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Technical Article

Effects of coal ash supplementation on aerobic granular sludge cultivated in a simultaneous fill/draw sequencing batch reactor

Efeitos da suplementação de cinzas de carvão mineral em lodo granular aeróbio cultivado em reator em batelada sequencial de alimentação/descarte simultâneos

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ABSTRACT

This study aimed to verify if coal ash, a residue from thermal power plants, could act as a granulation nucleus, cations source, and abrasive element to favor granules formation and stability in aerobic granular sludge (AGS) systems. Two simultaneous fill/draw sequencing batch reactors (SBRs) (R1 and R2) were operated with 6-h cycles, *i.e.*, the filling and drawing phases occurred simultaneously, followed by the reaction and settling phases. R1 was maintained as control, while R2 was supplemented with coal ash (1 g·L^{-1}) on the first day of operation. Granulation was achieved in both reactors, and no significant differences were observed in terms of settleability, biomass retention, morphology, resistance to shear, and composition of the EPS matrix. However, the ash addition did not change the settleability, biomass retention, granule morphology, shear resistance, and extracellular polymeric substances (EPS) content significantly. COD removal was high (≥ 90%), while nitrogen (~50%) and phosphorus (~40%) removals were low, possibly due to the presence of nitrate during the anaerobic phase. With granulation, microbial population profile was altered, mainly at the genus level. In general, the operational conditions had a more considerable influence over granulation than the ash addition. The possible reasons are because the ash supplementation was performed in a single step, the low sedimentation rate of this particular residue, and the weak interaction between the ash and the EPS formed in the granular sludge. These factors appear to have decreased or prevented the action of the ash as granulation nucleus, source of cations, and abrasive element.

Keywords: aerobic granulation; simultaneous fill/draw SBR; coal ash.

RESUMO

O objetivo deste estudo foi verificar se a cinza de carvão mineral, um resíduo de usinas termelétricas, poderia atuar como núcleo de granulação, fonte de cátions e elemento abrasivo em sistemas de lodo granular aeróbio (LGA) para favorecer a formação e estabilidade dos grânulos. Dois reatores em batelada sequencial (RBS) (R1 e R2) foram operados em regime de alimentação/descarte simultâneos com ciclos de 6 h, ou seja, as fases de alimentação e descarte do efluente ocorreram simultaneamente, seguidas das fases de reação e de decantação. O R1 foi mantido como controle, enquanto o R2 foi suplementado com as cinzas (1 g·L^{-1}) no primeiro dia de operação. A granulação foi alcançada em ambos os reatores, não havendo diferenças marcantes em termos de sedimentabilidade, retenção de biomassa, morfologia do grânulo, resistência ao cisalhamento e conteúdo de substâncias poliméricas extracelulares (SPE). A remoção de DQO foi alta (≥ 90%), enquanto as remoções de nitrogênio (~50%) e fósforo (~40%) foram baixas, possivelmente pela presença de nitrato na fase anaeróbia. Após a granulação, o perfil da comunidade microbiana mudou, especialmente em termos de gênero. Globalmente, as condições operacionais tiveram maior influência sobre a granulação do que a adição das cinzas, possivelmente porque elas só foram adicionadas uma vez e possuem baixa velocidade de sedimentação, bem como devido a uma fraca interação das cinzas com a matriz de SPE formada no lodo aeróbio. Esses fatores podem ter diminuído, ou mesmo impedido, a ação das cinzas como núcleo de grânulo, fonte de cátions ou elemento abrasivo.

Palavras-chave: granulação aeróbia; RBS com alimentação/descarte simultâneos; cinzas de carvão mineral.

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INTRODUCTION

Aerobic granulation is a process through which microorganisms (mainly bacteria) self-immobilize due to the influence of several selection pressures, such as short settling times and high aeration intensity (ROLLEMBERG *et al.*, 2018). The granules formed usually present a compact and robust structure, as well as a good settling ability, being also capable of withstanding high organic loadings and simultaneously removing carbon, nitrogen, and phosphorus (ADAV *et al.*, 2008; ADAV; LEE; LAI, 2009). Furthermore, when compared to conventional activated sludge (CAS), the aerobic granular sludge (AGS) technology reduces operational costs (20–25%), electricity demand (23–40%), and space requirements (50–75%) (ADAV *et al.*, 2008; BENGTSSON *et al.*, 2019; NEREDA, 2017).

AGS is commonly cultivated in sequencing batch reactors (SBR), which are operated in cycles, including the phases of filling, aeration, settling and decanting (BEUN *et al.*, 1999). Recently, researches have been conducted on simultaneous fill/draw SBR, also known as constant-volume SBR (DERLON *et al.*, 2016; WANG, Q. *et al.*, 2018). These studies are still incipient, even though the majority of full-scale AGS systems are operated in such a manner, *e.g.*, the Nereda® technology (NEREDA, 2017).

The biggest challenges of aerobic granulation are the long start-up period required and the maintenance of long-term granule stability. In order to solve these issues, studies have demonstrated that the addition of calcium (JIANG *et al.*, 2003), magnesium (LI *et al.*, 2009), and polyaluminum chloride (PAC) (LIU *et al.*, 2016) can lead to a faster granulation, thus improving sludge settleability. Supplementation with dried sludge micropowder also proved beneficial to granule stability, having eliminated extended filaments through several mechanisms, such as collision and friction against granules, which stimulate extracellular polymeric substances (EPS) secretion (LIU *et al.*, 2019).

In this context, the present research aims to investigate whether the addition of coal ash (a residue from thermal power plants) can shorten granulation time in AGS systems. Firstly, coal ash might behave as a granulation nucleus (ZHANG *et al.*, 2017). Secondly, it could function as a source of divalent cations. Finally, it is possible that the friction between coal ash and granules would lead to an increase in granule resistance to shear, making them more stable, especially when a long-term operational stability is considered. Furthermore, no similar studies are known to have been conducted in simultaneous fill/draw SBR with a low liquid upflow velocity.

MATERIALS AND METHODS

Sequencing batch reactors configuration and operation

The experiments were conducted in two cylindrical acrylic SBR with a diameter of 100 mm, a total height of 1 m and a working volume of 7.2 L. The reactors were operated at a simultaneous fill/draw regime (WANG, Q. *et al.*, 2018) of 6-h operating cycles, which were divided into 30 min of simultaneous filling and drawing, 90 min of anaerobic/anoxic phase, 210–235 min of aeration, 30–5 min of settling. To stimulate granulation, the sedimentation time was gradually decreased from 30 (Stage I) to 15 (Stage II), 10 (Stage III), and 5 (Stage IV) min. The time subtracted from the settling phase was added to the aeration phase. Each stage was maintained for approximately six weeks.

The fill/draw was performed using a Masterflex peristaltic pump model BTG 2344, which produced a volumetric exchange rate of 50% and a liquid upflow velocity of 0.92 $m \cdot h^{-1}$. Aerators were positioned at the bases of the reactors, providing an air upflow velocity of 2.12 $cm \cdot s^{-1}$.

Inoculum and feeding solution

The reactors were inoculated with aerobic sludge from a domestic wastewater treatment plant (WWTP) (Fortaleza, Ceará, Brazil) at an initial concentration of mixed liquor volatile suspended solids (MLVSS) of approximately 2 $g \cdot L^{-1}$, whose sludge volume index at 30 min (SVI_{30}) was 110 $mL \cdot g^{-1}$.

The reactors were fed with synthetic wastewater containing ethanol (800 mg $COD \cdot L^{-1}$), ammonium (100 mg $N\text{-}NH_4^+ \cdot L^{-1}$), phosphate (10 mg $P\text{-}PO_4^{3-} \cdot L^{-1}$), sodium bicarbonate as buffer (700 mg $CaCO_3 \cdot L^{-1}$), and micronutrients (DOS SANTOS, 2005). The synthetic wastewater was kept refrigerated at 4°C in order to prevent its degradation in the feeding tank.

To investigate the influence of coal ash addition on the granulation, a reactor (R1) was maintained as control, while the other (R2) was supplemented with coal ash at a concentration of 1 $g \cdot L^{-1}$ (~3.1 $mg \cdot L^{-1}$ of calcium and ~0.2 $mg \cdot L^{-1}$ of magnesium). It is important to emphasize that the coal ash application was performed only once, at the beginning of the operation. This decision was made in the light of the experiment conducted by Liu *et al.* (2016), which demonstrated that the long-term application of PAC in a conventional SBR did not produce significant differences in relation to the short-term application, although both accelerated the granulation in comparison with the control reactor. In addition, such a frequency of supplementation was chosen to avoid an excess of inert solids inside the reactor.

Analytical methods

The chemical oxygen demand (COD), the concentration of nitrogen in the forms of ammonia ($N\text{-}NH_4^+$), nitrite ($N\text{-}NO_2^-$), and nitrate ($N\text{-}NO_3^-$), and the concentration of phosphorus as phosphate ($P\text{-}PO_4^{3-}$) were measured to evaluate the removal efficiency of organic matter and nutrients. The analyzes were performed for the influent and effluent, twice a week, and according to the Standard Methods for the Examination of Water and Wastewater (APHA, 2012).

In order to characterize the sludge settleability, a dynamic SVI, which is a modified version of the SVI, was performed for 5, 10, and

30 min (SCHWARZENBECK; BORGES; WILDERER, 2005). The frequency of the analyses was also twice a week.

To characterize the coal ash added to R2, a solubilization test was performed according to NBR 10006:2004 (ABNT, 2004). During this analysis, 250 g of coal ash was added to 1 L of distilled water. The solution was stirred for 5 min and then left idling for 7 days. After this period, the sample was filtered with a membrane of porosity equal to 0.45 μm. The filtered solution was analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES) (Thermo Fisher iCAP 6000) to determine the concentrations of calcium and magnesium.

Characterization of mature granule

At the end of Stage IV, the mature granules were subjected to a physical resistance analysis (shear test) (NOR-ANUAR *et al.*, 2012). Samples of the granules (> 0.2 mm) were subjected to a shear force caused by a stirrer at approximately 200 rpm for 10 min. The fragmented fraction was expressed in terms of a stability coefficient (S), obtained by the ratio of the amount of total solids after and before the stirring of the sludge sample. This coefficient was classified into three categories: very stable (S < 5%), stable (5% ≤ S ≤ 20%) and not stable (S > 20%). Therefore, the lower the value of S, the greater the resistance of the aerobic granules to shear.

A scanning electron microscope (SEM) (Inspect S50 – FEI model) with a nominal resolution of 3 nm was utilized to obtain detailed images of the granules and to carry out semi-quantitative chemical analysis by energy-dispersive X-ray spectroscopy (EDX). The mature granules were also observed under an optical microscope (Opton). The preparation for such microscopy analyses was performed following the methodology proposed by Motteran, Pereira and Campos (2013). The analyses were performed at the Analytical Central of Microscopy of *Universidade Federal do Ceará*, Brazil. The granule size profiles of the mature granules were obtained. For this, sieving was carried out with sieves of 0.2, 0.6, and 1 mm openings. The depth of oxygen penetration in the granules was also estimated, according to Equations 1 and 2 (DERLON *et al.*, 2016; HENZE *et al.*, 2008).

$$z = \sqrt{\frac{2 \times D_F \times C_{LF}}{k_{O,F} \times X_F}} \quad (1)$$

$$k_{O,F} \times X_F = K_{O,O_2,H} \times X_{H,F} + K_{O,O_2,AUT} \times X_{AUT,F} \quad (2)$$

Where:

Z = Oxygen penetration depth (m);

D_F = Diffusion coefficient for oxygen ($175 \cdot 10^{-6}$ $m^2 \cdot d^{-1}$);

C_{LF} = Oxygen concentration at the surface of the granules (maximum = 8 mg $O_2 \cdot L^{-1}$);

$K_{O,F}$ = Zero-order constant of biomass oxygen consumption (g $O_2 \cdot$g $COD^{-1} \cdot d^{-1}$);

X_F = Total biomass concentration (10,000 g $COD \cdot m^{-3}$);

$K_{O,O_2,H}$ = Zero-order constant of heterotrophic bacteria oxygen consumption (7.2 g $O_2 \cdot$g $COD^{-1} \cdot d^{-1}$);

$X_{H,F}$ = Heterotrophic bacteria biomass concentration (assumed as 2% of the total biomass concentration: 0.02 × 10,000 = 200 g $COD \cdot m^{-3}$);

$K_{O,O_2,AUT}$ = Zero-order constant of autotrophic biomass oxygen consumption (18.8 g $O_2 \cdot$g $COD^{-1} \cdot d^{-1}$);

$X_{AUT,F}$ = Autotrophic bacteria biomass concentration (assumed as 98% of the total biomass concentration: 0.98 × 10,000 = 9800 g $COD \cdot m^{-3}$).

Extracellular polymeric substances (EPS) were also quantified. For this, initially, 5 mL of the mixed liquor was collected, from which the EPS were extracted under alkaline conditions (pH > 10, with the addition of 5 mL of a 1-M NaOH solution, followed by a water bath at 80° C for 30 min and sonication at 55 kHz for 5 min) (TAY; LIU; LIU, 2001). Then the obtained samples were filtered (filter paper porosity of 0.45 μm) and diluted twice. A modification of the Lowry method was used to calculate the protein content (PN), and the phenol-sulfuric acid method was used to determine the fraction of polysaccharides (PS) (LONG *et al.*, 2014). The total quantification of EPS was given by the sum of the fractions of PN and PS.

DNA sequencing of the 16S rRNA gene was performed by extracting 0.5 g (total wet weight) of the sludge samples collected at the aeration phase completion, at the end of Stage IV, using the PowerSoil® MOBIO Kit. DNA extraction was done in triplicate for each sludge sample. The quality and concentration of the extracted DNA were estimated using Nanodrop 1,000 (Thermo Scientific, Waltham, MA, USA). For the taxonomic profile of bacterial communities, the amplicon library of the 16S rRNA gene V4 region was prepared as described previously (ILUMINA INC., 2013) using the region-specific primers (515F/806R) (CAPORASO *et al.*, 2011). The data obtained by sequencing were analyzed using bioinformatics tools.

RESULTS AND DISCUSSION

Settleability and sludge retention

Figure 1 exhibits the values of SVI_5, SVI_{10}, SVI_{30}, and the concentrations of MLVSS obtained throughout all four stages of operation in both reactors. The drop in SVI values indicates an improvement of sludge settleability. The SVI_5 becomes gradually closer to the SVI_{30}, which indicates the evolution from flocculent to granular sludge (BASSIN *et al.*, 2012; CORSINO *et al.*, 2016). The values stabilized at Stages III and IV, thus marking granule maturation. The mature granules showed SVI_{30} between 30 and 80 mL·g^{-1}, which corresponds to the variation reported in AGS literature for both conventional and simultaneous fill/draw SBR (DERLON *et al.*, 2016; LIU *et al.*, 2010;

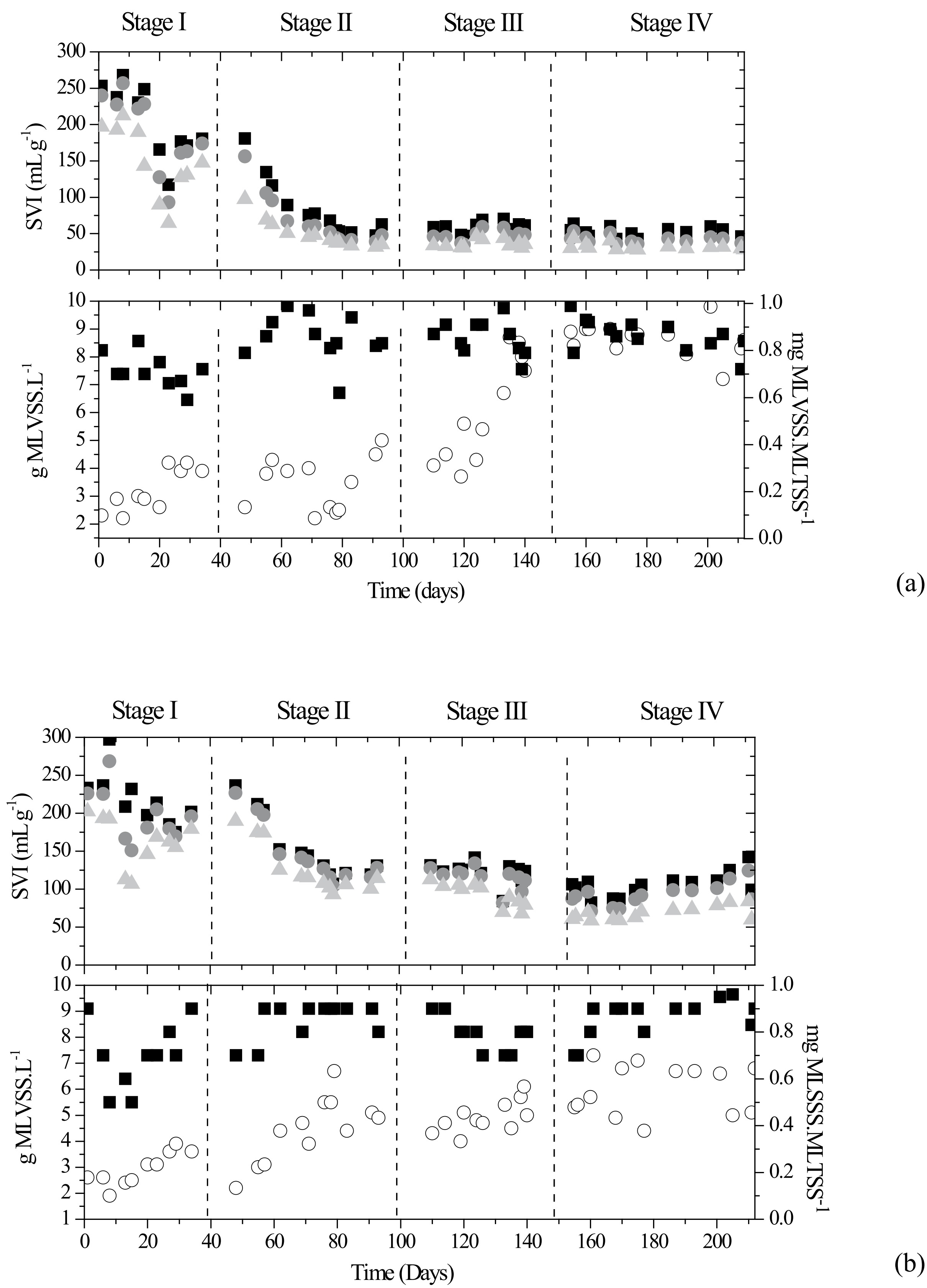

Figure 1 – Sludge volumetric index and mixed liquor volatile suspended solids values for simultaneous fill/draw SBR operated in the (A) absence (R1, control) and (B) presence (R2) of coal ash (■) SVI_5, (●) SVI_{10}, (▲) SVI_{30}, (○) MLVSS and (■) MLVSS/MLSS.

LONG *et al.*, 2014). When compared to the control reactor (R1), the coal ash addition in R2 did not lead to significant changes in the variables presented in Figure 1.

Concerning MLVSS concentrations (Figure 1), the values increased consistently, despite the gradual decrease of settling times. In conventional SBR, sludge retention tends to drop considerably due to the decrease of settling times or to the shear force generated by the feeding, which results in a selection of microbial communities with good settleability (KOŃCZAK; KARCZ; MIKSCH, 2014; PIJUAN; WERNER; YUAN, 2011). Therefore, it can be suggested that, under the simultaneous fill/draw regime, the influence of settling times on the microbial community selection in the SBR is diminished, resulting in the growth of biomass retention as previously reported.

It is also important to notice that coal ash addition promoted a decrease in the MLVSS/MLSS ratio in R2, reaching a value of 0.5. This result is compatible with the fact that the supplemented material behaves as fixed solids. However, the ratio starts to increase at day 20, and, at day 34, the same value is observed in both reactors. Considering that the two SBR had similar MLVSS at this point, this seems to indicate that the coal ash was eliminated from R2. Sedimentation tests conducted showed that, after 30 min, the coal ash would settle only partially. Since after its addition, the settling time was kept at 30 min, a considerable fraction of it would have been washed out in each cycle. After a certain number of cycles, its concentration would have decreased considerably, resulting in an increase of the MLVSS/MLSS ratio.

Characterization of mature granules

At the end of Stage IV, both reactors had similar granule size profiles, with granules with diameters larger than 0.2 mm representing 98% of the biomass, and granules larger than 1 mm accounting for 90%. These results confirm the tendency observed in SVI and MLVSS values.

Concerning the morphology of mature granules, Figure 2 presents the images obtained at the end of Stage IV by optical microscopy and SEM. SEM images show an absence of inorganic crystals or organic material other than the amorphous material which covered the granules' surfaces entirety. This is compatible with well-delineated surfaces, with only a few cavities. It is also important to notice that no trace of coal ash has been identified in the microscopic images.

A different outcome was obtained by Liu *et al.* (2019), whose optical microscopy images clearly showed the presence of micropowder, and even identify its interaction with the granules. Zhang *et al.* (2017) worked with the addition of biochar and obtained photographs in which the compound was identified as granule nucleus. The fact that SEM analysis did not indicate the presence of coal ashes suggests that they were washed out from R2. This evidence is in accordance with the MLVSS/MLSS ratios observed as well as with the results of the sedimentation tests conducted (see section "Settleability and sludge retention").

On the subject of resistance to shear, mature granules of reactors R1 and R2 demonstrated stability coefficients of 24.3 and 27.4%, respectively. According to the guidelines developed by Nor-Anuar *et al.* (2012), the granules can be classified as not strong. Xavier (2017), working with a pilot-scale conventional SBR for the treatment of real wastewater, also found stability coefficients around 25%. However, the author emphasizes that, since the shear force applied during the resistance test is usually much higher than the one actually maintained inside the reactors, the absolute values of the stability coefficient do not provide, on their own, relevant information about the systems. Nevertheless, they are relevant when comparing different systems. From this point of view, it is not possible to state that the mature granules of the present study are, in fact, fragile, only that both systems (R1 and R2) presented granules with similar strength. Therefore, coal ash addition did not have an influence on the resistance of the granules to shear.

Regarding EPS compositions, mature granules of R1 and R2 had similar values, with PN of 44 and 42 mg·g $MLVSS^{-1}$ and PS of 93 and 92 mg·g $MLVSS^{-1}$, respectively. The EDX results reinforce this outcome since the composition of the granules was quite similar: C (R1: 33.5%; R2: 40%) and O (R1:39.8%; R2: 25%), with small fractions of K, P, Na, Cl, and S.

Mechanisms of granule formation and maintenance in the presence of coal ash

Regarding the role played by coal ash in the formation and maintenance of granules, one possibility would be that coal ash might behave

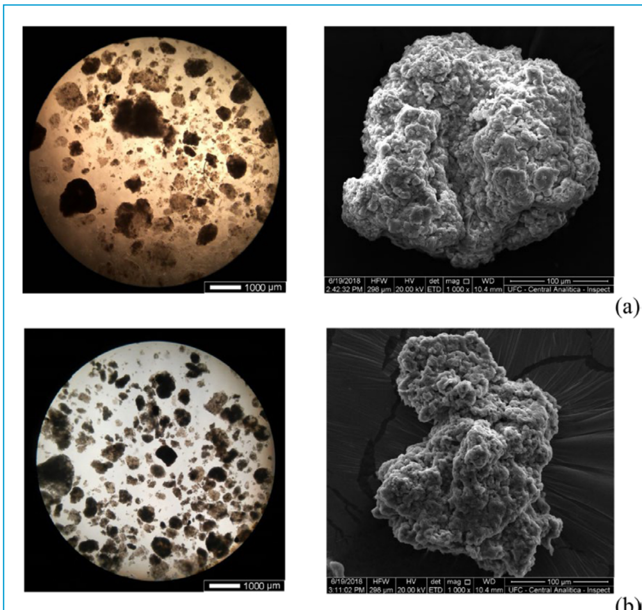

Figure 2 – Optical and scanning electron microscopy (SEM) micrographs, respectively, of the granules of simultaneous fill/draw SBR in the (A) absence (R1, control) and (B) presence (R2) of coal ash.

as a granulation nucleus, analogously to the biochar utilized by Zhang *et al.* (2017). Coal ash might also play one of the roles described by Liu *et al.* (2019), when working with dried sludge micropowder. The authors have mentioned that the ions (Si, Fe, Ca, and Mg) released from micropowder could have facilitated microbial aggregation. They have also suggested that the friction between micropowder and granules during aeration would promote the control of the filaments extending from the granules, causing them to break from granule surface. Such friction would also stimulate EPS secretion, which would work as a way of protecting the biomass against shear stress. Micropowder supplementation would have also increased the COD and particulate organic matter, kinetically disfavoring the filamentous bacteria in terms of substrate consumption.

In the present study, the increase in COD and particulate organic matter could not be a possible mechanism of granule stabilization, since coal ash was not constituted of organic compounds. Therefore, three mechanisms of granule formation and/or maintenance by the coal ash are proposed for further discussion. In essence, the ash might behave as granulation nucleus (hypothesis 1), it would function as a source of cations and anions (hypothesis 2), and it would be a friction source against the granules (hypothesis 3).

Regarding the first hypothesis, since none of the microscopy images seem to suggest it, coal ash did not work as a granulation nucleus. The composition of the EPS matrix obtained by EDX reinforces this conclusion.

Regarding the second hypothesis, the estimated concentrations in the reactor were ~3.2 $mg{\cdot}L^{-1}$ of calcium and ~0.2 $mg{\cdot}L^{-1}$ of magnesium. These concentrations are likely insufficient to produce significant changes in sludge, since they are much lower than those developed by researchers who reported meaningful results. For example, Jiang *et al.* (2003) worked with a conventional SBR with the addition of 100 mg $Ca^{2+}{\cdot}L^{-1}$ in every cycle and obtained lower SVI_{30} (control reactor: 150 $mL{\cdot}g^{-1}$, calcium reactor: 100 $mL{\cdot}g^{-1}$), greater sludge retention (control reactor: 2 g $SS{\cdot}L^{-1}$, calcium reactor: 7.9 g $SS{\cdot}L^{-1}$) and increased amounts of PS in the EPS (control reactor: 41 mg L^{-1}, calcium reactor: 92 mg L^{-1}). Li *et al.* (2009) also worked with a conventional SBR but supplemented it with 10 mg $Mg^{2+}{\cdot}L^{-1}$ in every cycle. They obtained greater sludge retention (control reactor: 6.8 g $MLSS{\cdot}L^{-1}$, magnesium reactor: 7.6 g $MLSS{\cdot}L^{-1}$), mature granules with larger sizes (control reactor: 1.8 mm, magnesium reactor: 2.9 mm) and increased PS production (control reactor: 35 mg L^{-1}, magnesium reactor: 70 mg L^{-1}). However, the SVI_{30} remained the same (between 20 and 25 $mL{\cdot}g^{-1}$).

Finally, regarding the third hypothesis, since extended filaments were not observed in the granules of the control reactor (R1), it is reasonable to assume that the granules cultivated in R2 would also not have developed this feature. In such an environment, the effects of friction would not be pronounced, since there might not have been filaments to be broken from the granule surface. This might explain why very few differences between the settling properties and retention ability of the sludges were found. Similar behavior was observed by Liu *et al.* (2019) when they applied micropowder over granules without extended filaments. The authors reported that SVI_{30} was kept nearly the same (decrease from around 55 to 45 $mL{\cdot}g^{-1}$), and stabilized biomass retention (increase from around 3.8 to 4 g $MLVSS{\cdot}L^{-1}$).

Furthermore, the ash washout evidenced by MLVSS/MLSS ratios and SEM analysis would have lowered the possibility of contact between the coal ash and the granules, reducing friction. Such a reduction, combined with the absence of extended filaments, would have made friction an ineffective mechanism of granule stabilization. In the absence of the friction promoted by coal ashes, EPS production would not be stimulated, which may explain the similar EPS composition obtained in the reactors. The coal ash washout becomes even more relevant because the last two mechanisms through which the ashes were considered to influence granulation (friction and ion supplementation) are highly dependent on the concentration of the agents used. However, since the coal ashes were present in the reactor for at least 20 days (see section "Settleability and sludge retention"), they would have still been able to act as a granulation nucleus. The fact that the coal ash could not act as such seems to indicate that this property is not inside their scope of capabilities.

Organic matter and nutrient removal

Table 1 summarizes the results regarding the removal of COD, nitrogen, and phosphorus, showing that the reactors performed similarly throughout the operation stages. It can be seen that COD removals remained above 90% for both reactors. These values correspond to those reported in the literature regarding the operation of conventional and simultaneous fill/draw SBR, for which COD removal is equal to or higher than 80% (DERLON *et al.*, 2016; LIU *et al.*, 2010; LONG *et al.*, 2014).

Concerning the nitrogen removal (Table 1), the small concentration of nitrite over nitrate in the effluent indicates the occurrence of complete nitrification. However, nitrate was accumulated, indicating that simultaneous nitrification and denitrification did not occur at considerable levels.

The phosphorus removal (below 60%) is considered low, especially for reactors operated with cycles containing an anaerobic phase, since the inclusion of this period is aimed at optimizing phosphorus removal (BASSIN, 2011). H. Wang *et al.* (2018), for example, worked with a conventional SBR with an anaerobic phase included (total cycle: 6 h, anaerobic phase: 2 h) and obtained phosphorus removals around 98%. However, in many cases, the high efficiencies of phosphorus removals reported in the literature are achieved by applying low concentrations of this compound. Therefore, its removal is due to the assimilative rather than dissimilative metabolism.

Table 1 – Operating performance of the simultaneous fill/draw SBR in the absence (R1, control) and presence (R2) of coal ash*.

Stage		R1				R2			
		I	II	III	IV	I	II	III	IV
COD	Influent ($mg \cdot L^{-1}$)	985 (123)	776 (107)	694 (86)	694 (149)	985 (123)	776 (107)	694 (86)	694 (149)
	Effluent ($mg \cdot L^{-1}$)	31 (24)	54 (38)	21 (17)	28 (15)	54 (10)	34 (25)	21 (13)	23 (12)
	Efficiency (%)	94 (2)	93 (5)	97 (2)	96 (3)	94 (1)	95 (3)	97 (2)	96 (3)
Nitrogen Fraction	Influent NH_4^+ (mg $N\text{-}NH_4^+ \cdot L^{-1}$)	114 (16)	96 (8)	93 (6)	92 (8)	114 (16)	96 (8)	93 (6)	92 (8)
	Effluent NH_4^+ (mg $N\text{-}NH_4^+ \cdot L^{-1}$)	3 (3)	13 (7)	10 (6)	10 (6)	0.4 (0.2)	9 (6)	8 (5)	9 (6)
	Effluent NO_2^- (mg $N\text{-}NO_2^- \cdot L^{-1}$)	8 (2)	8 (4)	13 (5)	4 (3)	4 (3)	8 (5)	8 (6)	4 (3)
	Effluent NO_3^- (mg $N\text{-}NO_3^- \cdot L^{-1}$)	45 (15)	26 (12)	26 (8)	23 (6)	57 (9)	28 (11)	27 (8)	23 (8)
	Nitrification efficiency (%)	96 (10)	98 (12)	91 (8)	83 (6)	99 (15)	90 (10)	91 (8)	89 (7)
	Total nitrogen removal efficiency (%)	51 (9)	51 (11)	47 (10)	59 (10)	46 (14)	53 (12)	53 (10)	60 (10)
pH	Influent	7.4 (0.5)	7.5 (0.3)	7 (0.5)	7.5 (0.2)	7.4 (0.5)	7.5 (0.3)	7 (0.5)	7.5 (0.2)
	Effluent	7.8 (0.4)	8.0 (0.2)	7.6 (0.3)	7.5 (1)	7.5 (0.8)	7.7 (0.6)	7.8 (0.3)	7.9 (0.1)
Total alkalinity	Influent (mg $CaCO_3 \cdot L^{-1}$)	694 (204)	783 (130)	631 (130)	723 (68)	694 (204)	783 (130)	631 (130)	723 (68)
	Effluent (mg $CaCO_3 \cdot L^{-1}$)	275 (172)	458 (157)	312 (116)	367 (165)	255 (178)	378 (81)	340 (106)	322 (83)
Phosphorus	Influent (mg $P\text{-}PO_4^{3-} \cdot L^{-1}$)	8 (1)	8 (1)	7 (1)	11 (2)	8 (1)	8 (1)	7 (1)	11 (2)
	Effluent (mg $P\text{-}PO_4^{3-} \cdot L^{-1}$)	5 (1)	3 (1)	4 (1)	9 (2)	4 (1)	5 (2)	4 (2)	9 (2)
	Efficiency (%)	39 (10)	56 (14)	38 (14)	23 (9)	45 (15)	39 (8)	40 (21)	18 (17)

*The standard deviation is shown in parentheses; COD: chemical oxygen demand.

The accumulation of nitrate could be explained by the absence of a large enough anoxic zone inside the granules. However, oxygen penetration depth was estimated at 194 µm. Since most granules had at least 1 mm of diameter (see section "Characterization of mature granules"), and considering that nitrification occurred at high levels, it is reasonable to assume that, in the aerobic phase, the anoxic zone would have comprised the granule volume without oxygen, which corresponds to a 306 µm radius. This invalidates the hypothesis of an non-existent or small anoxic zone. Therefore, it is likely that the low occurrence of denitrification is being driven by another factor: the scarcity of substrate at the end of the aeration phase (ZHONG *et al.*, 2013).

Furthermore, the low phosphorus removal may be associated with the accumulation of nitrate. With the nitrate accumulated inside the reactor, the filling would occur in an anoxic environment. This would allow substrate competition between denitrifying heterotrophic bacteria and polyphosphate-accumulating organisms (PAO). In this case, due to the kinetics of the removal reactions involved, PAO would lose the competition (WANG, Q. *et al.*, 2018). Therefore, complete denitrification of the nitrate accumulated during the previous cycle would be performed after feeding, impairing the action of PAO. Then, with the start of aeration, new amounts of nitrate would be produced. However, this portion would accumulate due to substrate scarcity to allow denitrification to take place at this point in the cycle. Thus, both denitrification and phosphorus removal efficiencies would be lowered.

Lastly, regarding other operational parameters, both the influent feed and the treated wastewater pH were close to neutral, varying between 6.5 and 8. Additionally, influent alkalinity (~700 mg $CaCO_3 \cdot L^{-1}$) was lowered by half due to nitrification.

Microbial community diversity

The metagenomic analysis found 1,731 individuals in the inoculum sludge, 2,739 in the control sludge, and 2,790 in the sludge exposed to coal ash. The diversity and richness of the microbial community (Table 2) were examined through five indices (Coverage, Gini-Simpson, Shannon, Chao1, and ACE). The Gini-Simpson and Shannon indices showed that community diversity in the reactors after granulation (Stage IV) was similar to that found in the inoculum sludge at the beginning of the experiment. However, the Chao1 and ACE indices indicated a relative difference in the richness of the microbial community between the inoculum sludge and the AGS, although the AGS presented similar values to one another. This indicates that the granulation process in simultaneous fill/draw SBR influenced the microbial community selection more than the use of coal ash.

Figure 3 shows that among microorganisms with an abundance greater than 10%, the most prominent at the phylum level were

Proteobacteria, Chlorobi, Bacteroidetes, Planctomycetes, Chloroflexi, and Verrucomicrobia. These phyla have already been shown to be relatively abundant in both granular aerobic and activated sludge systems (HE *et al.*, 2016; ZHANG *et al.*, 2017).

Proteobacteria was the main phylum of the microbial communities, with no significant difference between the communities developed in R1 and R2. These results indicate that coal ash did not promote a strong selection of microorganisms at the phylum level.

Rhodocyclaceae was one of the families present in the three sludges analyzed (inoculum = 4.5%, R1 = 6.9%, R2 = 5.7%). Some studies associate this family with the production of EPS (SZABÓ *et al.*, 2017). The presence of this type of microorganism is fundamental for the granulation process since the EPS formed mainly by PS, PN, and soluble microbial products (SMP) provides the aggregation of the various populations, especially when the selection pressure is limited. In addition, some bacteria, not only utilize the carbohydrates and proteins present in the medium but also are responsible for the degradation of SMP, as in the case of some species of the Saprospiraceae family (FU *et al.*, 2017; SACK; VAN DER WIELEN; VAN DER KOOIJ, 2014), which was favored by granulation in the present study (inoculum = 3.8%, R1 = 7.3%, R2 = 20.8%).

Table 2 – Indicators of richness and diversity of species of the microbial communities present in inoculum sludge and simultaneous fill/draw SBR in the absence (R1, control) and presence (R2) of coal ash.

Sample	Coverage (%)	Gini-Simpson	Shannon	Chao1	ACE
Inoculum	99.2	0.95	5.1	7.2	17.9
R1	98.5	0.95	5.0	29.5	27.3
R2	98.5	0.98	5.4	29.5	28.3

ACE: abundance-based coverage estimator

At the genus level, the granulation altered the population profile of the bacteria involved in the removal of nitrogen. In the inoculum sludge, *Candidatus Nitrososphera* (63%) was responsible for the oxidation of NH_4^+ to NO_2^-, and *Nitrospira* (4.5%), by the oxidation of NO_2^- to NO_3^-. However, after granulation, the first group did not adapt. The conversion of NH_4^+ to NO_2^- was performed by *Prosthecobacter* (R1 = 34% and R2 = 32%) (XU *et al.*, 2018), while *Nitrospira* continued to perform the conversion of NO_2^- to NO_3^- (R1 = 16% and R2 = 11%).

The genus *Thauera* also grew in abundance at the end of the experiment (inoculum = 3%, R1 = 15%, R2 = 8%). This denitrifying genus is characterized by absorbing a wide range of organic substrates under aerobic conditions, including glucose, acetate, propionate, pyruvate, oleic acid, amino acid blends, and ethanol. Under anoxic conditions, many substrates are consumed, with the exception of glucose and oleic acid (THOMSEN; KONG; NIELSEN, 2007).

CONCLUSIONS

Granulation was achieved in simultaneous fill/draw SBR operated with low upflow velocity. With the addition of coal ash, a residue from power plants, no significant differences were observed in terms of settleability, biomass retention, morphology, resistance to shear, and composition of the EPS matrix. COD removals were high (≥ 90%), while removals of nitrogen (~50%) and phosphorus (~40%) were low, possibly due to the presence of nitrate during the anaerobic phase. With granulation, the population profile of the microbial community was altered, mainly at the genus level. In general, it is verified that the operational conditions had a more considerable influence over granulation than the addition of coal ash. The possible reasons are because coal ash

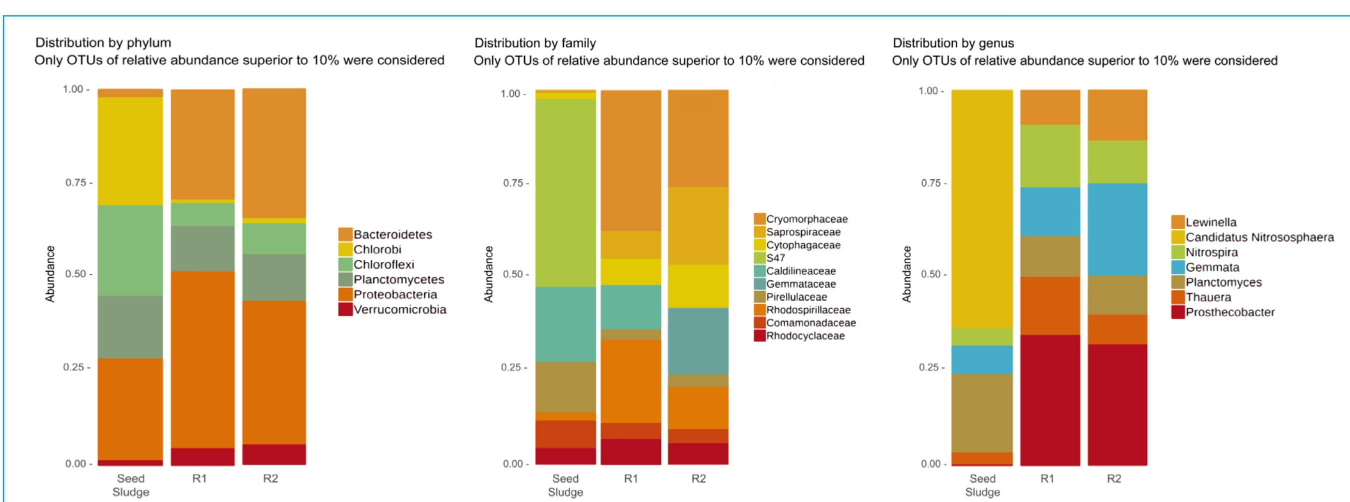

Figure 3 – Distribution of the microbial population at phylum, family, and genus levels, respectively, of the inoculum and samples collected in the systems operated in the absence (R1, control) and presence (R2) of coal ash.

supplementation was performed in a single step, the low sedimentation rate of this particular residue, and the weak interaction between the coal ash and the EPS formed in the granular sludge. These factors appear to have decreased or prevented the action of the ash as granulation nucleus, source of cations, and abrasive element.

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