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Research article

Impact of cycle type on aerobic granular sludge formation, stability, removal mechanisms and system performance

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ABSTRACT

This paper aimed to assess the impact of the cycle type on aerobic granular sludge (AGS) formation, stability and system performance. Six AGS reactors were operated either on A/O cycles (anaerobic followed by oxic phase) or A/O/A cycles (anaerobic, followed by oxic and anoxic phases), changing only the phase time distribution. Reactors with high percentage of aerobic phase (65% of the total cycle time) generated granules with better settleability and resistance, however denitrification was impaired. On the other hand, reactors with long anaerobic or anoxic phases presented excellent nutrients removals, but the granules were fluffy and unstable. The best results in terms of performance and stability were achieved in an A/O/A reactor with short anoxic phase (10% of the total cycle) and medium aerobic phase (55% of the total cycle). Therefore, in AGS reactors, it is indispensable to optimize the cycle, aiming at fast biomass formation, long-term granule stability and high-rate pollutants removal.

1. Introduction

For a long time, the conventional activated sludge system has been considered the main biological technology used in wastewater treatment plants (WWTPs) (Guven et al., 2019). However, a few decades ago, the aerobic granular sludge (AGS) process emerged (Tay et al., 2001), bringing a new alternative to the effluent treatment market. In comparison with activated sludge, several advantages can be found in AGS, such as simultaneous removal of C, N and P in the same tank, high settleability and biomass concentration, and significant reduction in surface area (75%) and power consumption (30–50%).

The aerobic granules are preferably grown in sequencing batch reactors (SBRs), and there are some factors which influence the granulation process, such as the reactor configuration (Rollemberg et al., 2019), which refers to the SBR's operating regime type, usually divided into the phases of filling, reaction (anaerobic, aerobic or oxic, and anoxic), settling and decanting (Rollemberg et al., 2018).

The anaerobic filling is usually accepted to be the best condition to select beneficial microorganisms for the granules, such as polyphosphate-accumulating organisms (PAOs) and glycogen-accumulating organisms (GAOs) (Liu and Liu, 2006). Another important point is to use settling times below 30 min to washout fast-growing and floc-forming organisms (Rollemberg et al., 2018). Nevertheless, both the type of the cycle (anaerobic-aerobic or anaerobic-aerobic-anoxic) and distribution of the reaction phases are not yet clear in literature, especially concerning their effect on granules stability (Pishgar et al., 2019; Filali et al., 2012).

The first experiments with AGS were conducted with A/O (anaerobic-oxic) systems aiming at the conversion of readily biodegradable substrate into polyhydroxyalkanoates (PHA), intracellular biopolymers associated with phosphorus removal (Filali et al., 2012). However, some studies have shown problems, such as nitrite (or nitrate) accumulation and, in other cases, low phosphorus removal, probably because of the competition between PAOs and denitrifying bacteria for the available organic carbon (Kishida et al., 2006). To solve this problem, an alternative cycle with three phases was proposed: anaerobic-oxic-anoxic (A/O/A), aiming to favor denitrifying PAOs (DPAOs) (Zeng et al., 2004), which have a lower growth rate, thus leading to a 20–30% reduction in sludge production (Murnleitner et al., 1997).

Although the A/O/A cycles present many advantages, some problems have been reported in literature. Recently, Pishgar et al. (2019), using an A/O/A SBR, observed that the granules formed were small, less resistant and had a very filamentous structure, probably due to the low shear stress imposed in the anoxic phase (different from what occurs in

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the aerobic phase when the granules are exposed to a higher shear stress caused by oxygen bubbles). Furthermore, investigations into A/O/A cycles resulted in low phosphorus removal because of the high presence of denitrifying GAOs (DGAOs), which compete directly with the DPAOs (Zhang et al., 2014).

In addition to the questions related to cycle condition (A/O or A/O/A), the phase time distribution is not yet well established in literature. For example, for the same total cycle of 6 h, He et al. (2018) used an aerobic phase of 60 min, while Wang et al. (2009) used 240 min. Therefore, the present work studied the impact of the cycle type (condition and phase time distribution) on aerobic granular sludge (AGS) formation, stability and system performance. Such optimization is important not only for a better performance of the system in terms of organic matter and nutrients removal efficiencies but also for the reduction in the aeration demand and hydraulic retention time/total cycle time.

2. Material and methods

In the evaluation of the cycle time distribution, six identical SBRs were operated under the same conditions, changing only the type of the cycle. The reactors were started up with the same inoculum. In the start-up (16 days), the settling time was 20 min (period I). Subsequently, it was reduced to10 min in period II (15 days, system granulation) and, then, to 5 min in period III (63 days, granules maturation) (Table 1). As described in literature, the reduction in the settling time is very important to achieve a good aerobic granulation in terms of efficiency and long-term stability (Rollemberg et al., 2018). The total duration of the experiment was 94 days, and the parameters used to identify when the reactors reached the steady state were MLVSS concentration (in the reactor and effluent), percentage of granules and biomass settleability.

2.1. Set-up and operational conditions of the SBRs

The experiments were carried out in column-type SBRs with working volume of 7.6 L, internal diameter of 10 cm and height of 100 cm. The air was injected (in the aerobic phase) into the bottom (air compressor Yuting SUN, China) of the reactors through fine-bubble porous diffusers. The dissolved oxygen (DO) concentration remained between 2 and 4 mg/L in the aerobic phase, and the aeration velocity was 1.2 cm/s, which was defined according to Tay et al. (2001). In the anoxic phase, the DO concentration was below 0.5 mg/L, and, in the anaerobic phase, there was no DO. The volumetric exchange ratio per cycle was 50%, and the hydraulic retention time (HRT) was 12 h.

The systems operation was automated using synchronized timers. The SBRs were subjected to mechanical stirring during the anaerobic and anoxic phases to prevent sludge settling (Magnetic Stirrer, WEA, 20 rpm). They were operated at room temperature, and the wastewater temperature inside the reactors was 28 ± 2 °C. No discharge of sludge was performed over the experiment, resulting in different sludge retention times (SRTs) in the systems due to natural sludge washout.

Table 1
Different phase time (in min) distributions for the investigated SBRs.

Phases	A/O systems			A/O/A systems		
	R1	R2	R3	R4	R5	R6
Filling (anaerobic)	35 (10%)	35 (10%)	35 (10%)	35 (10%)	35 (10%)	35 (10%)
Anaerobic	71 (20%)	107 (30%)	143 (40%)	89 (25%)	89 (25%)	89 (25%)
Aerobic	233 (65%)	197 (55%)	161 (45%)	197 (55%)	179 (50%)	161 (45%)
Anoxic	–	–	–	18 (5%)	36 (10%)	54 (15%)
Settling	20/10/5	20/10/5	20/10/5	20/10/5	20/10/5	20/10/5
Decanting	1	1	1	1	1	1

The percentage of some phases in relation to the total cycle is shown in parentheses.
In all systems, the idle time was the remaining value to complete the total cycle of 6 h.
A/O, anaerobic/oxic phases; A/O/A, anaerobic/oxic/anoxic phases.

2.2. Seed sludge and wastewater

The SBRs were inoculated with an aerobic sludge from an activated sludge wastewater treatment plant (Fortaleza, Ceará, Brazil) at the same concentration of mixed liquor suspended solids (MLSS) (~3 g/L). The reactors were fed at their bottom, during the anaerobic phase, with a synthetic wastewater containing 515 mg/L of sodium acetate (equivalent to a chemical oxygen demand, COD, of 550 mg/L), 50 mg/L of NH_4^+-N (ammonium chloride), 5 mg/L of PO_4^{3-}-P (potassium phosphate monobasic) and 1 mL/L of a trace elements solution as described by Rollemberg et al. (2019). The pH of influent wastewater was adjusted to about 7.5 using sodium bicarbonate.

2.3. Analytical methods

Influent and effluent samples were collected three times a week for analysis of COD, ammonium (NH_4^+-N), nitrite (NO_2^--N), nitrate (NO_3^--N) and phosphate (PO_4^{3-}-P), while mixed liquor samples were collected once a week for analysis of mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS) and sludge volumetric index (SVI).

COD, pH, NH_4^+-N, NO_2^--N, NO_3^--N, total phosphorus (TP), solids and SVI at 10 and 30 min (SVI_{10} and SVI_{30}) were determined according to APHA (2005), whereas DO was measured by a YSI Model 5000 m (YSI Incorporated, USA). Total inorganic nitrogen (TIN) was regarded as the sum of NH_4^+-N, NO_2^--N and NO_3^--N (Long et al., 2014). The extracellular polymeric substances (EPS) were extracted by a modified heat extraction method proposed by Yang et al. (2014). The protein (PN) content was determined by a modified Lowry method, and the polysaccharides (PS) content was analyzed using a phenol-sulfuric acid method (Long et al., 2014). The EPS were regarded as the sum of PN and PS. Granule formation and maintenance were evaluated by particle size analysis. The aerobic reactor is considered predominantly granular when more than 80% of the biomass has diameter greater than 0.2 mm (de Kreuk et al., 2007).

The physical resistance (shear test) analysis of the granules followed the methodology described by Nor-Anuar et al. (2012). Therefore, the granules were subjected to a shear force caused by a rotation of approximately 200 rpm for 10 min. The defragmented fraction identified (which passed through a 0.2-mm sieve) was expressed in terms of the stability coefficient (S). This coefficient was classified into three categories: very resistant ($S < 5\%$), resistant ($5\% \leq S \leq 20\%$) and non-resistant ($S > 20\%$). Therefore, the lower values of S (%), the higher stability of the aerobic granules.

2.4. Cycle test

To understand the mechanisms of SNDPR (simultaneous nitrification, denitrification and phosphorus removal), the processes which occurred during a cycle were evaluated. The cycle tests were conducted at the end of the experiment (period III), when the reactors achieved a pseudo-steady-state condition. The concentrations of COD, NH_4^+-N, NO_2^--N, NO_3^--N and PO_4^{3-}-P were analyzed, and dissolved oxygen (DO) concentrations were measured continually (data not shown) as described by Li et al. (2019). The EPS production and consumption were monitored according to Rollemberg et al. (2019) and He et al. (2018).

2.5. Statistical methods

The non-parametric Mann-Whitney test was used to compare the performance of the reactors at a confidence level of 95%, in which the data groups were statistically different when $p \leq 0.05$.

3. Results and discussion

3.1. Start-up and biomass concentration dynamics

The evolution of solids concentration and SVI_{30} of the sludge during the whole experiment is shown in Fig. 1. The reactors started with similar inoculum and showed the same suspended solids concentration and SVI after the start-up (3 g/L MLSS and SVI_{30} of 185 mL/g).

The granulation process occurred in different periods, showing the effect of the phase time distribution on the biomass change. At steady state, the mixed liquor solids concentration was very different in the systems. However, the VSS/TSS ratio was close to 90% in all reactors, which was also reported elsewhere (Corsino et al., 2017).

The sludge settling times of 20, 10 and 5 min resulted in an up-flow velocity in the reactors higher than 1.5, 3 and 6 m/h, respectively. Although a high loss of biomass was observed during the start-up (period I), the reductions in the settling time to 10 min (period II) and 5 min (period III) did not cause high losses of solids in the systems. As shown in Fig. 1, among all the systems, R1 had the highest retention of solids (>3.5 g/L) followed by R4 (≈3.2 g/L) and R2 (≈3 g/L). On the other hand, R5 (≈2.2 g/L) and, especially, R6 (≈1.8 g/L) and R3 (≈1.6 g/L) showed very low solids retention. In terms of settleability (Fig. 1), R1 had the best SVI_{30} result (<40 mL/L) and, as occurred with the solids concentration, R3 and R6 also obtained the worst settleability results (>80 mL/L).

The solids retention capacity was different among the reactors, and the settleability of the granules was directly related to the phase time distribution, in which the reactors with the highest aeration phase (R1, R2 and R4) were those which presented the best results. Such findings contrast those reported by He et al. (2018), who observed that the increase in the anoxic phase (and consequent reduction in the oxic phase) caused an improvement in the solids concentration and the biomass settleability. On the other hand, the present results are in agreement with the recent investigation carried out by Pishgar et al. (2019), who observed that the increase in the aerobic phase increased the solids concentration in the reactor and decreased the SVI_{30} values. According to these authors, low shearing activity obtained in anaerobic and anoxic phases could further favor the appearance of filaments in the granules. Literature reports that aeration is directly related to the increase in hydrophobicity, EPS production (microbial stress) and the specific weight of the granules (shear stress improving microbial aggregation) (Tay et al., 2001; Nancharaiah and Reddy, 2018).

For the type of wastewater used in the present work, it seems that a minimum aeration of 55% of the total cycle duration (197 min) is required for both the formation of an AGS with good settling capacity and good retention of solids (in this study, the optimum time was 233 min, i.e. 65% of the total cycle duration). This value is in agreement with those used by Nereda®, an aerobic phase of approximately 60–70% of the total cycle duration (van Haandel and van der Lubbe, 2012). Some studies on full-scale AGS systems also used longer aerobic phases than those reported in the present work (~70% of the total cycle duration) (Li et al., 2014; Pronk et al., 2015). Thus, the values found in the current investigation may be applied to full-scale systems.

It is worth mentioning a comparison between R2 and R4, since both reactors had the same proportion of aerobic phase (55%) (Table 1). However, R4 had a short anoxic phase (5% of the total cycle duration) with consequent reduction in the anaerobic phase. This change proved to be beneficial to the system in terms of total nitrogen (TN) removal (Table 2), probably for favoring the presence of DPAOs and DGAOs. Literature reports that the anoxic phase after the oxic phase may benefit these microbial groups (Zhang et al., 2014), which can store organic matter as PHA and, therefore, outcompete floc-forming organisms and filamentous bacteria, improving biomass settleability (Beun et al., 1999; Liu and Liu, 2006). However, the reduction in the anaerobic phase to include an anoxic phase should be done with caution because extended anaerobic reaction time might be the main reason for the excellent

Table 2
Removal of COD, nitrogen and phosphorus of the AGS systems after maturation (period III).

Parameter	A/O Systems			A/O/A Systems		
	R1	R2	R3	R4	R5	R6
COD_{inf} (mg/L)	581 ± 51	537 ± 57	584 ± 24	529 ± 39	546 ± 47	559 ± 43
COD_{eff} (mg/L)	42 ± 27	49 ± 31	59 ± 35	53 ± 21	49 ± 33	56 ± 27
COD removal (%)	91 ± 5	90 ± 8	89 ± 6	89 ± 8	91 ± 9	90 ± 4
NH_4^+-N_{inf} (mg/L)	53 ± 2	49 ± 5	53 ± 7	52 ± 4	55 ± 4	48 ± 6
NH_4^+-N_{eff} (mg/L)	4 ± 1	4 ± 5	13 ± 8	7 ± 3	11 ± 4	14 ± 6
NO_2^--N_{eff} (mg/L)	4 ± 1	3 ± 2	5 ± 1	1 ± 1	1 ± 1	1 ± 1
NO_3^--N_{eff} (mg/L)	7 ± 2	5 ± 1	2 ± 1	1 ± 1	0	0
NH_4^+-N removal (%)	92 ± 5	91 ± 5	75 ± 4	90 ± 5	78 ± 8	71 ± 7
TN removal (%)	71 ± 6	73 ± 6	63 ± 6	85 ± 4	78 ± 2	57 ± 4
PO_4^{3-}-P_{inf} (mg/L)	9 ± 2	10 ± 1	12 ± 2	10 ± 2	9 ± 1	11 ± 2
PO_4^{3-}-P_{eff} (mg/L)	3 ± 1	3 ± 1	4 ± 3	3 ± 2	4 ± 1	5 ± 1
TP removal (%)	68 ± 8	71 ± 4	68 ± 7	70 ± 5	50 ± 8	54 ± 4
MLVSS effluent (mg/L)	62 ± 19	58 ± 21	41 ± 13	56 ± 17	42 ± 17	41 ± 12

A/O, anaerobic/oxic phases; A/O/A, anaerobic/oxic/anoxic phases.

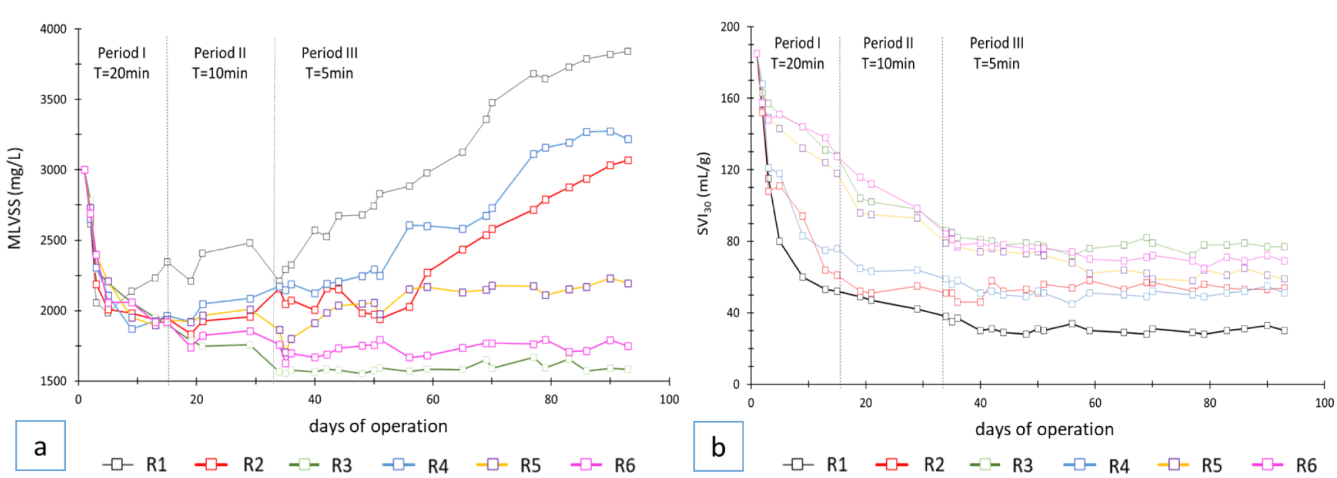

Fig. 1. MLSS concentration profile during the experiment (a) and evolution of the sludge volume index at 30 min (SVI_{30}) (b).

performance achieved. It is important to mention that the anaerobic phase is essential for the biological phosphorus removal mechanism because, during this phase, the phosphorus release process occurs (Wang et al., 2015).

3.2. Formation and stability

Aerobic granules are defined as microbial aggregates with minimum size of 0.2 mm. The granulation process is considered complete when AGS corresponds to 80% of the solids in the reactor (de Kreuk et al., 2007). The formation of AGS is related to the selection pressure imposed on the system, such as the settling time (Beun et al., 1999), hydraulic shear stress (Liu and Tay, 2002), substrate composition and organic loading rate (OLR) (Gao et al., 2011). Although all the parameters mentioned were the same in the reactors used (only the phase time distribution of the cycles was changed), the appearance of the first granules occurred in different periods, leading to the conclusion that the phase time distribution also has a direct impact on granules formation.

R1 was the system which showed the fastest formation of granules (9 days), followed by R2 and R4 (12 days). For these reactors, in less than 20 days, more than 50% of the granules already had diameter greater than 0.2 mm. On the other hand, a late formation of granules in reactors with longer anaerobic or anoxic phases, specifically in R3 and R6 (approximately 20 days), was verified.

The results found here were similar to those observed by Jiang et al., 2016. The authors introduced an anoxic phase after the aeration phase. They verified high removal of COD and nutrients (90% of COD, 88.5% of nitrogen and 90% of phosphorus). However, the AGS formation process was slow (almost 3 months were necessary to achieve complete granulation).

The systems R3 and R6 (reactors with short aerobic phase), besides requiring a longer time for granulation (~20 days), also presented instability of the granules during the experiment, with oscillation of the solids percentage for diameters higher than 0.2 mm. In the other reactors, after granule maturation (period III), it was observed that the percentage of granules greater than 0.2 mm was always above 90%.

Some studies also reported AGS instability with granules disintegration and biomass washout in the effluent (Wan et al., 2013). Currently, many authors consider instability as a major problem for the application of the AGS technology to full-scale WWTPs (Show et al., 2012; Zhang et al., 2016).

The explanation for instability observed in the R3 and R6 systems may be related to the long periods without DO. It seems that DO deficiency favors the filamentous microorganisms growth, which causes the breakage of aerobic granule (Gaval and Pernell, 2003; Rossetti et al., 2005). Studies show that oxygen deficiency or other stresses, when frequent in the operation, can be an important inducer for the development of dominant filamentous microorganisms during the biomass growth (Gaval and Pernell, 2003).

Another possible cause of the instability (disintegration) of the granules observed in R3 and R6 may be related to the size of the granules. The reduction in the aerobic phase and consequent increase in the anoxic phase reduces the shear stress (caused by the oxygen bubbles). As a result, an uncontrolled increase in the size of the granules is achieved, since the aeration also acts on the size control, breaking the filaments on the biomass surface (Franca et al., 2018). The filamentous outgrowth and pore clogging in large aerobic granule hinder the diffusion of dissolved oxygen, substrate and nutrients in the AGS. This condition (starvation) establishes an anaerobic environment in the AGS and causes decreased granules activity and, then, cell lysis (Toh et al., 2003).

3.3. Characteristics of the granules

The AGS formed had different diameters, color and physical strength (Table 3). Therefore, the phase time distribution of the cycle also affected the characteristics of the granules, probably due to the different microbial groups which are favored according to the type of the cycle time used (He et al., 2018).

The granules formed in the reactors which had a longer aeration phase had lighter color and a more rounded shape with a regular surface, differently from reactors with longer anaerobic and anoxic phases (R3 and R6), which formed darker granules with irregular surfaces (Fig. 2). Similar results were presented by Mosquera-Corral et al. (2005) and Val del Río et al. (2013), in which it was shown that long anaerobic or anoxic phases made fluffy and soft granules with irregular surfaces and presence of filaments.

Although the average size of the granules was similar, the settling velocities were quite different. The reactors with longer aeration phases formed denser granules with higher settling velocities, which is in agreement with Pishgar et al. (2019). The settling capacity of the formed granules was evaluated according to the SVI_{30}/SVI_{10} ratio, in which the closer to 1, the better is the biomass settleability (Rollemberg et al., 2019).

With regard to EPS, these substances play a key role in AGS structure (Nancharaiah and Reddy, 2018), in which PS and PN are responsible for aggregation and granule mechanical stabilization, respectively (Sarma et al., 2017). As expected, the reactors which had the longest aeration phase (R1, R2 and R4) presented a greater amount of EPS, confirming that the EPS production is stimulated by the stress caused by the aeration conditions (Liu et al., 2010). The higher amount of EPS may also have contributed to the better settleability of the granules of these reactors. The contents of EPS in aerobic granules with different phase time distribution are shown in Table 3.

A sharp increase in the EPS amount, especially the PN content, was related to be beneficial for AGS (Liu et al., 2010). In most studies on AGS, PN is the most abundant component of EPS in stable granules. To evaluate the relationship between PS and PN, the PN/PS ratio has been evaluated in AGS studies (Rollemberg et al., 2018). In this study, it was observed PN/PS ratios in R1, R2, R3, R4, R5 and R6 were 1.1, 1.1, 0.9, 1.2, 0.9 and 0.9, respectively. These results were similar to those reported in others studies on AGS, which found that aerobic granules with a PN/PS ratio close to 1.0 resulted in a system with good stability (He et al., 2018).

The results reported for reactor R4 are important to highlight, since the addition of a short anoxic phase increased EPS production and improved the PN/PS ratio. The possible explanation for this is the equilibrium between production and consumption of EPS which occurred in this type of cycle. As it is known, the production of EPS occurs mainly in the feast period and the consumption of these substances occurs in the famine period. The cycle in R4 (using a shorter aerobic phase) may have reduced the EPS consumption. However, in the reactors which had long anoxic phases and short aerobic phases (R5 and R6), the EPS production was low. These results show that there is an optimal aeration phase for the maximum production of EPS, after which the EPS will be continuously consumed. The most favorable situation pointed out by some authors is to reach a maximum production and to use this material as an electron donor for denitrification in the famine period (Wu et al., 2012).

Regarding the granules strength, the stability coefficient (S) was used, in which low values of S (%) are related to the presence of granules with high stability (Nor-Anuar et al., 2012). In this way, granules grown in R4 presented better results, and granules grown with long anoxic phases showed low resistance, possibly due to the low shear stress applied. The resistance results are in accordance with literature (Val del Río et al., 2013; Pishgar et al., 2019).

3.4. System performance

The reactors performance was evaluated in terms of COD, nitrogen and phosphorus removal (Table 2). In all systems, COD removal was high, but nitrogen and phosphorus removals were slightly different.

The phase time distribution did not appear to have a significant effect

Table 3
Characteristics of the granules formed in the reactors after maturation (period III).

Parameter	A/O systems			A/O/A systems		
	R1	R2	R3	R4	R5	R6
Mean diameter (mm) after maturation	1.3 ± 0.1	0.9 ± 0.2	1.1 ± 0.7	1.3 ± 0.3	0.9 ± 0.4	1.0 ± 0.6
Stability indicator (%)	39.4 ± 14.5	44.9 ± 17.3	47.2 ± 20.1	37.3 ± 9.2	44.5 ± 11.8	46.9 ± 19.7
Total EPS (mg/L)	903.8 ± 274.1	846.3 ± 198.6	382.8 ± 89.5	1273.1 ± 225.3	441.9 ± 160.1	613.87 ± 213.9
PN/PS ratio	1.1	1.1	0.88	1.2	0.89	0.91
PS/VSS (mg/mg VSS)	51.8	50.4	26.1	50.3	27.3	28.4
PN/VSS (mg/mg VSS)	56.9	55.5	24.5	60.3	24.2	25.6
Settling velocity (m/h)	41.7 ± 8.5	39.6 ± 11.3	18.6 ± 5.1	39.0 ± 12.9	36.3 ± 10.7	22.1 ± 6.0
SVI_{30}/SVI_{10}	0.98 ± 0.11	0.95 ± 0.13	0.88 ± 0.21	0.96 ± 0.12	0.89 ± 0.15	0.86 ± 0.19
Formation of the first granules (days)	9	12	20	12	15	20
Time of maturation (days)	21	22	>30	20	28	>30
Color	Light yellow	Yellow	Brown	Yellow	Yellow-brownish	Brown
Format	Granular and spherical	Granular and spherical	Granular with irregular surface	Granular and spherical	Granular with irregular surface	Granular with irregular surface

A/O, anaerobic/oxic phases; A/O/A, anaerobic/oxic/anoxic phases.

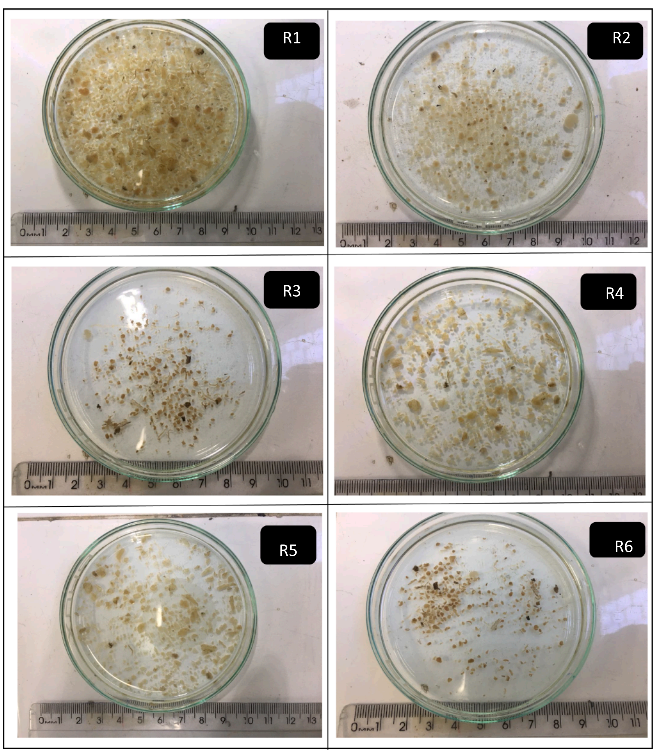

Fig. 2. Aerobic granules collected from the investigated reactors after maturation (period III).

on COD removal (p = 0.08), and, even in cycles with long anaerobic phases, a high removal of organic matter was observed. Thus, high COD removals can be obtained even in AGS systems with long anaerobic phases. Martins et al. (2004) reported that anaerobic filling phase could favor the hydrolysis of more complex substrates. However, it cannot be long enough to favor the presence of filamentous microorganisms (Wagner et al., 2015).

On the other hand, the removal of ammonium showed significant differences (p = 0.04). The reactors which had an aeration phase equal to or greater than 50% of the total cycle time (R1, R2 and R4) showed ammonium removal near 90% (Table 2). However, the reactors which had short aerobic phases showed ammonium removal below 80%. These results differ from those presented by He et al. (2018), in which approximately 90% ammonium removal was achieved with a 6-h cycle (60 min of aeration). However, in the above-mentioned work, the COD was only 200 mg/L, and ammonium was only 20 mg/L (low-strength wastewater), unlike the effluent used in the present study, which presented higher content of organic matter (550 mg/L COD) and ammonium (50 mg/L).

Therefore, for low-strength wastewaters, it seems that is possible to have low aeration time without compromising nitrification. However, in the case of medium-strength wastewater, a sufficient aerobic phase (at least 50% of the total cycle duration) is required to ensure nitrification. It is important to note that, at the beginning of aeration, the DO available in the medium is preferably used by ordinary heterotrophic bacteria in the oxidation of organic matter and, at the end, by the autotrophic bacteria in the nitrification metabolism.

For reactors with A/O/A cycles (R4, R5 and R6), the presence of nitrite and nitrate in the final effluent is very low (Table 2) as verified elsewhere (Zeng et al. al., 2004). The reactor which presented the best results in terms of total nitrogen (TN) removal was R4. Even in A/O/A reactors, it is still possible to have low TN removal if the aerobic phase is not long enough for ammonium nitrification. A solution for reactors with short aerobic and long anoxic phases would be Anammox granules. However, the characteristics of the wastewater (low ammonium) and lack of specific conditions for the enrichment of Anammox bacteria practically impaired the presence of these microbial groups (Lema and Suárez, 2017).

Regarding the removal of phosphorus, significant differences were also observed (Table 2). The removals in R1, R2, R3 and R4 were very close (around 70%) and statistically similar (p = 0.09). On the other hand, R5 and R6 presented significant differences (p = 0.04), and the longer anoxic phase increase caused a reduction in phosphorus removal. The explanation for this fact might be related to the competition between DPAOs and DGAOs. As it is known, the anoxic phase does not only favor the growth of DPAOs, but also it can favor denitrification via DGAOs. When the anoxic phase is short enough, only for sequestration of residual phosphorus via DPAOs, this group predominates. However, when the anoxic phase is long, DGAOs begin to act on the denitrification and to compete with DPAOs. They have similar metabolism, but DGAOs are not able to remove phosphorus (Kishida et al., 2006).

Finally, the reactors which had longer aerobic phases were those which generated more VSS in the effluent. This result can be explained by the cell growth rate. In the A/O cycle reactors, denitrification occurs mainly by denitrifying ordinary heterotrophic organisms (DOHOs), whereas, in the A/O/A reactors, the denitrification occurs preferentially by DPAOs and DGAOs (denitrifying bacteria capable of accumulating phosphorus or glycogen). The sludge production of DPAOs and DGAOs is about 20–30% lower than that of DOHOs (Murnleitner et al., 1997).

3.5. Cycle tests

In order to elucidate organic matter and nutrients removal in the mechanism of SNPDR, a full cycle analysis was carried out (Fig. 3).

Initially, approximately 50% of COD was fermented in the anaerobic phase, and that residual nitrite and nitrate from the previous cycle was denitrified as early as the first 30 min of filling. In both systems, the release of phosphorus was observed in the anaerobic phase, whose rates were similar to those reported by He et al. (2018). In the aerobic phase, during the first hour, the residual COD was oxidized. It is also noteworthy the occurrence of denitrification in the famine period. Because heterotrophic denitrification can use EPS as electron donor in the famine period (Wu et al., 2012), it is possible that the PS stored in the granules during the feast period was used at the end of the cycle as an electron donor in endogenous denitrification.

Due to the long aeration phase, R1 presented a slight nitrate accumulation. Although the simultaneous nitrification and denitrification (SND) may have occurred, the absence of COD at the end of the cycle complicated denitrification. On the other hand, for R3, the long anaerobic phase and consequent reduction in the aerobic phase caused low nitrification efficiency, thus causing accumulation of ammonium. The cycle which presented the best performance was that used in R4, since the aerobic phase was enough for nitrification, and the short anoxic phase added to the end of the cycle facilitated denitrification of both nitrite and the residual nitrate. Moreover, probably, the intracellular PHA might have been used as an electron donor for denitrification in the anoxic phase (Wang et al., 2015).

The cycle tests showed that a small reduction in the aeration time and the presence of an anoxic phase improved not only the removal of nitrite and nitrate, but also the removal of phosphorus. Such a finding is in accordance with other investigations who observed that, when the aeration time was reduced, the populations of denitrifying heterotrophic bacteria and DPAOs increased, which could compete for NO_3^--N and NO_2^--N (Liu et al., 2017; Zhang et al., 2016). Phosphorus-accumulating bacteria can use both nitrite and nitrate as electron acceptors (He et al., 2018). Therefore, the reduction in the aeration time can also positively influence the distributions of key functional groups.

The cycle tests showed that the nitrogen removal might have occurred through different mechanisms: exogenous denitrification, SND and endogenous denitrification. In the filling phase, the residual nitrite and nitrate from the previous cycle might have been rapidly denitrified using the readily available organic matter as electron donor (exogenous denitrification). In the aerobic phase, the ammonium oxidized to nitrite or nitrate might have been denitrified through the SND mechanism. Finally, in the anoxic phase, exclusively in the A/O/A SBRs, the remaining nitrite or nitrate might have been denitrified using the previously stored intracellular organic matter as the electron donor (endogenous denitrification).

A summary of the efficiencies achieved in terms of C, N and P, in comparison with other investigations which used different type of the cycles, is presented in Table 4. Evaluating the results regarding the removals of C, N and P, it was observed that the works which used very long aerobic phases had low removals of N and P. On the other hand, the insertion of anaerobic and anoxic phases improved the removal of these compounds. However, none of the studies cited evaluated the stability of the formed granules. The current investigation revealed that the presence of long anoxic phase impacts on the surface of the granules (they become irregular and with a high presence of filaments). Therefore, although A/O/A cycles assist nutrient removal, it is important that the anaerobic and anoxic phases be short (only sufficient to improve denitrification and biodesphosphatation) without affecting the granule settleability and long-term stability.

The optimal results achieved in R1 (A/O) and mainly in R4 (A/O/A) showed that the performance of these systems regarding the removal of organic matter and nutrients were close to the studies which reported good results in terms of SNDPR (Table 4). Once again emphasizing that, in the current work, the substrate had different concentrations in terms of C, N and P. Regarding the phosphorus, for example, He et al. (2018) used a concentration close to 3 mg/L, while the present work used 10 mg/L. This difference further reflects phosphorus removal by the selection of PAOs, since, at low phosphorus concentrations, one of the major mechanisms of removal is assimilation.

4. Conclusion

Considering the overall results, it seems that a minimum aeration time (55% of the total cycle) is necessary to obtain stable granules. A short anoxic phase helped the removal of nutrients without

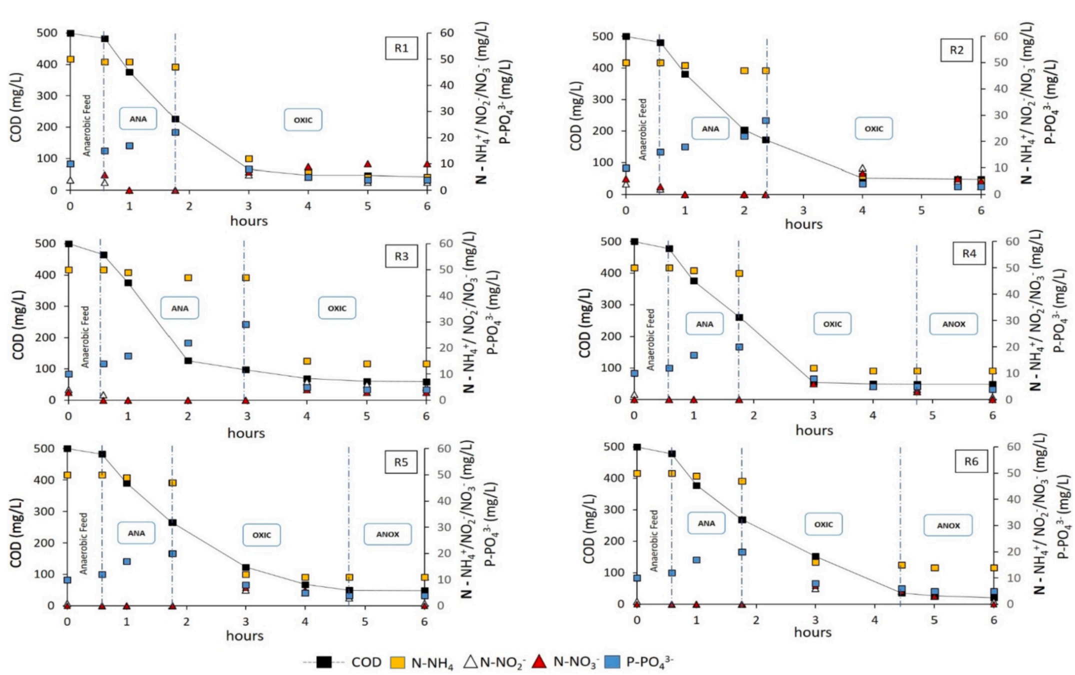

Fig. 3. C, N and P concentration profiles throughout the cycle tests under different phase time distributions.

Table 4
Comparisons of the best SNDPR systems with related works.

Removal (%)			Cycle type	Cycle phases (min)					Cycle duration (h)	Reference
COD	TN	TP		Filling (anaerobic)	Anaerobic	Aerobic	Anoxic	Settling		
>90	>75	>55	A/O/A	4	120	90	144	2	6	He et al. (2017)
>90	>80	>80	A/O/A	55	90	120	90	4	6	Zhang et al. (2015)
>90	>80	>80	A/O/A	6	90	120	140	1	6	Kishida et al. (2009)
>90	>80	>70	A/O/A	35	89	197	18	5	6	This study (R4)
>90	>50	>50	A/O	5	90	220	0	40	6	Lemaire et al. (2006)
>60	>50	>70	A/O	5	90	240	0	5	6	Wang et al. (2009)
>90	>70	>60	A/O	35	71	233	0	5	6	This study (R1)

In all systems, the idle time was the remaining value to complete the total cycle of 6 h.
A/O, anaerobic/oxic phases; A/O/A, anaerobic/oxic/anoxic phases.

compromising granule stability. Cycles with long anaerobic or anoxic phases can present instability in long periods due to the fragility of the granules formed, although they improve nutrients removal. The highest amount of EPS which was not oxidized in the aerobic phase may be the factor responsible for denitrification in anoxic phase in A/O/A cycles. The modification of the phase time distribution affected the formation, stability and system performance.

Author contributions section

Silvio Luiz de Sousa Rollemberg: Conceptualization, Formal analysis, Investigation, Writing - Original Draft. **Tasso Jorge Tavares Ferreira**: Investigation. **Paulo Igor Milen Firmino**: Writing - Review & Editing, Supervision. **André Bezerra dos Santos**: Resources, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

None.

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