence metal ions, such as calcium and magnesium, binding with phosphate to form different chemical precipitates (e.g., struvite and hydroxyapatite), which is believed to improve the stability of matured EBPR granules (Huang et al., 2015; Lin et al., 2012; Mañas et al., 2011).

Although the settling time was evaluated for the formation of aerobic granular sludge, the short settling time led to the inefficient nutrient removal performance. Additionally, the effects of hydrodynamic conditions on EBPR granules formation and the characterization of matured EBPR granules have rarely been studied. Therefore, the aims of this work were to investigate the role of hydrodynamic conditions on the EBPR granular sludge system without losing the EBPR capacity, and its structure. In addition, a combination of environmental scanning electron microscopy (ESEM) connected with energy dispersive X-ray (EDX) and the cold perchloric acid (PCA) extraction procedure were applied to characterize matured EBPR granule sludge.

## 2 | MATERIALS AND METHODS

### 2.1 | Experimental set-up

The experiment was carried out in a lab-scale sequencing batch reactor (SBR) during the period of over 8 months. The SBR had a working volume of 5 L with a working height of 44 cm and a diameter of 12 cm. H/D ratio was 3.7, which the H/D ratios in the range of 4 to 24 were reported as an insignificant effect on granulation (Kong et al., 2009). Effluent was discharged 19 cm above the reactor bottom with a volumetric exchange ratio of 60%. The reactor was operated in the cycle consisted of feeding (10 min), anaerobic phase (45 min), aerobic phase (120–175 min), and settling and decanting (10 min). The aerobic phase was reduced according to the improvement in the nitrogen and phosphorus removal efficiency. The settling time was firstly decreased to 5 min depending on the improvement in the sludge settling properties (Phase I) and then gradually reduced from 5 to 1 min to investigate the effect of settling velocity on granulation process without losing EBPR capacity (Phase II) as seen in Table 1. The programmable logic controller (PLC) was used to control the sequential operations of the reactors. The cycle times of 6, 4, and 3 h were applied corresponding the hydraulic residence times (HRTs) of 10, 6.7, and 5 h, respectively. A magnetic stirring (100 rpm) and a fine air bubble aerator were used to mix the reactor. Air was supplied at a flow rate of 300 L/h (superficial air velocity [SAV] = 0.78 cm/s) and 800 L/h (SAV = 1.96 cm/s). Dissolved oxygen (DO) and pH were monitored but not controlled, which were in the range of 8–8.5 mg $O_2$/L and 6.8–7.7. The reactor was operated at ambient temperature (controlled at 25 ± 5°C). The operating conditions were summarized in Table 1.

The synthetic wastewater used in this study composed of (mg/L): sodium acetate trihydrate (435), glucose (104), peptone (100), meat extract (64), starch (75), urea (42), $NH_4Cl$ (114), $K_2HPO_4$ (39.3), $KH_2PO_4$ (15.9), $MgSO_4{\cdot}7H_2O$ (50), $CaCl_2{\cdot}2H_2O$ (34), and $FeSO_4{\cdot}7H_2O$ (15), and a trace element solution was added 0.3 ml/L. The composition (g/L) of the trace element solution was as follows: $EDTAH_2Na_2{\cdot}H_2O$ (52.5), $ZnSO_4{\cdot}7H_2O$ (2.2), $MnCl_2{\cdot}7H_2O$ (3.97), $(NH_4)_6MoO_{24}{\cdot}H_2O$ (1.21), $CuSO_4{\cdot}5H_2O$ (1.57), and $CoCl_2{\cdot}6H_2O$ (1.61) (all chemicals were purchased from Merck Co.). Initial substrate concentrations for the influent were 650 mg COD/L, of 74 mg TN/L, and of 19 mg TP/L. The reactor was inoculated with floccular sludge taken from a municipal wastewater treatment plant in Istanbul. The SVI and MLSS/MLVSS ratio of seed sludge were 77 ml/g and 52%, respectively.

### 2.2 | Analytical methods

COD, ammonia ($NH_4^+$-N), nitrite ($NO_2^-$-N), total phosphorus (TP), orthophosphate ($PO_4^{3-}$-P), mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS), and sludge volume index (SVI) were analysed in accordance to Standard Methods for the Examination of Water and Wastewater (1999). Nitrate ($NO_3^-$-N) was determined with Nitrate test kit (Merck 1.14773). Volatile fatty acids (VFA) was measured by gas chromatography (GC) (Agilent Technologies-6890N) with column DB-FFAP 30 m × 0.53 mm × 1.0 μm (length × internal diameter × film) at 100°C while the injector and FID detector were operated at 260°C. Nitrogen was used as carrier gas at a flow rate of 10 ml/min; 0.9 ml of the filtered sample was transferred into a GC vial to which 0.1 ml of 10% phosphoric acid was added. The particle size was measured by a laser particle size analysis system (Mastersizer 2000, Malvern

**TABLE 1** Operating condition during the experiment

| Operational parameter | Start-up (0–39 days) | Phase I (40–97 days) | Phase II (98–250 days) |
|---|---|---|---|
| Settling time per cycle, $T_s$ (min) | 30-20 | 5 | 5-3-1 |
| Organic loading rate, OLR (kg COD/$m^3$d) | 1.2 | 1.8–2.4 | 2.4 |
| Superficial air velocity, SAV (cm/s) | 0.78 | 0.78 | 1.96 |

Instruments, UK) with a range of 0.02–2000 μm. System performance was monitored with samples taken at the end of the cycle three times a week, and cycle studies were performed weekly. To evaluate the fractions of chemically stored phosphorus in aerobic granules at the end of anaerobic or aerobic phase, the cold perchloric acid (PCA) extraction procedure was used (de Haas et al., 2000). The biomass samples collected from the reactor were homogenized by using ultra turax in the cold bath. The samples were centrifuged at 2000 *g* for 5 min, and the supernatant was separated. This was labelled as "SUP" and retained; 10 ml of 0.9% (m/v) NaCl was added to the pellet and resuspended. Subsequently, the sample was centrifuged at 2000 *g* for 5 min. The collected supernatant was added to the SUP labelled tube. Afterwards, the pellet was extracted three times with ice-cold 0.5-M PCA (20 ml) in a refrigerated water bath. Between extractions, the sample was centrifuged at 5°C and the collected supernatant after each centrifuge combined into one tube and labelled as "PCA." The pellet was resuspended by 50 ml of distilled water, and this was labelled as "RES." Orthophosphate and total phosphorus were analysed in both the SUP and PCA extract. The complex bio-phosphorus was calculated from the difference between the total phosphorus and the orthophosphate.

The microscopic observation was applied to the granular sludge samples that were first stained using Sudan Black stain at the end of anaerobic or aerobic phase and then investigated by bright field microscope (Olympus, CH30). To further visualize the structure of aerobic granules at a microscale level, ESEM (Philips XL30 ESEM) was employed without prior specimen preparation. Energy dispersive X-ray (EDX) analysis was used to obtain a quantitative analysis of the granule surface. For ESEM and EDX analysis, the biomass was harvested directly from the reactor and was kept at 4°C until analysis.

### 2.3 | Analysis of extracellular polymeric substances

The extracellular polymeric substances (EPS) were extracted by using the method proposed by Frølund et al. (1996). EPS was extracted by using DOWEX cation exchange resin; 0.5-g MLVSS were subjected to the 35-g DOWEX cation exchange resin. The MLVSS concentration of the sample was calculated by the MLVSS concentration of the sampling cycle. The extraction was carried on a magnetic stirrer, which was adjusted to 750 rpm, for 4 h. The protein content in EPS was determined using the modified Lowry method with bovine serum albumin as the standard (Walker, 1996). The polysaccharide content in EPS was measured using the Dubois method with alginic acid as the standard (Dubois et al., 1956).

## 3 | RESULTS AND DISCUSSION

### 3.1 | EBPR system performance

The ammonium and phosphate concentrations in the effluent and released phosphate concentrations at the end of anaerobic phase of every cycle were measured during the whole operating period (Figure 1). During the start-up period, the released phosphate at the end of anaerobic phase gradually increased and reached a value of 45 mg P/L, and phosphate and ammonium in the effluent was maintained at the very low level (less than 1 mg/L). Generally, the removal efficiencies of $PO_4^{3-}$-P and $NH_4^+$-N were over 97% except on the 119th, 152nd, and 187th days when the automation problems occurred that the magnetic stirrer did not switch off during the settling and decanting period.

A typical SBR cycle on Day-208 was illustrated in Figure 2. During anaerobic phase, the released phosphate increased and reached around 45 mg P/L, with a decrease of VFA. During the aerobic phase, the released phosphate was completely removed within 120 min, and the complete nitrification occurred at the same time. The layered structure of aerobic granular sludge allows the presence of an aerobic outer-layer and an anaerobic or anoxic core, where simultaneous nitrification and denitrification (SND) can be achieved, and SND was reported as the main nitrogen removal pathway in a full scale aerobic granular sludge system (Pronk et al., 2015). SND

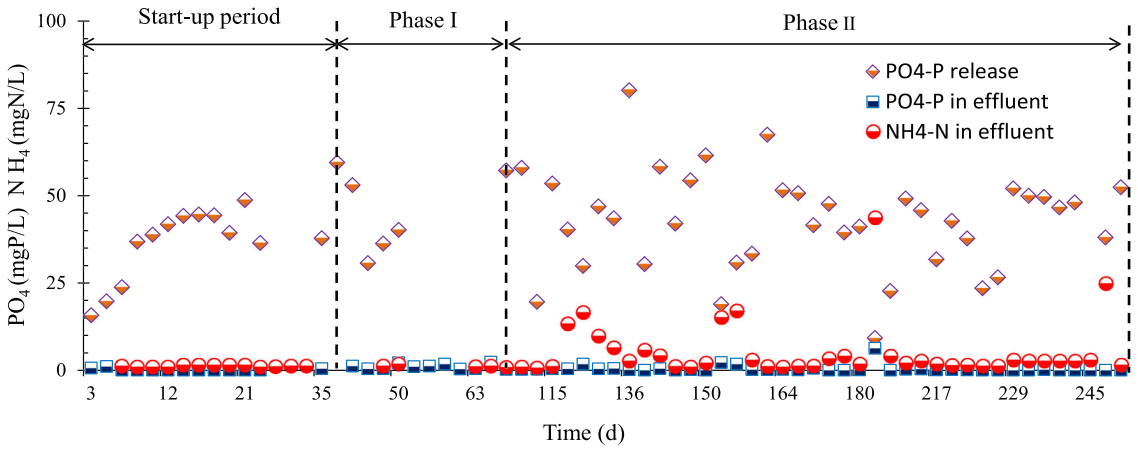



**FIGURE 1** $PO_4$-P and $NH_4$-N concentrations in the effluent, and $PO_4$-P concentrations at the end of anaerobic phase

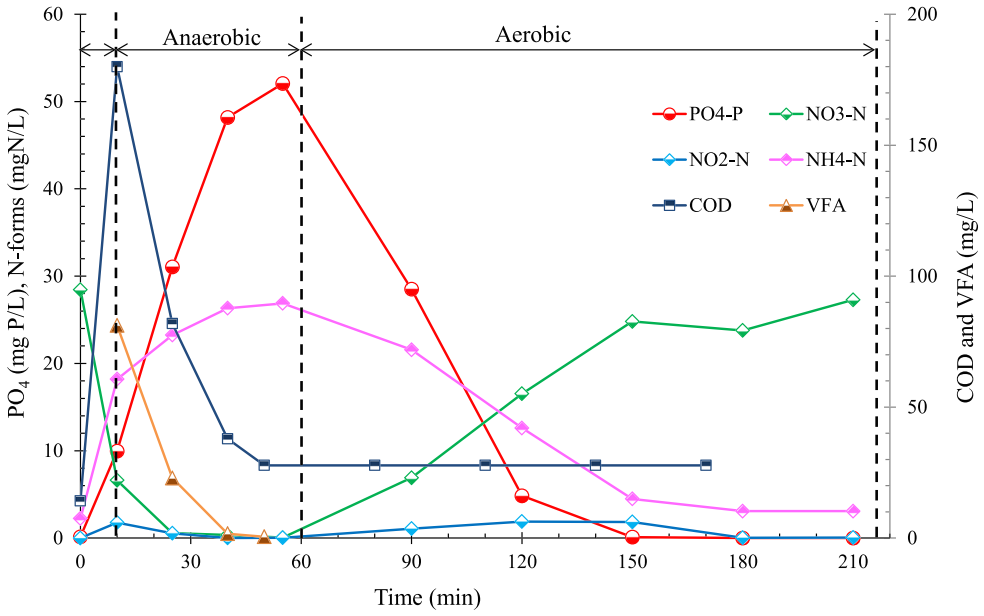



**FIGURE 2** A typical SBR cycle performed on Day-208; the two dotted lines delimit the feeding phase (10 min), the anaerobic phase (45 min) and the aerobic phase (135 min)

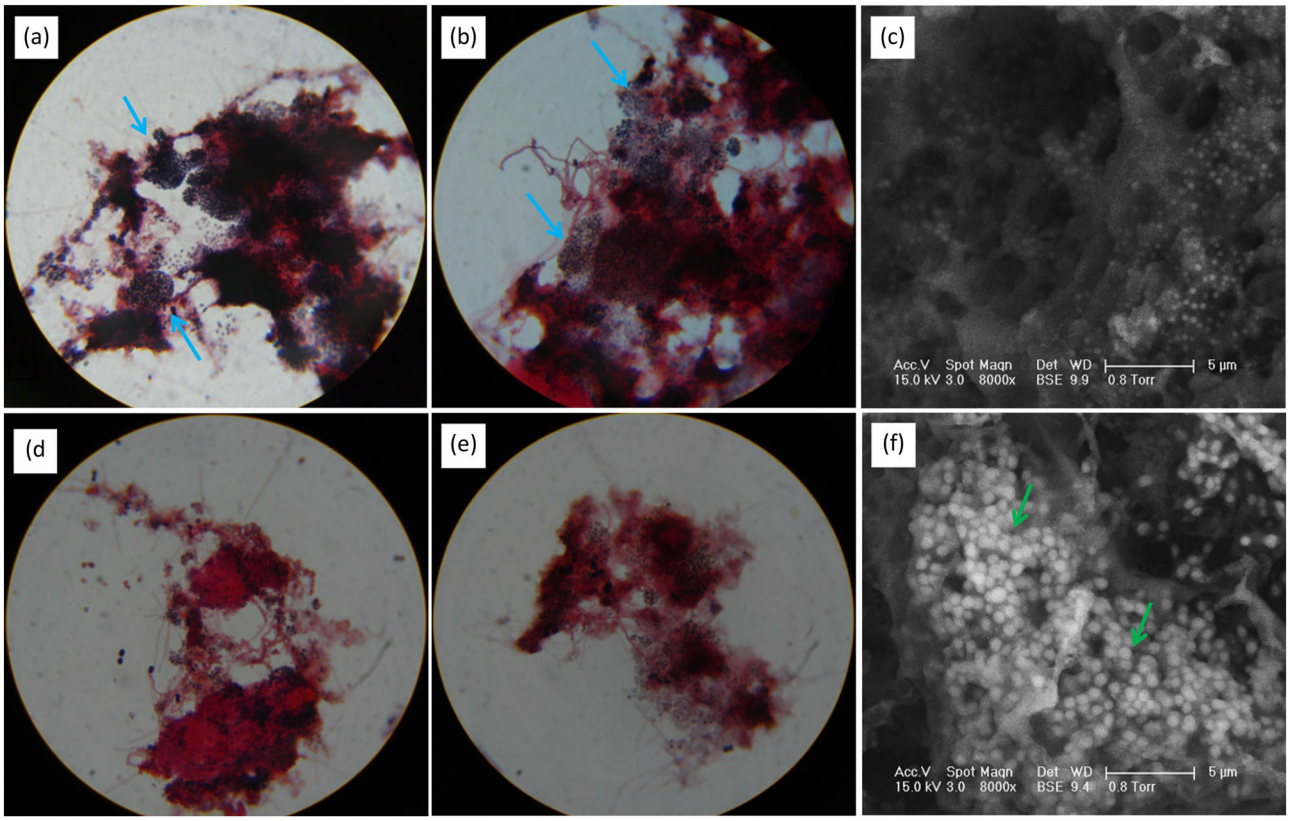



**FIGURE 3** Microscopic observation of the matured EBPR biomass at the end of the anaerobic phase (a–c) and at the end of the aerobic phase (d–f); a, b, d, and e were bright field micrographs stained by Sudan black stain (blue arrow indicated black granules were stained PHA); c and f were ESEM photos; green arrow indicated the presence of poly-P granules with white colour in the cells

was also observed in the presented system and evaluated in detail (Insel et al., n.d.).

Figure 3 underlines the variation of biomass in the EBPR system by visualizing PHA or poly-P granules in the cells at different phases. At the end of the anaerobic phase, PHA granules stained as blue-black granules were observed in Figure 3a,b, while disappeared at the end of the aerobic phase. Poly-P granules were observed in cells at the end of the aerobic phase (Figure 3f). The results clearly demonstrated that the enrichment of PAOs in the EBPR system.

## 3.2 | Formation of EBPR granular sludge

### 3.2.1 | Start-up period

The SBR inoculated with flocculent sludge and was acclimated to synthetic wastewater for 39 days. Most of the EBPR granular sludge reactors in previous literatures were fed with the easily degradable sole carbon source synthetic wastewater. In similar studies, the carbon sources used were acetate (Dulekgurgen et al., 2003; Liu et al., 2003), propionate (Wu et al., 2010), and glucose (Li et al., 2012). In this study, the synthetic domestic wastewater containing mixed carbon sources such as glucose, acetate, starch, peptone, and meat extract was fed to the reactor, in which the addition of acetate was used to enhance the biological phosphorus removal, while acetate (200 mg COD/L) and orthophosphate (10 mg P/L) in the influent was not as high as in previous studies (Dulekgurgen et al., 2003; de Kreuk, Heijnen & van Loosdrecht et al. 2005). The $P_{rel}/VFA_{up}$ ratio was reported between 0.32 and 0.66 Pmol/Cmol in fully EBPR systems (Oehmen et al., 2007), whereas this ratio was calculated as 0.27 Pmol/Cmol in our system. There are many factors effecting EBPR performance such as carbon source, influent COD/P ratio, pH, temperature, PAOs/GAOs competition, and precipitation. Also, the high initial $NO_x$ concentration in the anaerobic phase affected the $P_{rel}/VFA_{up}$ ratio in our study. Therefore, the quantity of released phosphate at the end of anaerobic phase during the whole experiment was lower than that reported in the literature (Dulekgurgen et al., 2003; de Kreuk, Heijnen & van Loosdrecht et al. 2005; Oehmen et al., 2007).

The seed sludge contained relatively low volatile suspended solids at a 52% MLVSS/MLSS ratio and had good settling characteristics ($SVI_{30}$ = 77 ml/g), while the sludge property was deteriorated in a week with $SVI_{30}$ as high as 152 ml/g (Figure 4a). The $SVI_{30}$ decreased to around 40 ml/g at the end of the start-up period when the settling time reduced from 30 to 20 min on Day 18. The MLSS and MLVSS/MLSS ratio increased to about 5.5 g/L and 70%, respectively. The average particle size stabilized at 150 μm (Figures 4b and 5a).

### 3.2.2 | Phase I

With the consideration that PAOs belong to slow-growing bacteria, a short SRT would certainly hinder the accumulation of PAOs in the system. Thus, a gradual reduction of the settling time ($T_s$) depending on the improvement of $SVI_{30}$ was implemented in order to prevent excess biomass washout from the reactor. On Day 39, the settling time ($T_s$) was reduced from 20 to 5 min when the $SVI_{30}$ was 40 ml/g (Figure 4a). MLSS was dropped due to the washout of slow settling flocs, while the average of particle size increased (Figures 4b and 5b). However, the loss of biomass did not affect the P-removal performance. The total cycle duration (Tc) decreased from 6 to 4 h with improvements in the $NH_4$-N and $PO_4$-P removal observed during weekly cycle studies. It means that the OLR increased correspondingly from 1.2 to 1.8 kg COD/$m^3$d as a result of the reduction of total cycle. The $SVI_{30}$ stayed stable around 35 ml/g over 4 weeks and increased slowly to 60 ml/g at the end of Phase I (Figure 4b). Moreover, the

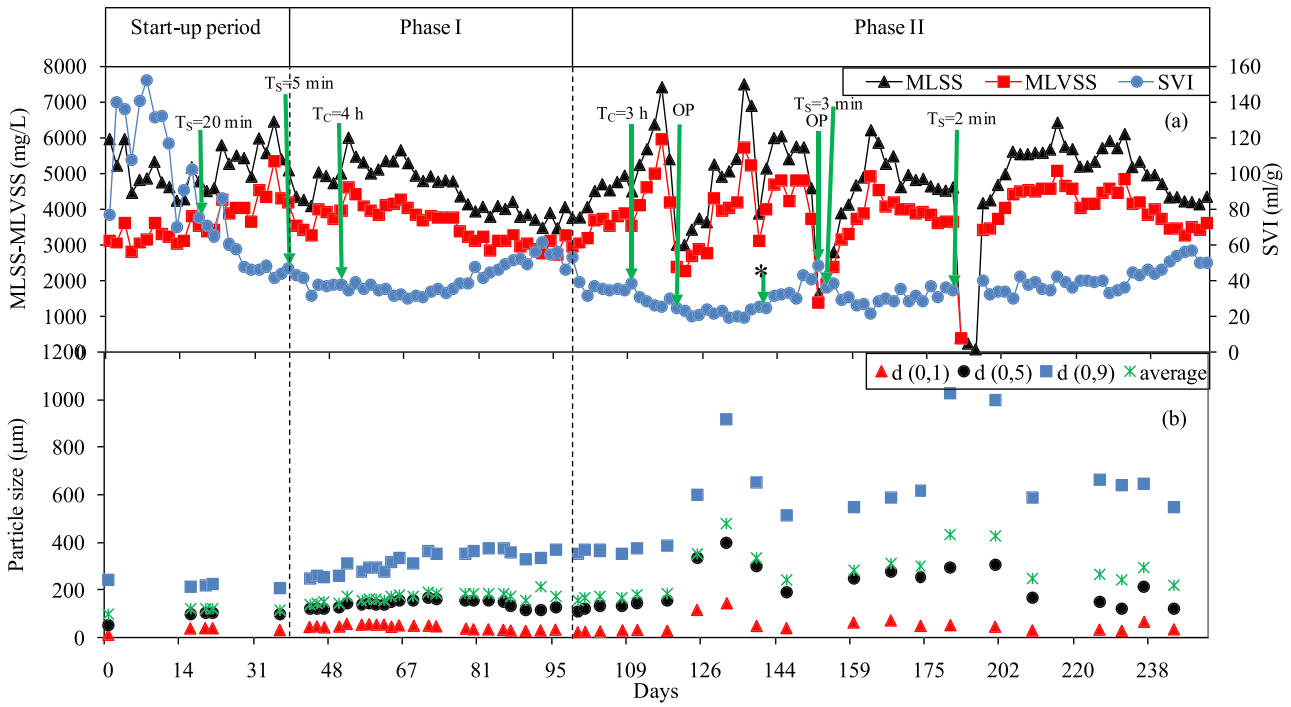



**FIGURE 4** (a) MLSS, MLVSS, and SVI; (b) distribution of granule size in the reactor. d(0.1), d(0.5), and d(0.9) are the 10th, 50th, and 90th percentiles of the distribution

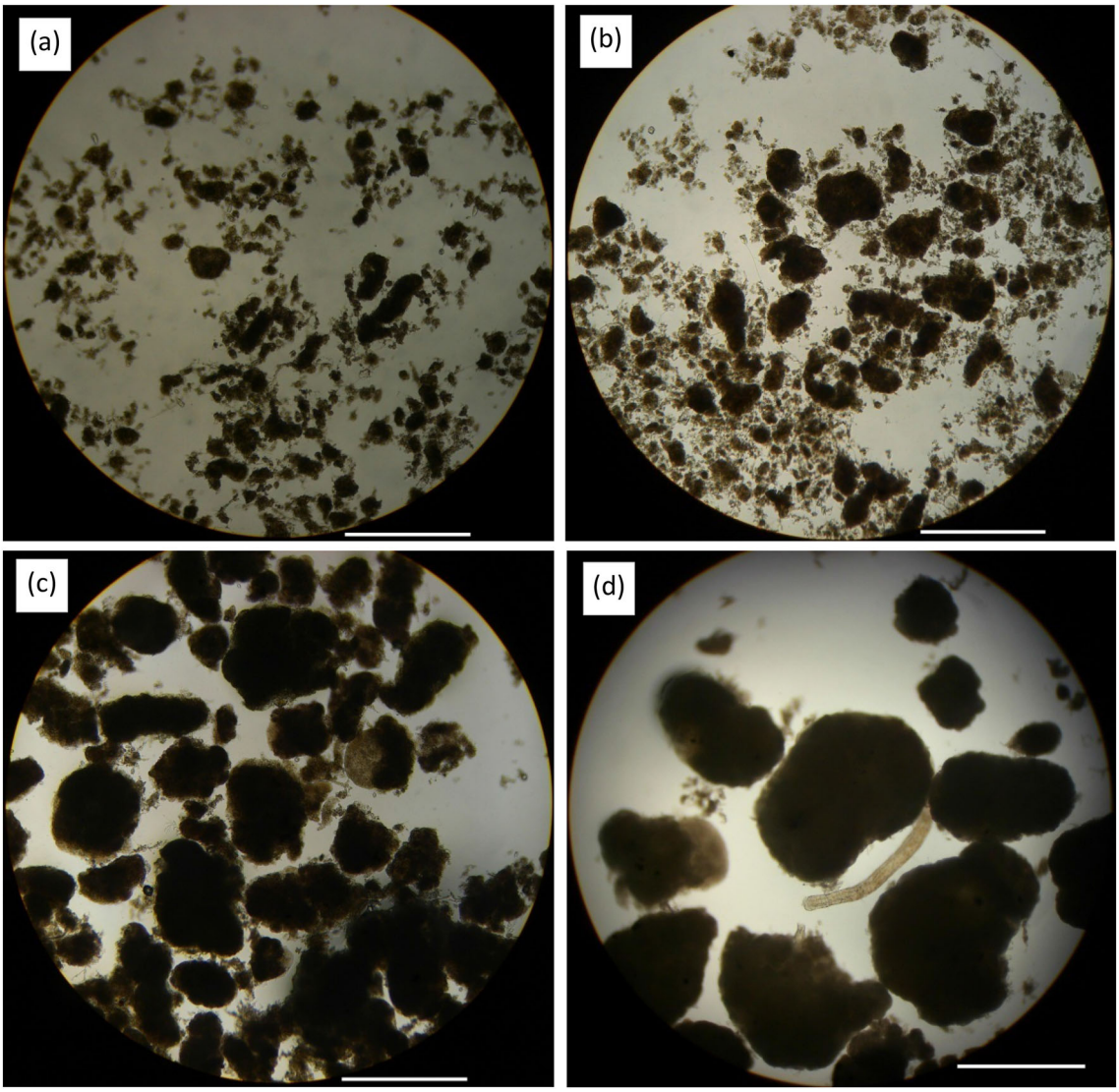



**FIGURE 5** Microscopic observation on different days in SBR system; (a) on Day 63; (b) on Day 104; (c) on Day 131; (d) on Day 181 (scale bar = 1 mm)

concentration of biomass decreased slowly, which indicated the inefficient wash-out of slow settling flocs and an out competition between microorganism under different growth rates at the relatively high OLR and the inefficient minimum settling velocity. The increase in $SVI_{30}$ was manifested by the decrease in biomass concentration as the granular sludge was denser and heavier than the floccular sludge.

The $SVI_5$, $SVI_{10}$, and $SVI_{30}$ measurements were proposed to be the main characterization of granular activated sludge (Schwarzenbeck et al., 2004; de Kreuk, McSwain et al. 2005). It is assumed that granulation is completed when the difference between $SVI_5$ and $SVI_{30}$ is less than 10%, and at the same time, a clear boundary of the granular activated sludge is observed (Liu & Tay, 2007). The $SVI_5/SVI_{30}$ ratio was approximately 2 at the end of Phase I, which is a relatively common value for floccular biomass (Wan & Spérandio, 2009). Furthermore, the microscopic observation showed the appearance of granular sludge at the end of start-up period (Figure 5a) and the coexistence of flocs and granular sludge during the Phase I (Figure 5b).

### 3.2.3 | Phase II

Positive effect of high hydrodynamic shear force in terms of SAV was proposed in previous studies (Liu & Tay, 2002). Therefore, SAV was increased from 0.78 to 1.96 cm/s in order to enhance the granulation from Day 97 (Phase II). Obviously, the concentration of biomass increased with the decrease of $SVI_{30}$, which was probably due to the densification of bioaggregates (Wan et al., 2009). The OLR was increased to 2.4 kg $COD/m^3d$ by decreasing the total cycle time to 3 h from Day 109, which induced a sharp rise for MLSS concentration in a few days (Figure 4a). On Day 119, the first automation problem occurred. This unexpected incident restrains the heavier granules and

to push numerous flocs and small granular sludge washed-out from the reactor. After this incident, the concentration of MLSS sharply decreased. However, the average granule size reached a value of 400 μm in 2 weeks (Figure 4b), and the percentage of sludge volume with the size over 200 μm gradually increased and reached at a value of 82% on Day 131. At the same time, the $SVI_5/SVI_{30}$ ratio and $SVI_{30}$ were approximately 1.0 and 20 ml/g, which reflected the typical characteristics of granular sludge (Figure 5c). The second automation problem happened again on Day 152 and the settling time was reduced to 3 min on Day 153. These applications stopped the further increase of flocculent sludge and maintained the stabilization in both $SVI_5/SVI_{30}$ ratio (1.0–1.2) and the granule size (average size = 350 μm) between Day 153 and 180 (Figure 4b).

From Day 180, the settling time was reduced from 3 to 1 min, which caused a sharp decrease of MLSS concentration in the reactor. A large amount of sludge was washed-out from the reactor and only approximately 10% of biomass with a larger size (Figure 4d) restrained in the reactor on Day 181. The rest of the biomass rapidly recovered and a large amount of aerobic granular sludge was formed with size more than 2 mm. During this period, the effluent phosphate was close to 5 mg P/L, which means that the performance of EBPR system on P removal was deteriorated, although the MLVSS/MLSS ratio was over 90%. In the rest of operating period, this MLVSS/MLSS ratio fluctuated between 75% and 85%. To maintain the EBPR performance of the reactor, the waste sludge collected from the wastage tank was returned to the reactor and operated at 3 min settling time until the end of the study.

## 3.3 | Effect of hydraulic conditions on granular formation

The settling velocity in the SBR reactor was determined by the settling time and the geometry of the reactor (different H/D and exchange ratio). A minimum settling velocity, $(V_s)_{min}$, was defined for bioparticles to be retained in the reactor (Jenkins et al., 1993). The typical settling velocity of suspended activated sludge is generally less than 4–5 m/h (Giokas et al., 2003). Ineffective washout of floccular sludge caused poor granulation, because the growing rate of floccular sludge is often higher than that of granular sludge due to the substrate transfer limitation. The competition on substrate utilization between floccular sludge and granular sludge represses the formation of granules. Therefore, to provide effective washout of floccular sludge, a $(V_s)_{min}$ value greater than the settling velocity of activated sludge should be selected. Thus, the minimum settling velocity applied in this study was lower compared with similar previous studies (de Kreuk, Heijnen & van Loosdrecht et al. 2005). The SBR system was operated at 3.2, 5.2, and 7.5 m/h of the minimum settling velocity when the settling times were 5, 3, and 1 min, respectively. Although the granular sludge was obtained under 3.2 m/h of the $(V_s)_{min}$, flocculent biomass could not be effectively washed out from the SBR; 5.2 m/h of the minimum settling velocity led to the formation of the EBPR granular sludge with the average size of 350 μm while the ratio of SVI5/SVI30 was in the range of 1.0 to 1.2. The further decrease of the settling time of 1 min ($(V_s)_{min}$ = 7.5 m/h) led to complete granulation but the aerobic granules obtained had no ability to remove phosphorus.

Tay et al. (2001b) investigated the effects of shear force on the formation, structure and metabolism of fully aerobic granules by operating four reactors at different SAV ranging from 0.3 to 3.6 cm/s. In the reactor operated in a low SAV (0.3 cm/s), aerobic granules did not form. However, aerobic granules formed in the reactors operated in superficial air velocities of 1.2, 2.4, and 3.6 cm/s. This implies that shear force, in terms of SAV, is favourable in the aerobic granulation process. It was also indicated that the size of aerobic granules decreased with the increase of SAV, whereas granules became rounder at high SAV (Liu & Tay, 2004) so the disintegration of matured granules at high hydrodynamic shear force was possible. Tay et al. (2001b) proposed that aerobic granulation occurs when SAV is over 1.2 cm/s. In this study, EBPR granular sludge was obtained at low aeration rate. The average granule size exceeded 200 μm both at SAV of 0.78 and 1.96 cm/s. Granules became rounder when increasing SAV from 0.78 to 1.96 cm/s in this study. It can be concluded that the selection of slow growing bacteria in granule may lead to reduce energy consumption because granules could be formed at low aeration rates.

## 3.4 | Characterization of matured EBPR granular sludge

The microscale structure of EBPR granule sludge was directly observed by using ESEM. Figure 6a,b showed that the surface of matured granule was not homogeneous, on which the green frame represented a lot of poly-P granules with bright white (Figure 6d). These results were confirmed by the previous observation as shown in Figure 3f. It was noticed that the size of these poly-P granules was close to 1 μm at the end of aerobic phase whereas the size of poly-P granules decreased to around 380 nm at the end of anaerobic phase. On the other hand, the numerous irregular products were probably the chemical precipitates as shown in Figure 6c.

The EDX combined ESEM was used to analyse the elemental composition in the three special areas (i.e., poly-P granules, bacteria community, and chemical precipitates) on surface of granules. Table 2 summarized the EDX analysis results carried out on the different areas of the granular sludge sampled at the end of the aerobic period. The weight of P, Mg, and Ca for the poly-P granules were almost two times of the bacterial community. The weight percentages of C and N in the chemical precipitate were considerably different from the granular sludge. The percentage of C in the chemical precipitate was almost half of other bacterial community and the poly-P granules, although the percentage of N in the chemical precipitate was four times higher than that of these parts. In addition to the percentage of P and N, the percentage of Mg in the poly-P granules and the chemical precipitation was two times higher than that of the bacterial

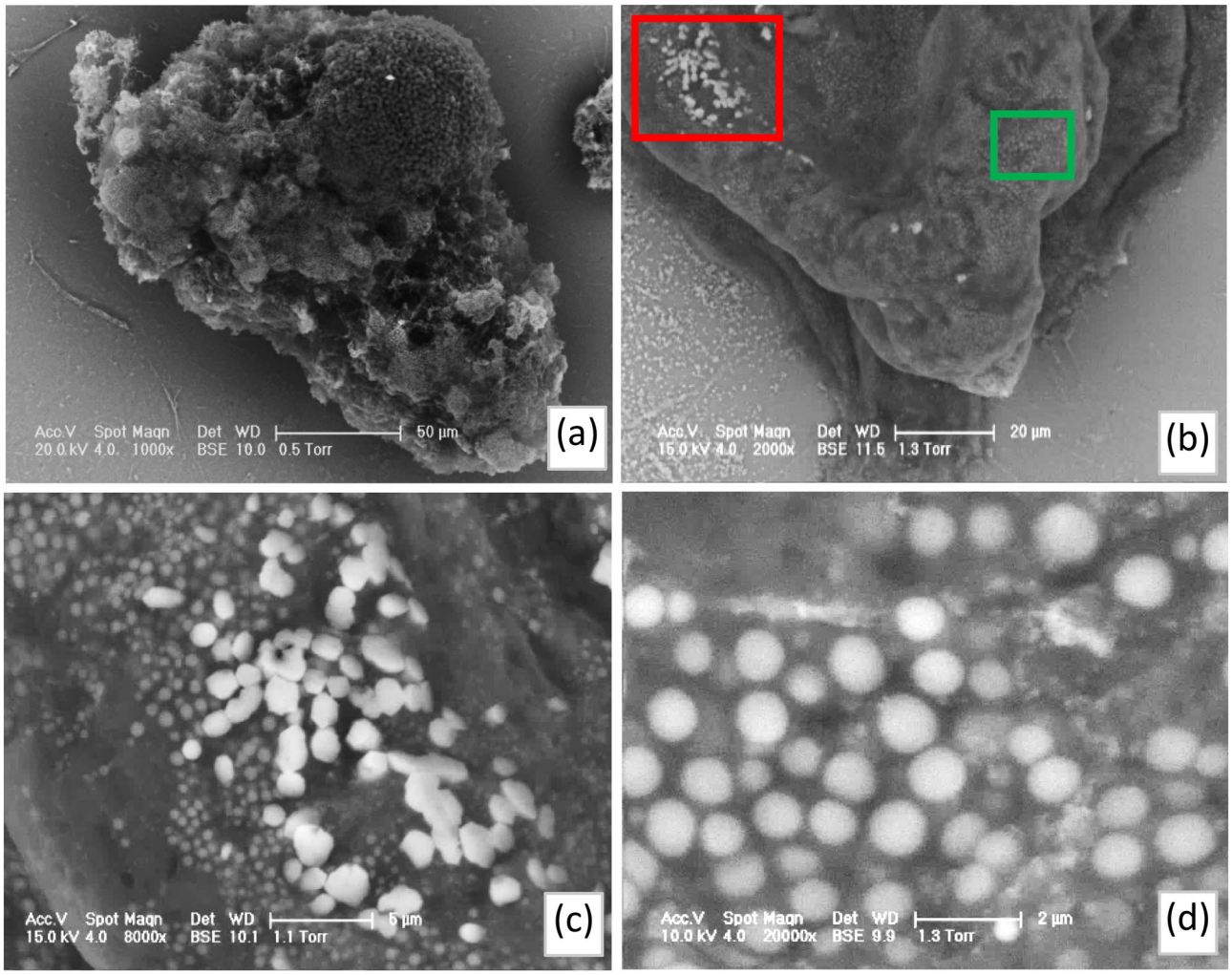



**FIGURE 6** ESEM images of the matured EBPR granular sludge samples; (a) a complete granule; (b) a part of granule, the red frame on the left side indicates chemical precipitates on the granule surface, the green frame indicates the poly-P granules in the cells; (c) enlarged red frame; (d) enlarged green frame

**TABLE 2** The EDX analysis of the EBPR granular sludge extracted at the end of the cycle

| | Of total weight (%) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Area | C | N | O | Na | Mg | Al | Si | P | S | Cl | K | Ca |
| Poly-P granules ($n = 2$)[a] | **40.6 (0.9)**[b] | **6.3 (0.6)** | 29.2 (2.1) | 1.9 (0.2) | **2.1 (0.3)** | 0.5 (0.1) | 7.3 (0.6) | **7.5 (0.5)** | 0.6 (0.1) | 0.3 (0.0) | 2.2 (0.4) | 2.6 (0.3) |
| Bacterial community ($n = 3$) | **43.4 (1.6)** | **7.9 (0.5)** | 26.9 (0.6) | 2.7 (0.3) | **1.0 (0.1)** | 0.6 (0.0) | 9.6 (1.0) | **3.9 (0.2)** | 0.7 (0.1) | 1.6 (0.1) | 0.3 (0.1) | 1.5 (0.2) |
| Chemical Precipitate ($n = 1$) | **24.3** | **30.4** | 25.4 | 2.3 | **2.0** | 0.6 | 5.4 | **5.4** | 0.7 | ND | 2.1 | 1.5 |

Abbreviation: ND, not detected.
[a]Number of sample analysed.
[b]Standard deviation.

community. In fact, Bassin et al. (2011) reported that considerable ammonium was adsorbed by aerobic granules, which probably induced the struvite formation as chemical precipitates on the surface of matured EBPR granules (Lin et al., 2012).

### 3.5 | Possible phosphorus forms in biomass

In this study, the cold PCA procedure was conducted both for homogenized and non-homogenized granular sludge and results showed that

**TABLE 3** Phosphorus fractions in biomass at the end of the anaerobic and aerobic phases

| Samples | Orto-P (% of sample TP) | Total P (% of sample TP) | Complex bio-P (% of sample TP) |
|---|---|---|---|
| End of anaerobic period ($n = 3$)[a] | | | |
| Supernatant | 24.3 (10.4)[b] | 28.6 (5.6) | 4.3 (4.7) |
| PCA | 8.3 (1.5) | 33.9 (14.8) | 25.6 (13.4) |
| Residue | - | 43 (21.7) | - |
| Recovery | - | 105.5 (2.3) | - |
| End of cycle ($n = 4$) | | | |
| Supernatant | 0 (0) | 2.2 (1.9) | 2.2 (1.9) |
| PCA | 8.5 (2.6) | 56.2 (17.4) | 47.7 (16.8) |
| Residue | - | 40.5 (3.7) | - |
| Recovery | - | 98.9 (4.7) | - |

[a]Number of the sample analysed.
[b]Standard deviation.

the difference of the chemical precipitation fraction between the homogenized and non-homogenized sludge was only 1% which was negligible. It is concluded that cold PCA procedure proposed by de Haas et al. (2000) is appropriate for evaluating the phosphorus fractions of aerobic granular sludge.

The previous studies reported that approximately 10% of the total phosphorus accumulation was the chemical precipitation at the aerobic granular sludge (Liu, Lin & Tay et al., 2005; Yilmaz et al., 2008). Yilmaz et al. (2008) demonstrated that the chemical precipitate could be in the form of struvite because of the high concentration of phosphate, ammonia and magnesium during the anaerobic period. The contribution of this mineral precipitation in the nutrient removal, however, was found negligible due to subsequent dissolution in the aerobic period. The chemical precipitation related to orthophosphate was determined 8.5% by the cold PCA procedure in this study (Table 3). The presence and percentage of Mg, N and P and the morphology of the precipitate indicate that this chemical precipitate could be in the form of struvite. The chemical precipitation could be monitored by using ESEM on the surface of the granule.

It is assumed that poly-phosphate granules have a chemical formula of $K_{1/3}Mg_{1/3}PO_4$ (Smolders et al., 1994). Therefore, phosphorus accumulating organisms release, uptake and store the poly-phosphate in that ratio. The other study reported that besides phosphorus, the metals Ca, Mg, and K were the main components of poly-phosphate granules, and it was suggested that in accordance with the proportions between the dominating cations, seven different types of granules could be defined (Schönborn et al., 2001). Based on this study, the results showed that the poly-P granules of the EBPR granule included mixed-type poly-phosphate granules according to the estimated molar ratios of magnesium, potassium, and calcium ($C_{Mg/P}$:$C_{K/P}$:$C_{Ca/P}$ = 0.3:0.2:0.3). The element Si was detected in all areas where the EDX analysis was made. The previous study reported that the element Si was found in the EPS structure of the EBPR floccular sludge in the range of 8.3% to 9.1% of the total weight but not detected in the cell clusters (Cloete & Oosthuizen, 2001).

ESEM and EDX analysis showed that the poly-P granules were observed in the EPS structure of the granules. The poly-P granules at the end of anaerobic period were bigger than that at the end of cycle. On the other hand, these granules could not be observed after the cold PCA procedure, which all chemical and complex P were dissolved by PCA without destroying bacteria. Hence, it is highly possible that bacteria may store the poly-P granules in the EPS structure.

## 4 | CONCLUSION

The strategy of the minimum settling velocity was found to be beneficial in maintaining granular biomass without losing the EBPR capacity. In this study, the minimum settling velocity for the EBPR granular sludge was found as 5.2 m/h. The chemical precipitates on the EBPR granule sludge was monitored by using ESEM combined with EDX. The percentage of chemical precipitation in the EBPR granule was 8.5%. The results of the EDX analysis on the chemical precipitates showed that the chemical precipitates occurred on the EBPR granule sludge could be in the form of struvite. The granules giving bright white reflection was thought to be poly-P granules based on ESEM analysis before and after the cold PCA procedure and EDX analysis. However, at this stage, it cannot be explained why the poly-P granules were stored extracellular in EPS structure.

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### DATA AVAILABILITY STATEMENT

The data used to support the findings of this study are included within the article.

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