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# Effect of calcium addition to aerobic granular sludge systems under high (conventional SBR) and low (simultaneous fill/draw SBR) selection pressure

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ABSTRACT

This paper investigated the effect of calcium addition on the formation and properties of aerobic granules under high (conventional SBR) and low (simultaneous fill/draw SBR) selection pressure. Additionally, the simultaneous removal of carbon, nitrogen, and phosphorus, and the operational stability were assessed. The conventional SBRs showed earlier granule development (20 days) than the simultaneous fill/draw SBRs. The effect of calcium on granulation was more accentuated in conventional SBRs, forming larger granules in a shorter interval of time due to the higher EPS production. Additionally, higher amounts of calcium were found in the EPS matrix, mainly during the formation of granules. The operation regime and the addition of calcium did not affect the removal of carbon, nitrogen, and phosphorus. However, they both influenced the granulation time, settleability characteristics, size, and granule composition.

## 1. Introduction

Aerobic granular sludge (AGS) is an evolution of activated sludge, being developed mainly in sequencing batch reactors (SBRs). These reactors can be operated in two configurations: conventional (variable volume), in which the filling, reaction, settling, and drawing phases occur sequentially; and simultaneous fill/draw (constant volume), i.e., the filling and drawing phases occur simultaneously, followed by the reaction and settling phases (Rollemberg et al., 2019). While conventional SBRs are widely used in laboratory research, simultaneous fill/draw SBRs are usually adopted in full-scale treatment plants, notably in Nereda systems (Derlon et al., 2016). However, there are still few scientific studies dealing with this latter configuration (Barros et al., 2020; Derlon et al., 2016; Rollemberg et al., 2019; Wang et al., 2018a,b).

In conventional SBRs, the settling time and exchange volume, which can be simultaneously expressed by the minimum settling velocity ($V_{ms}$), are the main hydraulic selection pressure factors that can induce granulation. With $V_{ms}$ lower than 3.8 m $h^{-1}$, flocs of activated sludge predominate in the system (Liu et al., 2005). In simultaneous fill/draw SBRs, the driving force for granulation is the upflow velocity of the liquid ($V_f$) during the filling phase, considering that only the microbial aggregates with a settling velocity lower than $V_f$ are eliminated from the reactors (Derlon et al., 2016). Upflow velocities around 1 m $h^{-1}$ proved to be able to promote the selection of granular biomass effectively (Barros et al., 2020; Derlon et al., 2016).

Accordingly, the type of SBR configuration influences the granulation time and characteristics of the biomass cultivated. In general, conventional SBRs present a shorter granulation time, forming larger granules with better settleability, than simultaneous fill/draw SBRs. However, biomass retention in conventional reactors is usually lower (Rollemberg et al., 2019). As an alternative to decrease the granulation time and improve the quality of the aerobic granules formed, both recurring problems in full-scale wastewater treatment plants (WWTP), the addition of some di and trivalent cations to the influent has been studied. The main mechanism that facilitates microbial aggregation is based on their effect of neutralizing the negative surface charges of microorganisms. In addition, cations are able to bond with the EPS structure, establishing a more structurally stable complex, and form precipitates that serve as a support medium for attached growth (Kończak et al., 2014). The main cations studied are the divalent cations $Ca^{2+}$ (very common in the literature) and $Mg^{2+}$, and the trivalent cations $Al^{3+}$ and $Fe^{3+}$, for which the literature presents different behaviors in relation to their effect on granulation (Ren et al., 2008; Sajjad and Kim,

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**List of abbreviations**

| | |
|---|---|
| AGS | aerobic granular sludge |
| COD | chemical oxygen demand |
| EDX | energy-dispersive X-ray |
| EPS | extracellular polymeric substances |
| MLVSS | mixed liquor volatile suspended solids |
| PAC | polyaluminum chloride |
| PN | proteins |
| PS | polysaccharides |
| SBR | sequencing batch reactors |
| SEM | scanning electron microscope |
| SVI | sludge volumetric index |
| $T_s$ | settling time |
| TSS | total suspended solids |
| $V_f$ | upflow velocity of the liquid |
| $V_{ms}$ | minimum settling velocity |
| VSS | volatile suspended solids |
| WWTP | wastewater treatment plant |

2015). For example, polyaluminum chloride (PAC) had a more positive impact on the granulation time, granule quality, and even EPS matrix composition in long-term operation than aluminum sulfate (Liu et al, 2016, 2019).

Interestingly, the SBR configuration also appears to have an effect on the extent to which the presence of divalent cations is able to influence the characteristics of the granules (Barros et al., 2020). For instance, adding 100 mg $Ca^{2+}$ $L^{-1}$ to a conventional SBR, Jiang et al. (2003) found granules with considerably larger average sizes (control: 2 mm; calcium-supplemented: 2.8 mm), higher biomass retention (control: 2 g MLVSS $L^{-1}$; calcium-supplemented: 7.9 g MLVSS $L^{-1}$), and higher production of extracellular polysaccharides (PS) (control: 41 mg g $MLVSS^{-1}$; calcium-supplemented: 92 mg g $MLVSS^{-1}$). The granules enriched with calcium also showed significantly higher resistance to shear strength (measured by the turbidity method).

In contrast, using a simultaneous fill/draw SBR with the same calcium addition, Barros et al. (2020) reported that calcium did not significantly alter the granulometric distribution, biomass retention (around 8 g MLVSS $L^{-1}$), production of extracellular polysaccharides (PS) and proteins (PN) (control: 93 ± 8 mg PS g $MLVSS^{-1}$, 44 ± 5 mg PN g $MLVSS^{-1}$; calcium-supplemented: 87 ± 8 mg PS g $MLVSS^{-1}$, 50 ± 5 mg PN g $MLVSS^{-1}$), and resistance of the granules (measured by the S coefficient method). In addition, the calcium content in the granules found by energy-dispersive X-ray (EDX) analysis was low (1.1%) when compared to that observed by Ren et al. (2008) (~14%) in a conventional SBR enriched with only 40 mg $Ca^{2+}$ $L^{-1}$.

However, Barros et al. (2020) worked only with simultaneous fill/-draw SBRs, with no conventional SBR for a direct comparison concerning the effect of calcium addition on the formation and characteristics of AGS. In other works, the operational conditions, type of substrate, concentration of calcium, period of time that calcium was supplemented, inoculum, etc. were quite different. Therefore, a comparison among them may be hindered or lead to false interpretations. Consequently, an experimental investigation under the same conditions is necessary to better elucidate the effect of divalent cations. Thus, the present paper aimed to investigate the effect of calcium addition on the formation and properties of AGS granules under high (conventional SBR) and low (simultaneous fill/draw SBR) selection pressure. Additionally, the simultaneous removal of C, N, and P, and the operational stability were assessed.

## 2. Materials and methods

### 2.1. Configuration of the systems and operational conditions

Four sequencing batch reactors (SBRs) with working volumes of 7.2 L, 1 m high, and 100 mm in diameter were operated to cultivate aerobic granular sludge using an activated sludge inoculum (3.6 L; 2.1 g VSS $L^{-1}$). The reactors R1 (control) and R2 (supplemented with calcium) were operated as simultaneous fill/draw SBRs ($V_f$ of 1 m $h^{-1}$) at 6-h cycles, divided into: filling/drawing (30 min), anaerobic/anoxic reaction without agitation (60 min), aerobic reaction (250 min), and settling (20 min). The reactors R3 (control) and R4 (supplemented with calcium) were operated as conventional SBRs at 6-h cycles, divided into: filling (30 min), anaerobic/anoxic reaction without agitation (60 min), aerobic reaction (249 min), settling (20 min), and drawing (1 min). The characteristics and operational parameters were defined according to previous work (Barros et al., 2020; Rollemberg et al., 2019).

The settling time ($T_s$) of the reactors was gradually reduced throughout three stages to induce granulation as follows: stage I, $T_s$ of 20 min for 14 days; stage II, $T_s$ of 10 min for 25 days; and stage III, $T_s$ of 5 min for 24 days. The change from one stage to the next one was determined by the stabilization of the sludge volumetric index (SVI) values in the reactors. The time subtracted from the settling phase was added to the aerobic reaction phase. It is important to note that, although the settling time is not an important selection pressure in simultaneous fill/draw SBRs, the R1 and R2 reactors were also exposed to the aforementioned operational procedure in order to maintain the conditions of the four reactors as close as possible.

The reactors were fed with a synthetic feed solution containing acetate (~500 mg COD $L^{-1}$), ammonium (~50 mg $NH_4^+$-N $L^{-1}$), phosphate (~7 mg $PO_4^{3-}$-P $L^{-1}$), micronutrients solution (~1 mL $L^{-1}$), whose composition was adapted from dos Santos et al. (2005), and sodium bicarbonate (1 g $L^{-1}$), used as a buffer to keep the pH close to 7.0. In addition, reactors R2 and R4 were supplemented with 100 mg $Ca^{2+}$ $L^{-1}$. The feed solutions were stored at 4 °C to avoid their degradation, while the reactors were operated at room temperature (27–30 °C).

### 2.2. Systems monitoring

COD, ammonium, total suspended solids (TSS), and volatile suspended solids (VSS) were determined according to the Standard Methods for the Examination of Water and Wastewater (APHA, 2012). Nitrite, nitrate, and phosphate were determined by a Dionex™ ICS-1100 ion chromatograph equipped with a Dionex™ IonPac™ AG23 pre-column (2 × 50 mm), a Dionex™ IonPac™ AS23 column (2 × 250 mm), and a Dionex™ AERS™ 500 suppressor (2 mm) (Thermo Scientific, USA). 5 μL of the filtered sample (0.45 μm) was injected and then eluted by an aqueous solution containing 4.5 mM sodium carbonate and 0.8 mM sodium bicarbonate at a constant flow of 0.25 mL $min^{-1}$. The oven temperature was 30 °C, the applied current was 7 mA, and the running time was 30 min.

These analyses were performed for the influent and effluent samples, 3 times a week. The measurement of the dynamic sludge volumetric index (SVI) for 5 and 30 min (Schwarzenbeck et al., 2005) was also performed at the same frequency. Granulometric distribution of the biomass was carried out every fifteen days by passing the mixed liquor through sieves with 0.2 and 1 mm openings, and the dry weights of the total sample and the aliquots that passed through each of the sieves were recorded.

EPS were also quantified every fifteen days. To extract them, 5 mL of mixed liquor were added with 5 mL of 1 M NaOH solution, heated in a water bath at 80 °C for 30 min, maintained in a 55-kHz ultrasonic bath for 5 min, filtered (0.45 μm), and diluted (dilution factor of 2) (Tay et al., 2001). To quantify proteins (PN) and polysaccharides (PS), a modification of the Lowry method and the phenol-sulfuric acid method, respectively, were used (Long et al., 2014). With the same frequency, the

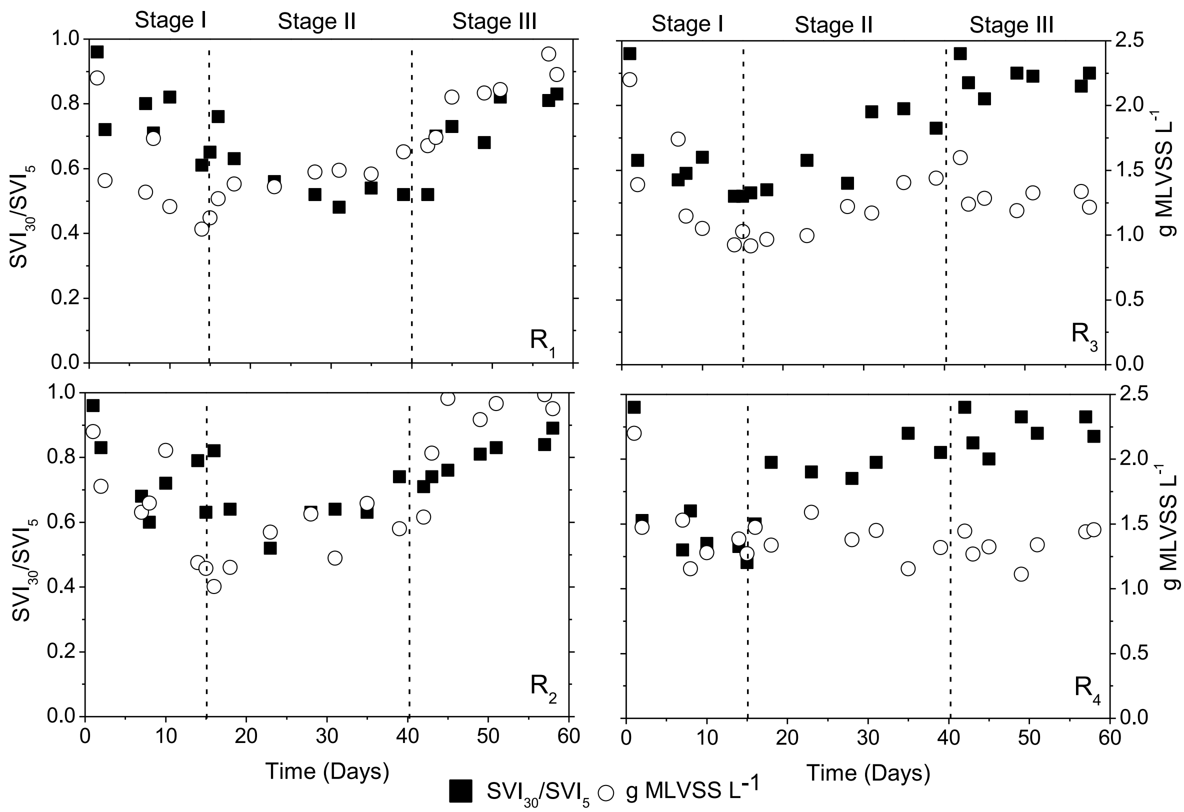


**Fig. 1.** $SVI_{30}/SVI_5$ ratio and mixed liquor volatile suspended solids (MLVSS) achieved in simultaneous fill/draw SBRs R1 (control) and R2 (supplemented with $Ca^{2+}$) and conventional SBRs R3 (control) and R4 (supplemented with $Ca^{2+}$).

granulometric distributions were also determined by optical microscopy images of the cultivated biomass.

At the end of the last operation stage, the shape and composition of the granules formed were analyzed in a scanning electron microscope combined with an energy dispersive X-ray detector (SEM-EDX) (Inspect S50 - FEI). Sample preparation was carried out according to Motteran et al. (2013).

### 2.3. Statistical methods

The Kruskal-Wallis non-parametric test, which does not require a specific data distribution, was used to compare the performances of the reactors during the experiment at a 95% confidence level.

## 3. Results and discussion

### 3.1. Granule formation, settleability, and size

Initially, after inoculation, the $SVI_{30}$ of the sludge was 166 mL $g^{-1}$, and the biomass concentration was 2.1 g MLVSS $L^{-1}$. Then, throughout stage I, the four systems showed a drop in their MLVSS concentration, which must be related to a biomass adaptation to the new operational conditions (Fig. 1). Wang et al. (2018a,b), using a simultaneous fill/draw SBR with an upflow liquid velocity of 0.67 m $h^{-1}$, also found a biomass loss (washout) on the first days of operation. According to those authors, it was attributed to the denitrification during the filling phase, since the bubbles of nitrogen gas washed out part of the sludge (fraction with lower settleability). However, in the present study, this was not evident (bubbles were not observed).

At stages II and III, the solids in R3 and R4 tended to stabilize, while, in R1 and R2, an accumulation was observed, reaching a concentration close to 2.5 g MLVSS $L^{-1}$ at the end of the experiment. It is important to remind that systems operated with low selection pressure as R1 and R2 (simultaneous fill/draw SBRs) tend to maintain a higher amount of biomass (Liu et al., 2005; Nancharaiah and Reddy, 2018). This was clearly observed by Rollemberg et al. (2019) when comparing these two reactor configurations for AGS cultivation. At the end of 125 days of operation, those authors found 7 g MLVSS $L^{-1}$ in the simultaneous fill/draw SBR and only 1.5 g MLVSS $L^{-1}$ in the conventional one. The reactor configuration may also affect the solid retention time (SRT) (sludge age) of the system, with simultaneous fill/draw SBRs reaching higher SRTs than conventional SBRs. In the present study, R1 and R2 had SRTs of 15–20 days, while R3 and R4 had SRTs of 9–12 days. Such results also corroborate those found by Rollemberg et al. (2019). Concerning the effect of calcium, no considerable difference was observed between R1 and R2 (simultaneous fill/draw SBRs) and between R3 and R4 (conventional SBRs), in terms of biomass concentration, during the entire experiment.

It is worth mentioning that the operation time may also have a considerable influence on the amount of biomass in simultaneous fill/draw SBRs. For instance, Barros et al. (2020) operated a simultaneous fill/draw SBR with a liquid upflow velocity of 0.92 m $h^{-1}$ for 200 days and reached concentrations of 8 g MLVSS $L^{-1}$ at the end of the experiment. On the other hand, Wang et al. (2018a,b) operated a reactor for 80 days under similar conditions and reached approximately 3 g MLVSS $L^{-1}$, values close to those found in R1 and R2 in this study.

The behavior of the $SVI_{30}/SVI_5$ ratio (settleability indicator) in R1 (simultaneous fill/draw SBR) was different from that in R3 (conventional SBR). While R3 had this ratio increased already at stage II, R1 had its settleability improved only at stage III (Fig. 1). At the end of the experiment, R1 reached an $SVI_{30}/SVI_5$ ratio of 0.8, and R3 presented a ratio above 0.9, i.e., a better settleability (Bassin et al., 2012; de Kreuk et al., 2007; Liu et al., 2005). Regarding the effect of calcium, R2 had a behavior very similar to that of R1. Conversely, the $SVI_{30}/SVI_5$ ratio of R4 increased faster than that of R3. On the 20th day of operation, the ratios in R3 and R4 were 0.76 and 0.65, respectively, reaching values

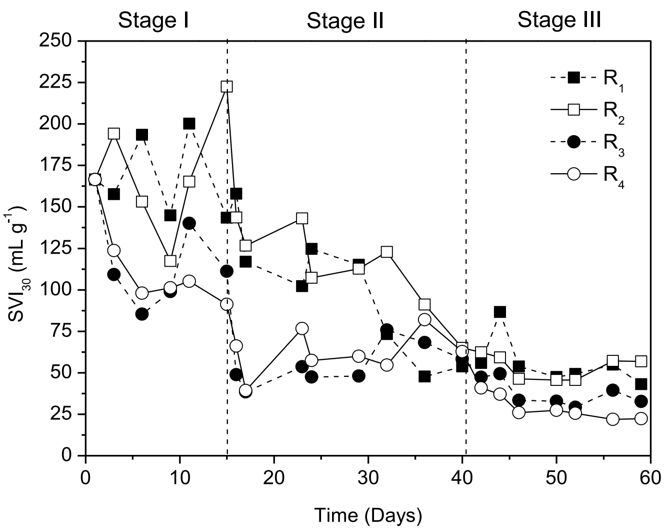


**Fig. 2.** Sludge volumetric index at 30 min ($SVI_{30}$) for the simultaneous fill/draw SBRs R1 (control) and R2 (supplemented with $Ca^{2+}$) and conventional SBRs R3 (control) and R4 (supplemented with $Ca^{2+}$).

above 0.9 at stage III (Fig. 1). Therefore, calcium may have a more significant impact on the sludge settleability of conventional SBRs.

When analyzing only the $SVI_{30}$ profile (Fig. 2), the operation regime of R3 and R4 favored a more pronounced $SVI_{30}$ drop than that of R1 and R2. Such observations were already reported by Rollemberg et al. (2019), who evaluated only the decrease in the settling time from 20 to 10 min in cycle configurations similar to those tested in this work. According to those authors, the physical mechanism of granule selection in the conventional SBR was directly linked to the minimum settling velocity of the biomass during the settling time of the cycle. On the other hand, in the simultaneous fill/draw SBR, the physical mechanism of biomass selection was the sludge settling velocity, which was higher than the liquid upflow velocity during filling, and not the settling time itself. However, as the $SVI_{30}$ of R1 and R2 (simultaneous fill/draw SBRs) decreased markedly from stage II to III (Fig. 2), the decrease in the settling time can intensify the selection pressure in this type of SBR, thus improving the settleability.

Concerning calcium supplementation, its effect on $SVI_{30}$ was not evident (Fig. 2) as in previous studies (Barros et al., 2020; Jiang et al., 2003). Jiang et al. (2003), in an experiment with simultaneous fill/draw SBRs with a settling time of 2 min, found a much lower $SVI_{30}$ in the reactor supplemented with 100 mg $Ca^{2+}$ $L^{-1}$ (73 $\pm$ 7 mL $g^{-1}$) than in the control reactor (172 $\pm$ 10 mL $g^{-1}$). Although less expressively, Barros et al. (2020) also observed a positive effect of calcium on the $SVI_{30}$ of a simultaneous fill/draw SBR with a settling time of 5 min. The $SVI_{30}$ of the reactor supplemented with calcium (100 mg $L^{-1}$) was lower than 50 mL $g^{-1}$, while the $SVI_{30}$ of the control reactor was between 60 and 80 mL $g^{-1}$. It is worth reminding that, in the present study, although calcium did not affect the $SVI_{30}$ of the reactors regardless the operation configuration, it improved the settleability of the conventional ones, since they presented better $SVI_{30}/SVI_5$ ratios as discussed above. That is, the settling velocity was improved by calcium.

Regarding the granule sizes, R3 (conventional SBR) presented larger granules than R1 (simultaneous fill/draw SBR) throughout the entire experiment (Fig. 3). For instance, around the 20th day of operation, R3 had more than 25% of its biomass larger than 1 mm, while R1 had only 5%. As for the calcium-supplemented reactors (R2 and R4), their granules were larger than those of their respective control reactors (R1 and R3). Accordingly, the calcium-supplemented conventional SBRs also showed larger granules than the simultaneous fill/draw SBRs. With only 10 days of operation, more than 30% of the sludge of R4 was greater than 1 mm, while only 5% of the sludge of R2 achieved this size.

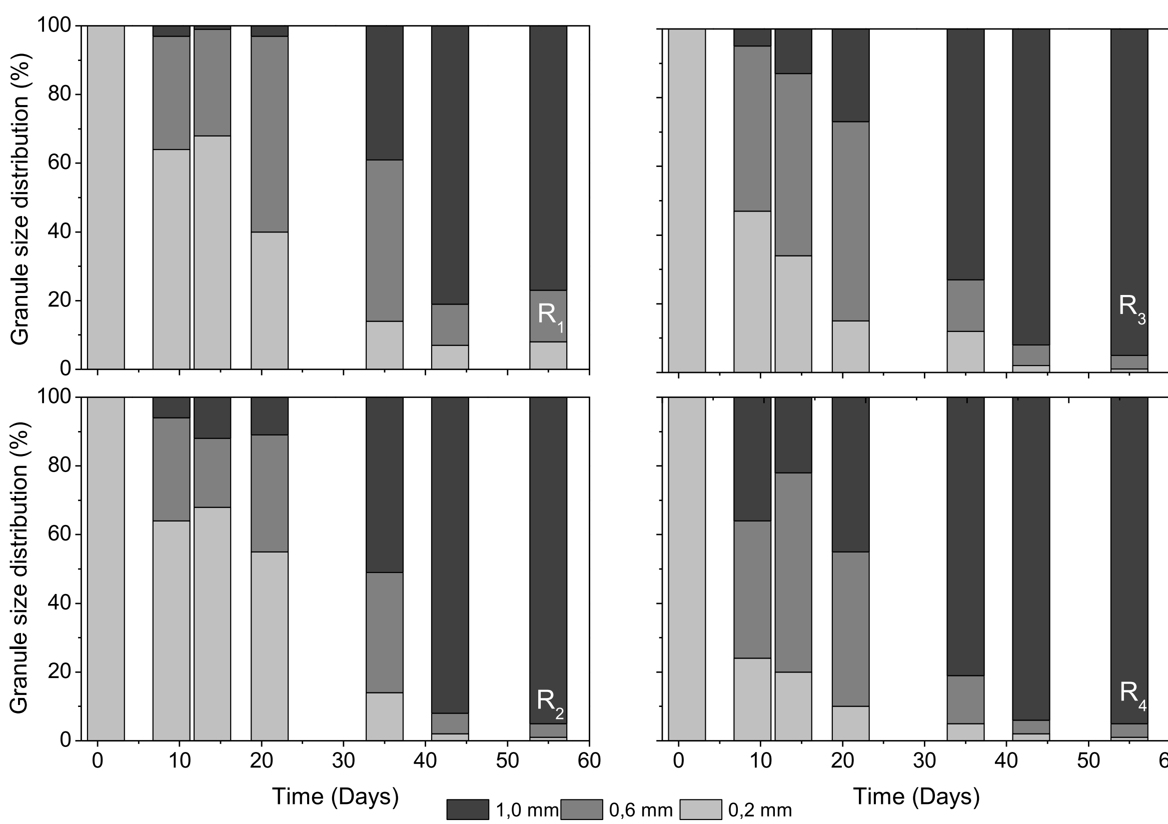


**Fig. 3.** Size of the granules obtained in the simultaneous fill/draw SBRs R1 (control) and R2 (supplemented with $Ca^{2+}$) and conventional SBRs R3 (control) and R4 (supplemented with $Ca^{2+}$).

**Table 1**
Operational performance achieved in the simultaneous fill/draw SBRs R1 (control) and R2 (supplemented with $Ca^{2+}$) and conventional SBRs R3 (control) and R4 (supplemented with $Ca^{2+}$).

| Reactor | | R1 | | | R2 | | | R3 | | | R4 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Stage | | I | II | III | I | II | III | I | II | III | I | II | III |
| **COD** | Influent (mg $L^{-1}$) | 582 (53) | 551 (37) | 554 (37) | 550 (65) | 560 (31) | 564 (59) | 582 (53) | 551 (37) | 554 (37) | 550 (65) | 560 (31) | 564 (59) |
| | Effluent (mg $L^{-1}$) | 57 (30) | 46 (9) | 54 (11) | 53 (25) | 47 (6) | 49 (12) | 30 (14) | 47 (16) | 34 (9) | 63 (16) | 61 (25) | 50 (19) |
| | Efficiency (%) | 90 (5) | 91 (2) | 90 (2) | 89 (6) | 91 (1) | 91 (3) | 94 (2) | 91 (6) | 93 (2) | 88 (2) | 90 (5) | 91 (4) |
| **N** | Influent $NH_4^+$-N (mg $L^{-1}$) | 54.6 (2.3) | 48.1 (3.0) | 47.0 (4.3) | 55.0 (3.9) | 43.8 (1.1) | 46.3 (4.2) | 54.6 (2.3) | 48.1 (3.0) | 47.0 (4.3) | 55.0 (3.9) | 43.8 (1.1) | 46.3 (4.2) |
| | Effluent $NH_4^+$-N (mg $L^{-1}$) | 9.4 (8.5) | 4.6 (1.4) | 2.2 (0.7) | 8.2 (5.5) | 13.5 (8.4) | 2.7 (0.8) | 11.5 (6.5) | 8.9 (5.9) | 2.9 (0.9) | 3.1 (0.4) | 2.4 (0.4) | 2.5 (0.7) |
| | Effluent $NO_2^-$-N (mg $L^{-1}$) | 3.8 (1.7) | 2.1 (0.7) | 1.1 (0.4) | 3.0 (1.1) | 2.2 (1.0) | 1.7 (0.6) | 4.1 (1.4) | 2.6 (0.8) | 1.1 (0.5) | 3.5 (1.1) | 2.5 (0.4) | 1.1 (0.2) |
| | Effluent $NO_3^-$-N (mg $L^{-1}$) | 6.9 (1.8) | 4.7 (0.4) | 5.2 (0.5) | 5.8 (1.5) | 4.6 (0.5) | 4.9 (0.4) | 5.6 (2.7) | 4.6 (0.8) | 3.5 (0.5) | 5.6 (1.5) | 4.6 (1.0) | 3.3 (0.2) |
| | Nitrification efficiency (%) | 82 (18) | 90 (3) | 92 (3) | 85 (9) | 69 (19) | 93 (2) | 78 (13) | 82 (19) | 93 (2) | 94 (1) | 95 (1) | 94 (2) |
| | Total nitrogen removal (%) | 62 (16) | 76 (3) | 78 (3) | 69 (8) | 53 (19) | 79 (3) | 61 (12) | 67 (10) | 83 (5) | 77 (4) | 78 (4) | 84 (2) |
| **P** | Influent $PO_4^{3-}$-P (mg $L^{-1}$) | 7.9 (0.8) | 8.4 (0.6) | 9.1 (0.9) | 8.1 (0.9) | 8.5 (0.5) | 9.1 (0.5) | 7.9 (0.8) | 8.4 (0.6) | 9.1 (0.9) | 8.1 (0.9) | 8.5 (0.5) | 9.1 (0.5) |
| | Effluent $PO_4^{3-}$-P (mg $L^{-1}$) | 4.6 (1.3) | 2.5 (0.5) | 2.5 (0.4) | 4.4 (1.2) | 2.6 (0.4) | 2.3 (0.6) | 3.6 (1.1) | 2.6 (0.2) | 1.7 (0.3) | 3.5 (1.0) | 2.5 (0.3) | 1.6 (0.5) |
| | Efficiency (%) | 41 (17) | 69 (7) | 72 (5) | 45 (12) | 69 (6) | 74 (6) | 55 (12) | 68 (3) | 80 (5) | 55 (12) | 73 (5) | 82 (7) |

The standard deviation is shown in parentheses.

Considering that 80% of its biomass must be greater than 0.2 mm to be considered granular sludge (Adav et al, 2007, 2009; Liu and Tay, 2004), R4 was the first to granulate, after 14 days of operation, followed by R3, after 20 days, and then by R1 and R2, after approximately 30 days. Thus, both the addition of calcium and the conventional operation had a positive impact on the size of the granules formed. At the end of the experiment, the granules were mostly larger than 1 mm in the four reactors (Fig. 3).

Usually, the literature reports that, in simultaneous fill/draw SBRs, granules smaller than 1 mm predominate, even in long-term operations. For instance, Derlon et al. (2016), operating a SBR with an upflow liquid velocity of 1 m $h^{-1}$, 50% volumetric exchange ratio, settling time of 3 min and fed with low-strength real sewage, observed that 60% of the granules were between 0.25 and 0.63 mm, while only 15% were larger than 0.63 mm, after 317 days of operation. Wang et al. (2018a,b) operated two simultaneous fill/draw SBRs with 90% volumetric exchange ratio and fed with real sewage (R1) and synthetic wastewater containing acetate (R2). These authors observed that the granules formed in the reactor that treated real sewage had larger diameters on the 56th day of operation (R1: 0.2–0.8 mm; R2: 0.2–0.6 mm), possibly related to the composition of the substrate as reported by Barros et al. (2020).

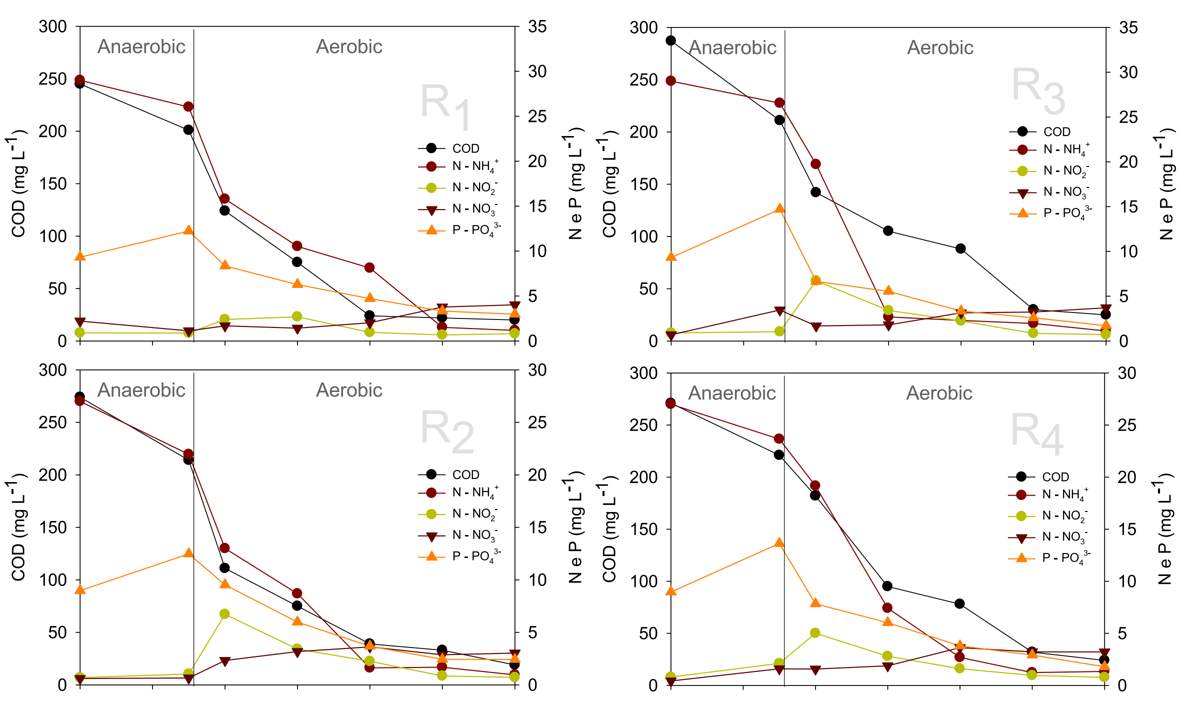


**Fig. 4.** Operational profile of simultaneous fill/draw SBRs R1 (control) and R2 (supplemented with $Ca^{2+}$) and conventional SBRs R3 (control) and R4 (supplemented with $Ca^{2+}$).

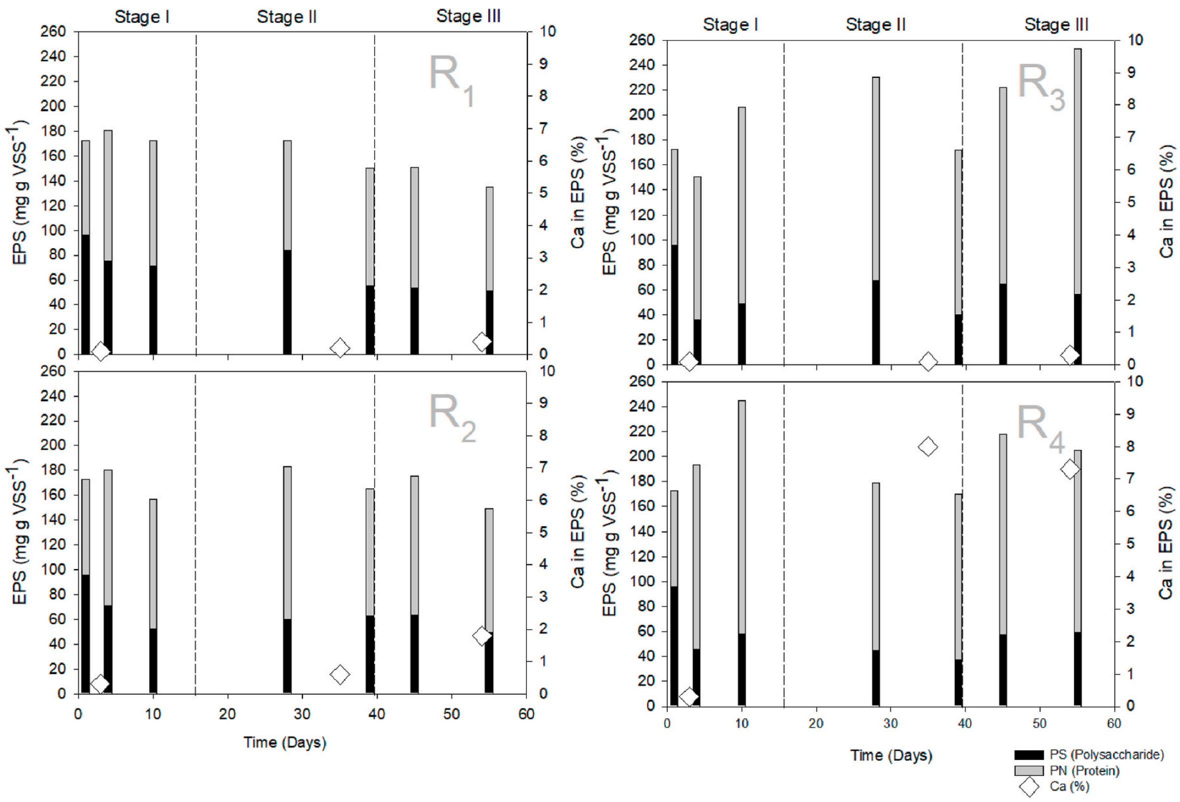


**Fig. 5.** EPS composition during the operational periods of the simultaneous fill/draw SBRs R1 (control) and R2 (supplemented with $Ca^{2+}$) and conventional SBRs R3 (control) and R4 (supplemented with $Ca^{2+}$).

Most of the studies with calcium supplementation report granules with similar sizes to those found in the present work (Barros et al., 2020; Jiang et al., 2003; Ren et al., 2008; Wan et al., 2015). In fact, Liu et al. (2010), comparing the effect of $Ca^{2+}$ and $Mg^{2+}$ (supplemented with 40 mg $L^{-1}$ each), observed that approximately 60% of the granules with calcium were in the range of 1.3–2.0 mm, and less than 16% in the range of 0.2–1.0 mm, with compact and regular morphology after 105 days of operation. On the other hand, the granules formed in the presence of magnesium were smaller (61% were between 0.3 and 1.3 mm) and looser.

### 3.2. Systems performance during the granulation process

In terms of COD, all reactors showed high and similar removal efficiencies throughout the entire experiment ($p_I = 0.570$, $p_{II} = 0.086$, $p_{III} = 0.076$) (Table 1). Additionally, there were not significant variations among the values obtained at the different operational stages ($p_{R1} = 0.690$, $p_{R2} = 0.898$, $p_{R3} = 0.366$, $p_{R4} = 0.478$). Therefore, neither the SBR configuration nor calcium addition affected COD removal regardless the maturation state of the granules.

Concerning nitrogen removal, in general, the efficiencies increased over time, with average values ranging between 78 and 84% at stage III, depending on the reactor (Table 1). These data are consistent with several AGS studies, mainly with those that used acetate as a COD source (Henriet et al., 2016; Wang et al., 2018a,b; Wang et al., 2014). In fact, such an increasing trend was already expected as the granulation process occurred. With the increase of the granule size, there is the formation of different redox zones inside the granule, favoring the simultaneous nitrification and denitrification (SND), which is reported to be a key process for nitrogen removal in AGS systems (Rollemberg et al., 2018).

By comparing the control reactors (R1 and R3), although, at stage III, the total nitrogen removal efficiency in R3 (83%) was higher than in R1 (78%), this difference was not statistically significant ($p = 0.059$) (Table 1). Thus, the operation regime did not significantly influence nitrogen removal. Regardless the SBR configuration, the addition of calcium may have not had a significant impact on nitrogen removal either, since similar efficiencies were obtained in the simultaneous fill/draw SBRs (R1: 78%, R2: 79%, $p = 0.883$) and in the conventional ones (R3: 83%, R4: 84%, $p = 0.645$) at stage III (Table 1). In contrast, Liu et al. (2019), analyzing the impact of $Al^{3+}$ in the form of polyaluminum chloride (PAC) and aluminum sulfate in single doses in conventional SBRs, identified that the removal of ammoniacal and total nitrogen was improved due to a higher biomass retention in the reactors supplemented with the cations.

Regarding the nitrogen transformation during the cycles, nitrification occurred efficiently in the four systems, evidenced by the sharp drop and low concentrations of ammonium after 5 h in the cycle (Fig. 4). At the beginning of the aerobic phase, there was a sharp increase in nitrite concentration, which decreased gradually until the end of this phase in all reactors. On the other hand, nitrate concentration was higher at the end of the cycle than at the beginning of the aerobic phase (Fig. 4). Evaluating the concentrations of $NO_2$-N and $NO_3$-N at the end of the cycle, it is possible to conclude that part of the oxidized nitrogen fractions was reduced (denitrification) even in the presence of oxygen (aerobic phase) through the SND process, which is related to the granule size and the consolidation of the anoxic zone in the granule core as discussed above.

Concerning phosphorus, the removal efficiency also increased considerably over time, reaching values between 72% and 82% at the last operational stage, depending on the reactor (Table 1). However, differently from nitrogen removal, phosphorus removal is not influenced by the granule size but by the redox condition alternation during the cycle. Comparing the control reactors R1 (simultaneous fill/draw SBR) and R3 (conventional SBR), after reaching the maturation stage (stage III), the conventional operation regime ensured significantly higher

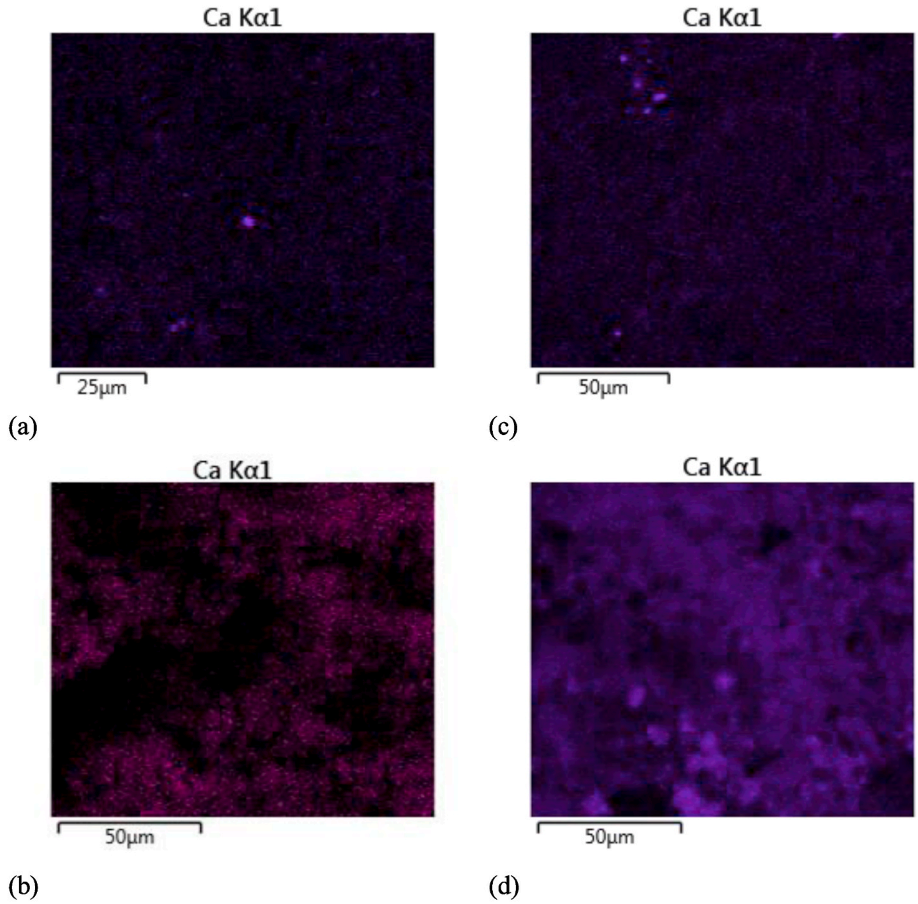


**Fig. 6.** Composition of granules by EDX and the amount of calcium in the EPS matrix obtained in the simultaneous fill/draw SBRs R1 (control) and R2 (supplemented with $Ca^{2+}$) and conventional SBRs R3 (control) and R4 (supplemented with $Ca^{2+}$).

removal efficiencies ($p = 0.007$) (Table 1), which may be due to the lower sludge age reached (section 3.1). In fact, biological phosphorous removal consists of an initial accumulation of P in the cell, followed by the removal through sludge discharge (poly-P biomass). Thus, higher sludge ages result in a lower cell production and then a lower phosphorus removal (Bassin et al., 2012). Conversely, the addition of calcium did not have a significant impact on phosphorus removal in both types of SBR configuration, reaching similar efficiencies at stage III (R1 and R2: $p = 0.443$, R3 and R4: $p = 0.442$). Therefore, in contrast to a previous study (Mañas et al., 2011), phosphorus removal in the reactors supplemented with calcium (R2 and R4) may have occurred mainly through biological processes and not through physical-chemical processes (e.g., precipitation).

### 3.3. Granule characteristics

One of the primary factors for the production of granules is the stimulation of EPS production (Liu and Sun, 2011; Nancharaiah and Reddy, 2018; Zhu et al., 2012). However, the EPS production behavior varied considerably among the reactors (Fig. 5). In conventional SBRs, the production was more pronounced during the first ten days of operation, while, in simultaneous fill/draw SBRs, there was a small increase on the third day of operation, and then EPS production fell and remained practically stable throughout the experiment. These factors can be directly related to the selection pressure imposed, which affects the granulation time and the granule size.

At the end of stage I, the conventional SBRs R3 (control) and R4 (supplemented with $Ca^{2+}$) had more than 50% of the granules greater than 0.6 mm. At the end of stage III, it appears that the addition of calcium did not influence either the total amount of EPS (mg $gVSS^{-1}$) (R3: 221.0 ± 22.0; R4: 218.7 ± 11.7) or the PN/PS ratio (R3: 2.9 ± 0.2; R4: 2.9 ± 0.1). In the simultaneous fill/draw SBRs, the addition of calcium in the R2 reactor was associated with an increase in the amount of EPS (mg $gVSS^{-1}$) (R1: 143.0 ± 11.3; R2: 162.5 ± 18.5), without impacting the granulation time.

By the analysis of EDX, a different behavior was observed between the reactors supplemented with calcium regarding their proportion in the EPS. At the beginning of the experiment, the amounts of calcium in the sludge were similar (0.3% in both systems). However, with 30 days of operation and granules already well-formed, R4 presented 8% of calcium in the EPS composition, and R2 only 0.6%. At the end of the experiment (55th day of operation), R4 remained with a high percentage (7.3%), and R2 presented 1.8% (Fig. 5). These data show that the higher selection pressure and EPS production tend to favor the presence of calcium in the EPS matrix (Fig. 6). In fact, the literature reports higher proportions of calcium in reactors with higher selection pressure. For example, Barros et al. (2020) and Ren et al. (2008) found, respectively, a calcium percentage of 1.1% (100 mg $Ca^{2+}$ $L^{-1}$) and 14% (40 mg $Ca^{2+}$ $L^{-1}$) in the biomass formed in simultaneous fill/draw and conventional SBRs, respectively, both supplemented with calcium.

As for the interaction of calcium with EPS, the literature reports that the ion tends to preferentially bind to the EPS (Jiang et al., 2003; Liu et al., 2010; Ren et al., 2008). Sajjad and Kim (2015) analyzed the interaction of $Ca^{2+}$ and $Mg^{2+}$ in reactors supplemented with cations (25 mg $L^{-1}$) with the NH, CN, and CO groups of proteins and the OH group of polysaccharides. They observed that the addition of $Ca^{2+}$ had a strong interaction with the OH groups and little interaction with the NH and CN groups. However, for the reactor supplemented with $Mg^{2+}$, there was a

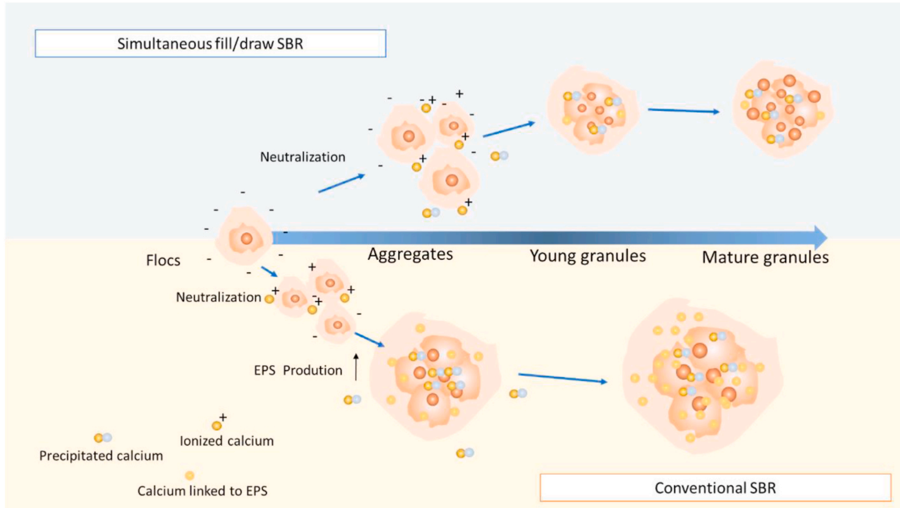


**Fig. 7.** Schematic of calcium action on the formation of granules in simultaneous fill/draw and conventional SBRs.

more significant interaction with the CN group of the protein-peptide bond. The work also revealed that the CN and NH groups of the proteins were more involved than the CO group, suggesting that nitrogen had a more significant relationship with divalent cations. However, it should be noted that the binding of calcium to proteins could also be frequently involved in bacterial adhesion to the surface, and subsequent cell-cell aggregation and colony formation (Rose, 2000).

One of the factors that are most related to the presence of calcium in the aerobic granules is the precipitated crystals that can favor nucleation, mainly in the form of calcium carbonate ($CaCO_3$) and calcium phosphate ($Ca_3(PO_4)_2$) (Liu et al., 2010; Ren et al., 2008; Zhou et al., 2013). Thus, the calcium ion can be linked to the soluble phosphate ion to form chemical precipitates in the form of polyphosphate, as shown in the following chemical reactions:

$$PO_4^{3-} + Ca^{2+} + H^+ \rightarrow CaHPO_4 \quad (1)$$

$$PO_4^{3-} + Ca^{2+} \rightarrow Ca_3(PO_4)_2 \quad (2)$$

However, in the pH range (7–8) applied during the operation of the systems, the phosphate was present in the form of $H_2PO_{4^-}$ and $HPO_4^{2-}$, which have high and intermediate solubility, respectively. Alkalinity was present in the form of bicarbonate, which is highly soluble, with no formation of carbonate ions at a pH below 8.3 (Girard, 2013).

### *3.4. Mechanism of action and immobilization of calcium in the EPS matrix of the aerobic granular biomass*

As previously mentioned, the main mechanism that facilitates microbial aggregation is based on the calcium effect in neutralizing the negative surface charges of microorganisms. Calcium is still able to bond to the EPS structure, establishing a more structurally stable complex, in addition to forming precipitates that serve as a support medium for attached growth (Kończak et al., 2014; Li et al., 2017; Lin et al., 2010; Liu et al., 2010; Ren et al., 2008; Sajjad and Kim, 2015; Xu et al., 2006; Ye et al., 2016). Furthermore, the type of SBR configuration influences the granulation time and characteristics of the biomass cultivated. As a result, different granulation times were obtained, for instance, 35 and 20 days for reactors R1 and R3, respectively. However, with the addition of $Ca^{2+}$, the settling of the biomass formed in R4 increased significantly when compared to R3. This result demonstrated the predominant role of the cation in improving the physical characteristics of the AGS when using a higher selection pressure as that of conventional SBR.

The action of the calcium ions had different effects on the two SBR strategies (Fig. 7). Initially, the ionized calcium tended to neutralize the charges of the flocs and bacterial cells, in which a portion remained in suspension driven by the high air injection in the reactor. However, the precipitation and interaction of calcium with the proteins and polysaccharides of EPS formed in the conventional SBRs were higher, which led to a greater capacity for calcium immobilization in this type of system, verified in mature granules.

## 4. Conclusions

Conventional SBRs showed earlier granule development (20 days) than the simultaneous fill/draw SBRs.

The effect of calcium on granulation was more accentuated in conventional SBRs, forming larger granules in a shorter interval of time due to higher EPS production. Additionally, higher amounts of calcium were found in the EPS matrix, mainly during the formation of granules.

The operation regime and the addition of calcium did not affect the removal of carbon, nitrogen, and phosphorus. However, they both influenced the granulation time, settleability characteristics, size, and granule composition.

## Credit author statement

**Antônio Ricardo Mendes Barros**: Conceptualization, Formal analysis, Investigation, Writing - Original Draft. **Clara de Amorim de Carvalho**: Investigation. **Paulo Igor Milen Firmino**: Writing - Review & Editing, Supervision. **André Bezerra dos Santos**: Conceptualization, Resources, Writing - Review & Editing, Project administration, Funding acquisition.

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## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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