Bioresource Technology 278 (2019) 195–204

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Effects of carbon source on the formation, stability, bioactivity and biodiversity of the aerobic granule sludge

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ARTICLE INFO

Keywords:
Aerobic granules
Carbon source
Microbial groups
Long-term operation

ABSTRACT

Three aerobic granular sludge systems were operated as sequencing batch reactors (SBR) with acetate, ethanol and glucose as carbon source. The SBR cycle was 6 h, with an anaerobic phase followed by an aerobic phase. The acetate granules (> 1.5 mm) had the greatest microbial diversity and better results in terms of removal efficiency for carbon and nutrients (TN ≈ 72% and TP ≈ 42%) and also in the resistance tests. However, partial disintegration was observed. On the other hand, when ethanol was the substrate, the granules were stable, good nitrogen removal was achieved (TN ≈ 53%), but phosphorus removal was not favored (TP ≈ 31%). Glucose presented the lowest efficiency values for nitrogen (TN ≈ 44%) and phosphorous removal (TP ≈ 21%), and the granules formed (< 1 mm) had the lowest microbial diversity. Therefore, the carbon source had a high impact on the characteristics of the granules.

1. Introduction

Aerobic granular sludge (AGS) is an innovative technology to remove simultaneously carbon, nitrogen and phosphorus (simultaneous nitrification, denitrification and phosphorus removal, SNDPR) from wastewater using bacterial granules, which is considered one of the most promising biological treatment technologies in the 21st century (Nancharaiah et al., 2006). One of the main challenging issues for the AGS technology is the long startup period when using real wastewaters. Therefore, many researchers have recommended the use of aerobic mature granules as inoculum in order to make shorter the startup period (Pijuan et al., 2011). This strategy has also been used in full-scale wastewater treatment plants, in which a pilot reactor is used for growing stable and mature granules that further, will be used as inoculum (Li et al., 2014).

A stress that induces a change in the behavior of a microbial population, being a key driving force for successful AGS cultivation, is called selection pressure. Distinct feast-famine periods during operation, short settling time, and high aeration intensity are examples of selection pressure. Additionally, many factors influence the properties of granules, such as substrate composition, organic loading, solids retention time, among others (Rollemberg et al., 2018).

Some studies observed that granules grown on different carbon sources might have diverse microbial communities, granule morphology and internal structures. Therefore, the carbon source influences the AGS stability and removal efficiencies, especially when carbon and nutrients removal are evaluated together (Li et al., 2008). In previous studies, glucose often led to filamentous granules, while acetate led to spherical and dense granules (Tay et al., 2002; Du et al., 2011). Phenol or pyridine could yield strong granules, although the cultivation time was longer (Adav et al., 2007). Finally, the use of propionate as carbon source led to stable and dense granules, but needed prolonged time to achieve a mature granule (Wan et al., 2014).

Competition between functional groups involved in the removal of organic carbon, nitrogen and phosphorus is also affected by substrate (Pijuan et al., 2004). For example, methanol and ethanol are commonly used in many wastewater treatment plants for enhancing denitrification, however they had insignificant or negligible effects on biological phosphorus removal. On the other hand, acetate is the most common volatile fatty acid (VFA) in lab and full-scale as a typical carbon source for enhanced biological phosphorus removal (EBPR). Other VFAs used for EBPR purposes are propionate, butyrate, valerate, isovalerate and lactate (Oehmen et al., 2004; Pijuan et al., 2004).

Regarding EBPR systems, glucose has been widely studied to replace VFAs as carbon source, although some studies have shown that this substrate may be detrimental to the process (Chen et al., 2015).

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https://doi.org/10.1016/j.biortech.2019.01.071
Received 30 November 2018; Received in revised form 14 January 2019; Accepted 18 January 2019
Available online 19 January 2019
0960-8524/

Although, other works found that EBPR could be induced and maintained when glucose was the sole carbon source, provided that VFAs were formed and further converted into polyhydroxyalkanoates (PHA) under anaerobic conditions (Kumar and Chaudhari, 2003).

In addition to the impact on granule stability, carbon source is important for AGS performance, extracellular polymeric substances (EPS) production, competition between glycogen-accumulating organisms (GAOs) and polyphosphate-accumulating organisms (PAOs) as well as between PAOs and denitrifiers (He et al., 2018).

Due to variation in the results found in the literature, as well as the lack of an integrated evaluation, this work aimed to contribute to the understanding of the impact of some important carbon sources (acetate, ethanol and glucose) on the formation, stability, activity and characteristics of the granule.

2. Material and methods

The research was divided into four main steps: (i) AGS growth on different substrates and evaluation of systems performance; (ii) evaluation of SNDPR mechanisms during an operation cycle; (iii) evaluation of the stability of the mature granules during organic and salt shocks, and (iv) evaluation of the bacterial communities of the granules.

2.1. Experimental setup

Three identical sequencing batch reactors (SBRs), with diameter of 100 mm, height of 1 m, working volume of 7.2 L and a height to diameter ratio (H/D) of 10, were used. The hydraulic retention time (HRT) was 12 h with a volume exchange ratio of 50%. The SBR cycle was 6 h, which consisted of filling (15 min), anaerobic reaction (54 min), aerobic reaction (270–285 min), settling (20–5 min) and decanting (1 min). During the anaerobic reaction, a mechanical stirring was used and, in the aerobic phase, an air compressor (*Yuting air pump* model ACO-003) was used at an aeration rate of 10.0 L/min, resulting in a superficial gas velocity of 1.0 cm/s. The SBRs were operated at room temperature of 28 ± 2 °C during the three phases, in which the length of the settling period was reduced gradually from 20 min (phase I) to 10 min (phase II) and, then, to 5 min (phase III). Accordingly, in order to keep a 6-h cycle, the subtracted time was added to the aerobic reaction period. This protocol was based on previous studies that used similar reactor configuration (SBR) and effluents (Rollemberg et al., 2018; Nancharaiah et al., 2006).

At the end of phase III, after achieve granules maturation, three cycles of each SBR were monitored over the time in order to understand the removal mechanisms. Finally, the resilience of the granules formed in the systems were tested. The effects of organic and salt shock loads (2,000 mg/L of COD and 15 g/L of NaCl, respectively) on the mature granules were evaluated individually. Three consecutive one-cycle shocks were applied to the reactors and the interval adopted between the shocks was four cycles, equivalent to a period of 1 day. The choice of these values was based on other studies that also evaluated the strength of the granules (Wang et al., 2017).

2.2. Seed sludge and wastewater

The SBRs were inoculated with activated sludge collected from an extended aeration activated sludge system of a wastewater treatment plant located in Fortaleza, Ceara, Brazil. Approximately 3.6 L was introduced into the SBRs, resulting in an initial concentration of mixed liquor suspended solids (MLSS) of about 3,200 mg/L. The sludge volume index at 30 min (SVI_{30}) during start-up was 198.1 mL/g.

The carbon sources tested in the experiment were acetate (R1), glucose (R2) and ethanol (R3). The synthetic wastewater used was compound of: 600 mg/L COD of carbon source, 110 mg/L of NH_4^+ (provided by ammonium chloride, NH_4Cl) as nitrogen source, 10 mg/L of PO_4^{3-} (provided by potassium phosphate monobasic, KH_2PO_4) as phosphorus source, and 1 mL of a trace element solution, which contained (in mg/L): H_3BO_3 (50), $ZnCl_2$ (50), $CuCl_2$ (30), $MnSO_4{\cdot}H_2O$ (50), $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (50), $AlCl_3$ (50), $CoCl_2{\cdot}6H_2O$ (50), and $NiCl_2$ (50).

The concentrations of C, N and P in the influent were used to simulate the values found in domestic effluents. It should be emphasized that the COD:N:P ratio is within the recommended values for the simultaneous presence of heterotrophic and autotrophic bacteria in the AGS (Wu et al., 2012; Luo et al., 2014).

2.3. Analytical methods

COD, pH, NH_4^+-N, NO_3^--N, NO_2^--N, total phosphorus (TP), MLSS and sludge volume index at 10 and 30 min (SVI_{10} and SVI_{30}) were determined according to APHA (2005), whereas DO was measured by a YSI 5000 m. Total inorganic nitrogen (TIN) was regarded as the sum of NH_4^+-N, NO_3^--N and NO_2^--N (Long et al., 2014). EPS were extracted by a modified heat extraction method proposed by Yang et al. (2014). Protein (PN) content was determined by a modified Lowry method, and polysaccharides (PS) content was analyzed using a phenol-sulfuric acid method (Long et al., 2014). EPS was regarded as the sum of PN and PS. The physical resistance (shear test) analysis of the granules followed the methodology described by Nor-Anuar et al. (2012).

2.4. DNA extraction, 16S rRNA gene amplicon sequencing and data processing

Samples from mixed liquor (at the end of the aeration reaction) were collected (maturation period, end of phase III), and DNA was extracted using PowerSoil® DNA isolation kit (MoBio Laboratories Inc., USA) based on the manufacturer's instruction. The amplicon library of the 16S rRNA gene V4 region was prepared as previously described (Ilumina, 2013), using the region-specific primers (515F/806R). After indexing, the PCR products were cleaned up using Agencourt AMPure XP—PCR purification beads (Beckman Coulter, Brea, CA, USA) based on the manufacturer's instruction and quantified using the dsDNA BR assay Kit (Invitrogen, Carlsbad, CA, USA) on a Qubit 2.0 fluorometer (Invitrogen, Carls- bad, CA, USA). The libraries were sequenced using the 300-cycle MiSeq Reagent Kits v2 chemistry (Ilumina, 2013) with a MiSeq Desktop Sequencer (Illumina). The data obtained by the sequencing was analyzed with bioinformatics tools as follows. All reads were trimmed using vsearch v2.8.1, with parameters -fastq_maxee 0.8 -fastq_trunclen 250. All reads were clustered into OTUs using QIIME script pick_open_reference_otus.py with 99% identity, using Greengenes 16S rRNA database (release 13_8). The BIOM file was used in PICRUSt in order to infer functional categories associated with taxonomic composition using KEGG (Kanehisa and Goto, 2000) metabolic pathways. Copy number normalization of 16S for each OTU was calculated using the PICRUSt script normalize_by_copy_number.py and contributions of various taxa to different KOs were computed with the script metagenome_contributions.py. The abundance-based coverage estimator (ACE), Chao1, Simpson, Shannon and coverage were calculated using Mothur software.

2.5. Statistical methods

Statgraphics Centurion XV computer software was used for the statistical analysis of the data. It was applied the Mann-Whitney Rank Sum and Kruskal-Wallis ANOVA on Ranks tests to compare the performance of the reactors. The results of the tests were evaluated according to the p-value. If $p \leq 0.05$, the null hypothesis is rejected, i.e. the data groups are considered statistically different.

3. Results and discussion

3.1. Inoculation and startup

The systems started in phase I (acclimation) and a reduction of the initial mixed liquor volatile suspended solids (MLVSS) concentration was observed in all systems. Due the low settleability of the inoculum sludge, the biomass was wash out from the reactor and the concentration of MVLSS after the first cycle was above 500 mg/L.

In phase II (settling time reduction from 20 min to 10 min), the MLVSS was greatly affected in R3 (glucose), and the mean values observed were 1303 ± 10 mg/L in R1 (acetate), 1178 ± 84 in R2 (ethanol) and 870 ± 284 mg/L in R3. In the last phase (settling time of 5 min), the mean values were close to those of phase II.

MLVSS reduction in R3 is possibly due to the type of granules cultivated. With a high sedimentation time (20 min), the bacteria grown in R3 were able to stay in the system. However, with a shorter sedimentation time (10 min and 5 min), part of the cultivated bacteria were washed out because of their low sedimentation velocity.

3.2. Formation and stability of the granules

The formation of the granules occurred at different times (Fig. 1). In R1 (acetate), aerobic granulation was reached 14 days after the startup. On the other hand, in R2 (ethanol) and R3 (glucose), granulation occurred after 40 and 60 days after the startup, respectively. The results showed in Fig. 1 suggest that either acetate or ethanol favored solids retention with high sedimentation capacity, whereas the system fed with glucose showed continuous washout, indicating the formation of a biomass with low sedimentation capacity. This problem could be associated with the growth of filamentous bacteria dispersed in the liquid and not associated with the granules (Du et al., 2011). Thus, the granules grown on acetate presented the highest SRT value (8–12 days), and the granules grown on glucose presented the lowest (5–7 days).

A rapid granule formation was observed in the R1, and stability was achieved nearly two months after granulation. On the 72nd day of the operation, an increase in the sludge volumetric index (SVI_{30}), as well as a reduction in the concentration of volatile suspended solids (VSS), were found, indicating solids disintegration. During this period, there was a reduction of approximately 50% of VSS in the reactor, and the percentage of granules above 1 mm was reduced from 95% to 40%. After about one month, the system returned to stabilization and, in less than 15 days, presented back low values of SVI_{30} and VSS close to 1500 mg/L. After reintegration, the percentage of granules above 1.0 mm was 90%.

Although the granulation in R2 was higher than R1, the maturation of the granules was definitively reached on the day 80 of operation and remained until the end of the experiment. On the day 120 of operation, there was a small VSS reduction, which was quickly reestablished. There was no granules disintegration during the operation period.

In R3, the granules formation was obtained after 60 days of operation. Solids washout was observed in this reactor when the sedimentation period was decreased from 20 to 10 min (change from phase I to phase II). This fact occurred because, most likely, glucose might have favored the growth of filamentous bacteria in phase I, compromising the sludge settleability. Then, in phase II, part of the solids were wash out from the reactor. At this moment, SVI_{30} decreased because only the solids with better settleability remained.

The mechanisms of disintegration observed in R1 probably occurred as follow:

- Step 1: Formation of granules with large diameters (> 3 mm), especially near the bottom of the reactor, similar to that reported by Verawaty et al. (2013);
- Step 2: The diffusivity of oxygen and carbon became difficult in these granules due to a thick layer formed in the biofilm. Because of the low penetration of oxygen, the anaerobic layer was larger, and, due to the low penetration of carbon, the main bacterial metabolisms in the regions near the core were fermentation and endogenous respiration (Adav and Lee, 2011; Verawaty et al., 2013).
- Step 3: The formation of anaerobic products (fermentation) and cell lysis (endogeny) decreased the cohesive forces in the core, causing granule disintegration and subsequent solids washout (Franca et al., 2018).

Other studies using acetate as carbon source have observed granules disintegration over long operation periods (Long et al., 2015), which were related to the granule size. In view of this, some researchers have established that the average size of the granules should be below 2–3 mm (Wang et al., 2007).

Several strategies to avoid AGS disintegration were proposed by Franca et al. (2018), which might have avoided the problem of disintegration in R1. For instance:

(a) selective sludge discharge – it was observed that a portion of the sludge retained in the reactor had a flocculent form and may be related to disintegration. Some researchers proposed sludge discharge as a solution to improve AGS stability, by maintaining the biomass in a particular size (Verawaty et al., 2013).
(b) aeration rate adjustment – as previously mentioned, the formation of granules with large diameters (> 3 mm) and filamentous characteristics was observed. In these cases, some researchers suggest a hydrodynamic shear stress increase to suppress the overgrowth of large granules (Adav and Lee, 2011; Verawaty et al., 2013). Therefore, an adjustment of aeration (and consequent agitation) could have controlled granule size not only in the formation but also in the maintenance over the operation time, therefore helping the system stability.

3.3. Characteristics of the granules

The characteristics of the granules formed during maturation period are presented in Table 1. The granules grown on acetate had the best settleability characteristics. However, in the presence of glucose, the granules had the greatest resistance. Regarding extracellular polymeric substances (EPS), as it is known, these are biopolymers consisting of polysaccharides, proteins and others substances, which act as a “biological glue” for granule formation and stability (Rollemberg et al., 2018).

The granules cultivated in R1 showed higher values of PS (40.8 mg PS/g MLVSS) and PN (66.6 mg PN/g MLSS). In terms of total EPS, granules cultivated in R1 presented the highest values (107.4 mg EPS/g MLVSS) followed by the granules formed in R2 (96.6 mg EPS/g MLVSS) and R3 (47.5 mg EPS/g MLVSS) (See Table 2).

In general, PN is the most abundant substance in the EPS of stable aerobic granules. Therefore, it is important to evaluate the PN/PS ratio (Rollemberg et al., 2018). The PN/PS ratios in R1, R2 and R3 were 1.6, 1.0 and 0.6, respectively. The granules formed in the presence of glucose were the only ones that presented higher protein content than polysaccharides. Therefore, the carbon source impact on the amount of PS and PN of the granules was clearly demonstrated. Other studies that used glucose as carbon source showed similar results, indicating that glucose does not favor the production of EPS (Liu et al., 2014).

The resistance of aerobic granules is presented using the stability coefficient (S) and the percentage of change in granule diameter before and after shear test. The lower the values of S (%), the higher the stability of aerobic granules (Nor-Anuar et al., 2012). The values obtained were 46.2%, 44.9% and 63.8% for R1, R2 and R3, respectively, which indicated that all granules formed were not considered strong, especially those cultivated in R3.

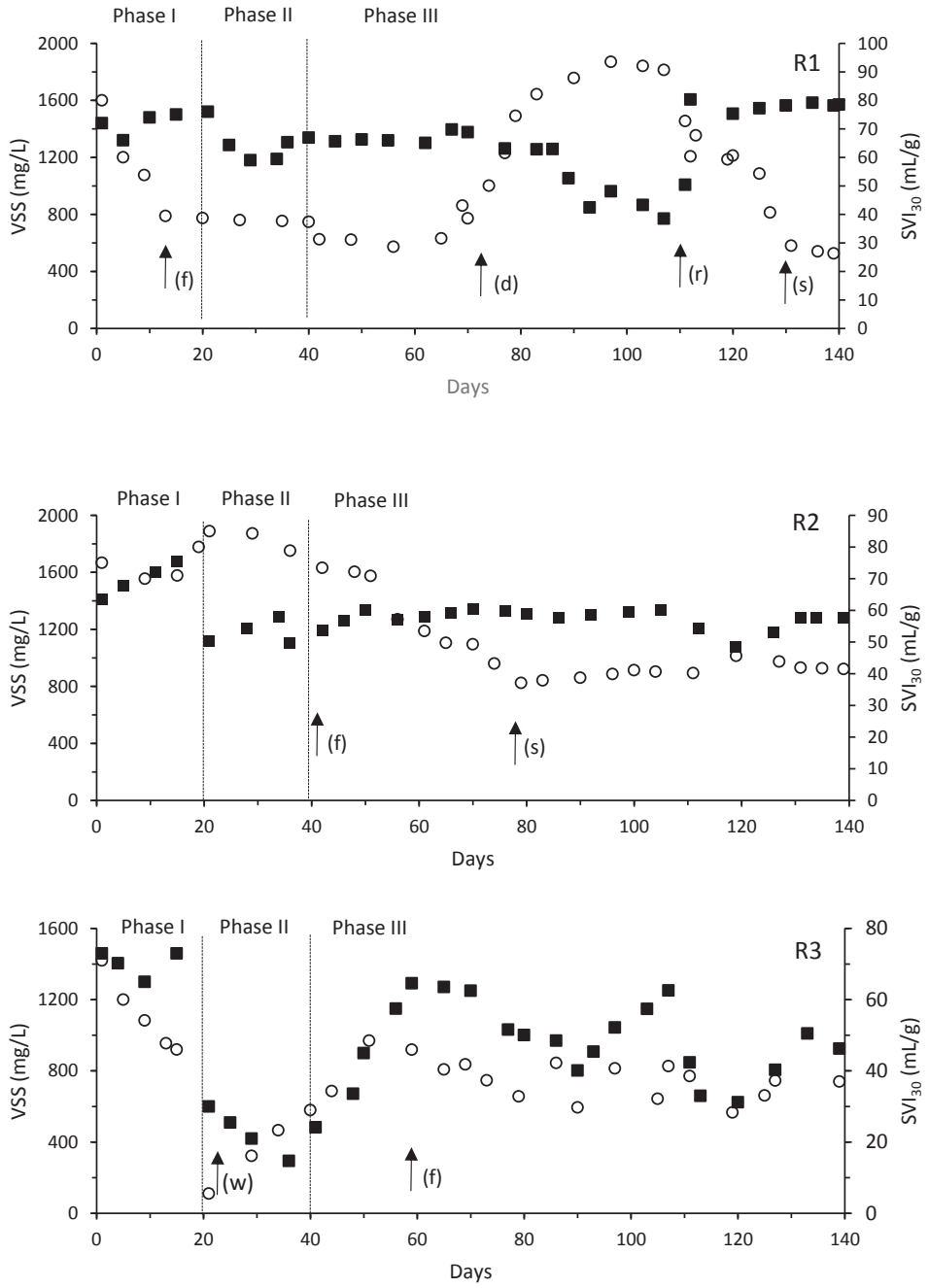

Fig. 1. Stability, in terms of VSS (filled square) and SVI_{30} (empty circle), of the AGS systems fed with the substrates acetate (R1), ethanol (R2) and glucose (R3) in the phases I (20 min), II (10 min) and III (5 min). (f) Granule formed, (d) start of disintegration, (r) granules reintegration, (s) system stability, (w) solids washout.

3.4. Reactor performance

In all systems, COD removal had high values (~90%) and there was no statistically significant difference ($p = 0.08$), but, in the last phase (maturation), it was observed that R1 and R2 presented mean values higher than R3 (Table 3).

In R1 (acetate), the ammonia removal was high throughout the period of operation, indicating that nitrifying bacteria (ammonia oxidizing bacteria, AOB and nitrite oxidizing bacteria, NOB) were stable in the system. However, during granules disintegration, NH_4^+ removal was reduced, possibly due to the loss of nitrifying bacteria that were present in the broken granules. Although, within a few days, the removal of ammonia was restored. The removal of phosphorus presented a great oscillation. After the startup, an average removal above 50% was observed. However, after granules maturation the removal values were about 30%. During the process of disintegration and subsequent granules reintegration, the system returned to higher removal values (~50%). Although, after stabilization, the typical values of removal were achieved.

In R2, the removal of total nitrogen was slightly lower than in R1 ($p = 0.01$), reaching values of around 55% during maturation phase. Among the three systems evaluated, R2 presented the lowest values of NO_2^- and NO_3^- accumulation, indicating better denitrification rates. This result corroborates some studies that suggest ethanol as one of the best electron donors for heterotrophic denitrification (Peng et al., 2007).

In R3 (glucose) presented the lowest N ($p < 0.01$) and P ($p = 0.05$) removal performance among the evaluated systems. Besides presenting

Table 1
Granules characteristics of the AGS systems fed with the substrates acetate (R1), ethanol (R2) and glucose (R3).

Characteristics	R1	R2	R3
SVI_{30} (mL/g)	33.7 ± 3.9	42.7 ± 12.3	41.6 ± 10.7
SVI_{10} (mL/g)	34.4 ± 4.2	44.2 ± 12.9	46.6 ± 16.8
SVI_{30}/SVI_{10}	~0.98	~0.96	~0.89
TSS > 1 mm (%)	97.1 ± 2.9	93.6 ± 1.8	90.6 ± 2.5
Mean diameter (mm)	1.5	1.2	0.7
Stability indicator (%)	46.2 ± 6.6	44.9 ± 3.9	63.8 ± 11
SRT (d)	8–12	6–10	5–7
PS (mg/g MLVSS)	40.8 ± 2.9	48.0 ± 8.7	29.1 ± 9.8
PN (mg/g MLVSS)	66.6 ± 3.2	48.6 ± 4.8	18.4 ± 3.3
PN/PS	1.6	1.0	0.6
Settling velocity (m/h)	35.7 ± 2.1	28.3 ± 4.7	21.7 ± 9.2
Color	Yellow	Brown	Light yellow
Format	Granular with irregular surface	Granular with irregular surface	Granular and spherical

the lowest values of removal, R3 was the system that took longer to present significant values of ammonia removal. The removal of total nitrogen was approximately 55%. Concerning phosphorus removal, mean values of 20% were observed.

The factors that may have influenced the low phosphorus removal in the systems studied are:

(i) absence of controlled solids discharge – Although all systems have presented relatively low values of SRT, it is important to note that there was no controlled discard of sludge. The explanation for this fact is due to the removal of solids together with the reactor effluent (thus resulting in low SRT). In this context, some authors (Bassin et al., 2012; Rollemberg et al., 2018) had proposed the controlled disposal of sludge to improve the removal of phosphorus. The main strategies reported are: to discharge a portion of the bottom sludge aiming at the removal of the saturated bacteria in P, thus improving the process of luxury uptake of phosphorus in the next cycles and the selective removal of floating sludge, which is reported to be rich in GAOs (these bacteria compete directly with PAOs);
(ii) short anaerobic period (1 h), affecting the metabolism of phosphorus release and subsequent sequestration in the aerobic period. Some studies with focus on EBPR suggest 2–3 h anaerobic period (He et al., 2018);
(iii) in R1 and R2, one of the possible causes may have been the competition between PAOs and denitrifiers. Although nitrite and nitrate concentration have been relatively low in the system, it is possible that residual nitrite and nitrate in the reactor after the cycle are denitrified in the anaerobic phase. In this competition, denitrifying bacteria may be favored because of the high growth rate (Kishida et al., 2006). Another (and most likely) cause is the presence of GAOs, which have a mechanism similar to PAOs, but do not remove phosphorus (Rollemberg et al., 2018).
(iv) The low phosphorus removal when glucose was the substrate can be related to the absence of the anaerobic conversion of the substrate into storage polymers, such as polyhydroxyalkanoates (PHA) (Chen et al., 2015).

3.5. Removal mechanisms

The simultaneous nitrification, denitrification and phosphorus removal (SNDPR) processes were evaluated over a complete cycle (Fig. 2). In this experiment, it was observed that dissolved oxygen (DO) values was between 1 and 3 mg/L during the first two hours of aeration, and, at the end of the aeration period, DO was above 4 mg/L. Regarding the famine period, it can be identified when there is no more COD available, and, by evaluating the cycles, it is observed that this moment occurred after 2 h of aeration (approximately). This time coincided with the moment when there is an increase in DO, since the bacteria enter the endogenous phase and require a lower oxygen value for their metabolism. It was also observed a low pH variation (6.4–7.7) in all systems during the cycle, probably due to the SND (simultaneous nitrogen and denitrification) process, which consumes (nitrification) and produces (denitrification) alkalinity.

In R1 (acetate), the maximum concentration of PS and PN (feast period) were 51 mg PS/g VSS and 70 mg PN/g VSS, respectively. At the end of the cycle (famine period), the concentration of PS was reduced to 42 mg PS/g VSS, and that of PN remained close to the previous value (67 mg PN/g VSS). Considering that practically all COD was consumed in the first hour of aeration, possibly the PS stored during the feast period was used as an electron donor for denitrification that occurred at the end of the cycle. Heterotrophic denitrification using EPS as electron donor in the famine period is reported in literature (Wu et al., 2012). Still in relation to R1, it was verified that the main mechanism of nitrogen removal occurred via SND during the aerobic period, and there was no considerable accumulation of nitrite and nitrate. This statement can be made through studies conducted by Wagner et al. (2015), who observed that, after granule formation, nitrogen assimilation was less than 5% of the total influent nitrogen. The results indicate that part of the nitrite formed (partial nitrification) was denitrified, and another portion was oxidized (complete nitrification) and, then, denitrified again. The low removal of ammonia during the anaerobic period indicates the small amount or absence of Anammox (anaerobic ammonia oxidation) bacteria.

In the R2 system, the maximum concentrations of PS and PN (feast period) were 57 mg PS/g VSS and 49 mg PN/g VSS, respectively. At the end of the cycle (famine period), the concentration of PS was reduced to 50 mg PS/g VSS, whereas that of PN remained almost the same (47 mg

Table 2
Removal of COD, nitrogen and phosphorus of the AGS systems fed with the substrates acetate (R1), ethanol (R2) and glucose (R3).

Parameters	Phase I			Phase II			Phase III		
	R1	R2	R3	R1	R2	R3	R1	R2	R3
COD_{inf} (mg/L)	621 ± 51	600 ± 50	620 ± 46	629 ± 59	617 ± 67	609 ± 54	590 ± 45	590 ± 51	612 ± 46
COD_{eff} (mg/L)	82 ± 50	71 ± 15	69 ± 35	53 ± 10	59 ± 12	85 ± 27	42 ± 16	47 ± 22	85 ± 40
COD removal (%)	87 ± 7	88 ± 1	89 ± 5	91 ± 2	90 ± 2	86 ± 3	93 ± 2	92 ± 4	86 ± 6
$N\text{-}NH_{4inf}^{+}$ (mg/L)	108 ± 6	104 ± 5	108 ± 10	115 ± 6	115 ± 7	112 ± 8	110 ± 6	108 ± 8	108 ± 5
$N\text{-}NH_{4eff}^{+}$ (mg/L)	22 ± 16	46 ± 28	34 ± 21	26 ± 3	37 ± 1	49 ± 19	9 ± 5	34 ± 17	32 ± 18
$N\text{-}NO_{2eff}^{-}$ (mg/L)	1 ± 1	9 ± 6	13 ± 7	3 ± 2	7 ± 4	6 ± 5	12 ± 7	10 ± 8	31 ± 12
$N\text{-}NO_{3eff}^{-}$ (mg/L)	0	0	2 ± 1	1 ± 1	1 ± 1	6 ± 4	6 ± 2	4 ± 3	8 ± 7
TN removal (%)	78 ± 15	48 ± 16	55 ± 13	72 ± 3	56 ± 5	45 ± 5	75 ± 14	55 ± 18	34 ± 18
$P\text{-}PO_{4inf}^{3-}$ (mg/L)	8 ± 2	11 ± 1	10 ± 2	8 ± 2	11 ± 1	10 ± 2	8 ± 2	11 ± 1	10 ± 2
$P\text{-}PO_{4eff}^{3-}$ (mg/L)	4 ± 1	4 ± 1	6 ± 5	5 ± 3	9 ± 1	10 ± 1	6 ± 2	8 ± 2	8 ± 2
TP removal (%)	40 ± 26	48 ± 24	40 ± 37	53 ± 30	22 ± 8	7 ± 4	33 ± 10	24 ± 13	18 ± 6

Table 3
Removal of COD, nitrogen and phosphorus, and granules characteristics of the AGS systems fed with the substrates acetate (R1), ethanol (R2) and glucose (R3) during the resilience tests.

Parameters	Before shocks (end of phase III)			After organic shocks			After salt shocks		
	R1	R2	R3	R1	R2	R3	R1	R2	R3
COD removal (%)	91.9	91.2	86.5	91.9	92.7	91.0	90.2	77.3	72.5
TN removal (%)	76.8	59.8	30.1	76.3	61.5	49.1	64.8	33.6	18.2
TP removal (%)	33.2	23.7	19.4	32.8	31.7	21.7	14	3.1	8.1
TSS > 1 mm (%)	97.1	93.6	70.6	97.3	94.2	94.8	92.4	90.8	94.3
VSS (mg/L)	1286	1273	978	2823	1548	1110	1105	1038	856
PS (mg PS/g VSS)	40.8	48.0	29.1	41.1	48.5	32.5	20.1	28.2	17.6
PN (mg PN/g VSS)	66.6	48.6	18.4	67.2	48.9	19.1	30.7	26.5	5.8
PN/PS	1.6	1.0	0.6	1.6	1.0	0.6	1.5	0.4	0.2

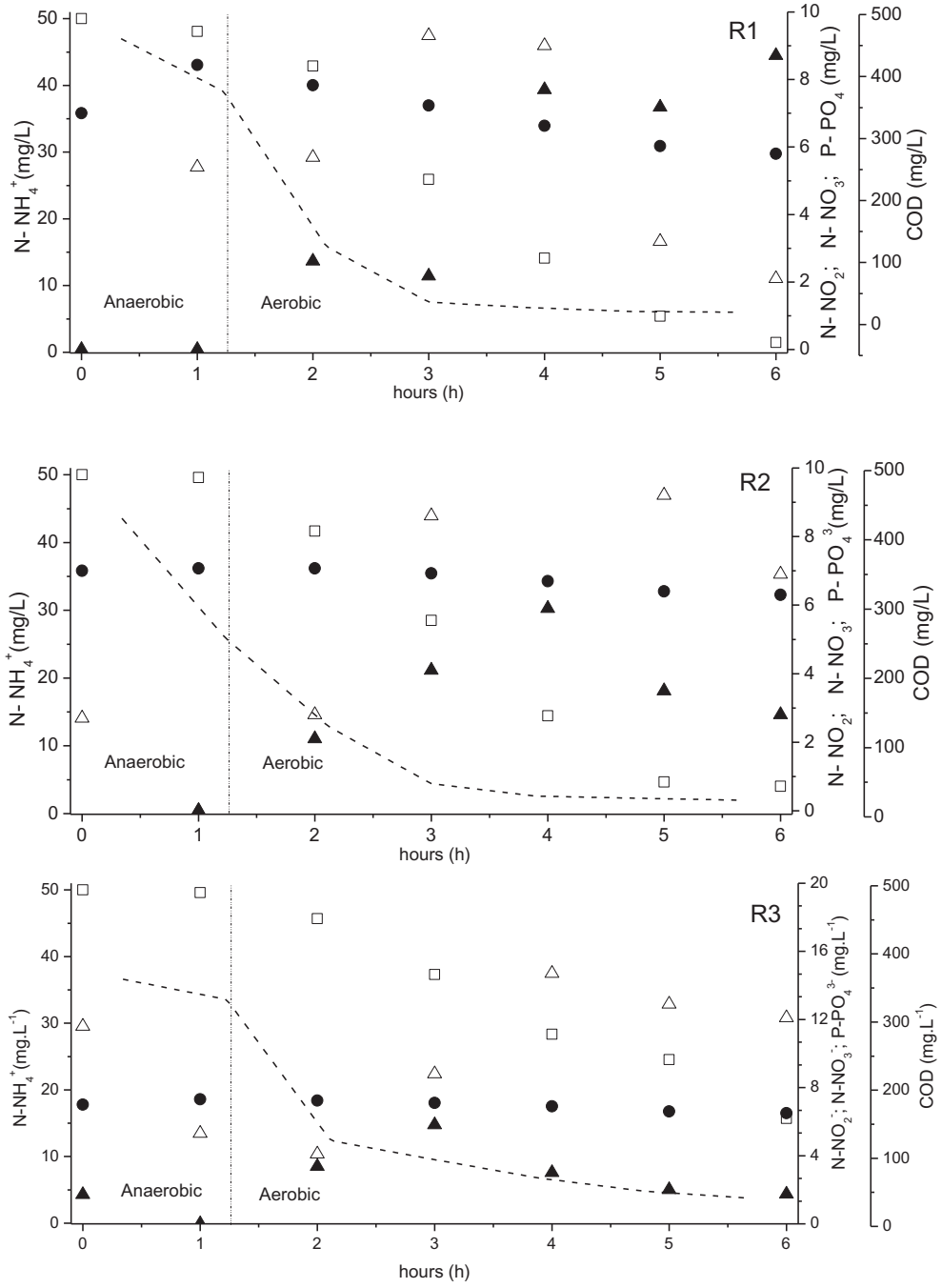

Fig. 2. Evaluation of simultaneous nitrification and denitrification and phosphorus removal in the AGS systems fed with the substrates acetate (R1), ethanol (R2) and glucose (R3). (dashed line) COD, (empty square) N-NH_4^+, (empty triangle) N-NO_2^-, (filled triangle) N-NO_3^-, (filled circle) P-PO_4^{3-}.

PN/g VSS). The SND results obtained in R2 (ethanol) were close to those obtained in R1. The main difference is related to nitrification. In R1, the presence of nitrite and nitrate was observed, but, in R2, mainly nitrite was found.

For R3 (glucose), the cycle analysis shows that some of the ammonia present in the influent was not oxidized. The maximum concentration of PS and PN (feast period) were 31 mg PS/g VSS and 18 mg PN/g VSS, respectively. At the end of the cycle (famine period), no difference was observed (30 mg PS/g VSS and 18 mg PN/g VSS). This system showed accumulation of nitrite and low denitrification rates in the famine period (after 2 h of aeration), indicating that denitrification via PHA (accumulated in the feast period) might have not occurred. The absence of variation in the PS value in R3 indicates the low presence of EPS-producing bacteria and GAOs, since these microorganisms contribute to the amount of polysaccharides and proteins in the granules (Adav and Lee, 2011).

3.6. Resilience test of the granules

In the organic shock load test, the same COD concentration of 2000 mg/L was used in all reactors, and the ammonium and phosphate concentration was the same as that used during the experiment (110 mg/L of NH_4^+ and 10 mg/L of PO_4^{3-}). Regarding the salt shock load test, 15 g/L of NaCl was added to the regular feeding solution (used in phases I–III).

The results showed that the organic shock load did not affect remarkably the removal of COD and nutrients (Table 3). In the presence of salt, the granules grown in R1 maintained COD removal capacity, while the granules grown in R2 and R3 showed a significant COD removal reduction. All reactors showed reduction in terms of nitrogen and phosphorus removal. In fact, literature reports that salt (15 g/L NaCl) can significantly affect nutrient removal due to inhibition of nitrifying bacteria and organisms that accumulate phosphate (PAOs) (Wang et al., 2017).

The greatest reductions occurred again in R2 and R3, concluding that the granules grown in R1 were the most resilient in the presence of saline shock. All reactors showed significant reductions in terms of EPS content (about 50%). The hypothesis for the performance of the granules (in the presence of salt) may be related to the amount of EPS, mainly the PN, responsible for the structure and stability of the AGS (Corsino et al., 2017). In this way, the amount and composition of the EPS had a vital role in the granules resilience during the saline shock, thus justifying the performance of the granules of R1 in the tests.

3.7. Microbial population dynamics

3.7.1. Diversity of microbial communities

The species richness and diversity estimators of microbial populations of R1, R2 and R3 are shown in Table 4. A total of 40016–61827 sequence tags were retrieved from the aerobic granules that were assigned to 1348–1899 OTUs. The numbers of sequences were similar to the previous aerobic granular samples with acetate and glucose as sole carbon sources (He et al., 2018).

The results showed that acetate is the substrate that favored the largest quantity of species and provided the greatest richness among the granules formed. The granules cultivated in R2 presented inferior variety to those cultivated in R1, but presented superior richness when compared to the granules grown in R3. The results were similar to those obtained by He et al. (2018), in which the richness indices Ace and Chao decreased when glucose was the substrate.

3.7.2. Overall taxonomic microbial populations

The identified bacterial structures and relative abundances of aerobic granules samples with different carbon sources are shown in Fig. 3 at phylum, class, family and genus levels, respectively.

At phylum level, Bacteroidetes and Proteobacteria were dominant in R1 and R2, whereas, in R3, only Bacteroidetes dominated. Less abundantly, the phyla Chlorobi and Verrucomicrobia were found. The Verrucomicrobia functioned in heterotrophic metabolism and were capable of degrading complex polysaccharides (Luo et al., 2014). The Chlorobi played vital roles in the carbohydrates and cellular materials biodegradation, and both nitrification and denitrification (Zhou et al., 2015).

At class level, Alphaproteobacteria had relative abundance of 69% in R1, 50% in R2 and 38% in R3. This class is related to a variety of bacteria capable of EPS secretion (Ramos et al., 2015). This fact may be a possible explanation for the low EPS production in R3.

At family level, the abundance of Saprospiraceae was observed in R3. This family include several different bacteria, and many of them are associated with protein hydrolysis. The bacteria of this family usually are filamentous (Nielsen et al., 2010), which matches with the morphological characteristic of the granules formed in the reactor.

Among the genera and families found, the following stand out: Xanthomonadaceae and Rhodocyclaceae (EPS production), *Flavobacterium* (OHO), *Paracoccus* (related to degradation of recalcitrant compounds, such as pyridine), and *Thauera* (DOHO) (Szabó et al., 2017).

The absence of the anaerobic ammonia oxidation process in the specific tests of the cycles was confirmed by the non-identification of Anammox bacteria.

3.7.3. Key functional groups

Taxonomic affiliation of each OTU was used to infer functional content related to the key functions, such as GAOs, PAOs, AOB, NOB and denitrifying bacteria (DNB) (Fig. 4). The values show that R1 and R2 presented greater abundance of AOB and NOB bacteria, unlike system R3, thus justifying the low oxidation of ammonia in the reactor fed with glucose. It was also observed that R1 showed a greater diversity of nitrifying species, confirming what was observed in other studies (Tay et al., 2002; Du et al., 2011), where it was verified that the acetate favors the growth of nitrifying bacteria and others), since glucose favors the growth of heterotrophic filamentous bacteria. Regarding denitrifying bacteria, both systems presented similar abundance results. However, a greater diversity of denitrifying bacteria was observed in R1.

A greater diversity of phosphorus-accumulating microorganisms was found in R1 and R2 when compared to R3. Nevertheless, a predominance of GAOs over PAOs was observed in all reactors due to the operating conditions, mainly in R1, since acetate is a preferential substrate. Although acetate is also the preferential substrate for PAOs, the results indicate that adequate strategies are needed to promote the predominance of these microorganisms over GAOs. In the context of strategies favoring PAOs, several authors observed that none of the GAOs bacteria could survive in an EBPR system with propionate as the

Table 4
Species richness and diversity indicators of microbial populations the AGS systems fed with the substrates acetate (R1), ethanol (R2) and glucose (R3).

Reactor	Sequences	OTUs	Good's Coverage	Richness	Ace	Chao	Shannon	Inverse Simpson
R1	61,827	1899	99.66519	1899	2004.848	1929.286	1.146	3.021
R2	28,349	1493	98.65956	1493	1813.214	1650.571	1.706	4.853
R3	40,016	1348	99.17283	1348	1597.061	1486.617	1.669	4.833

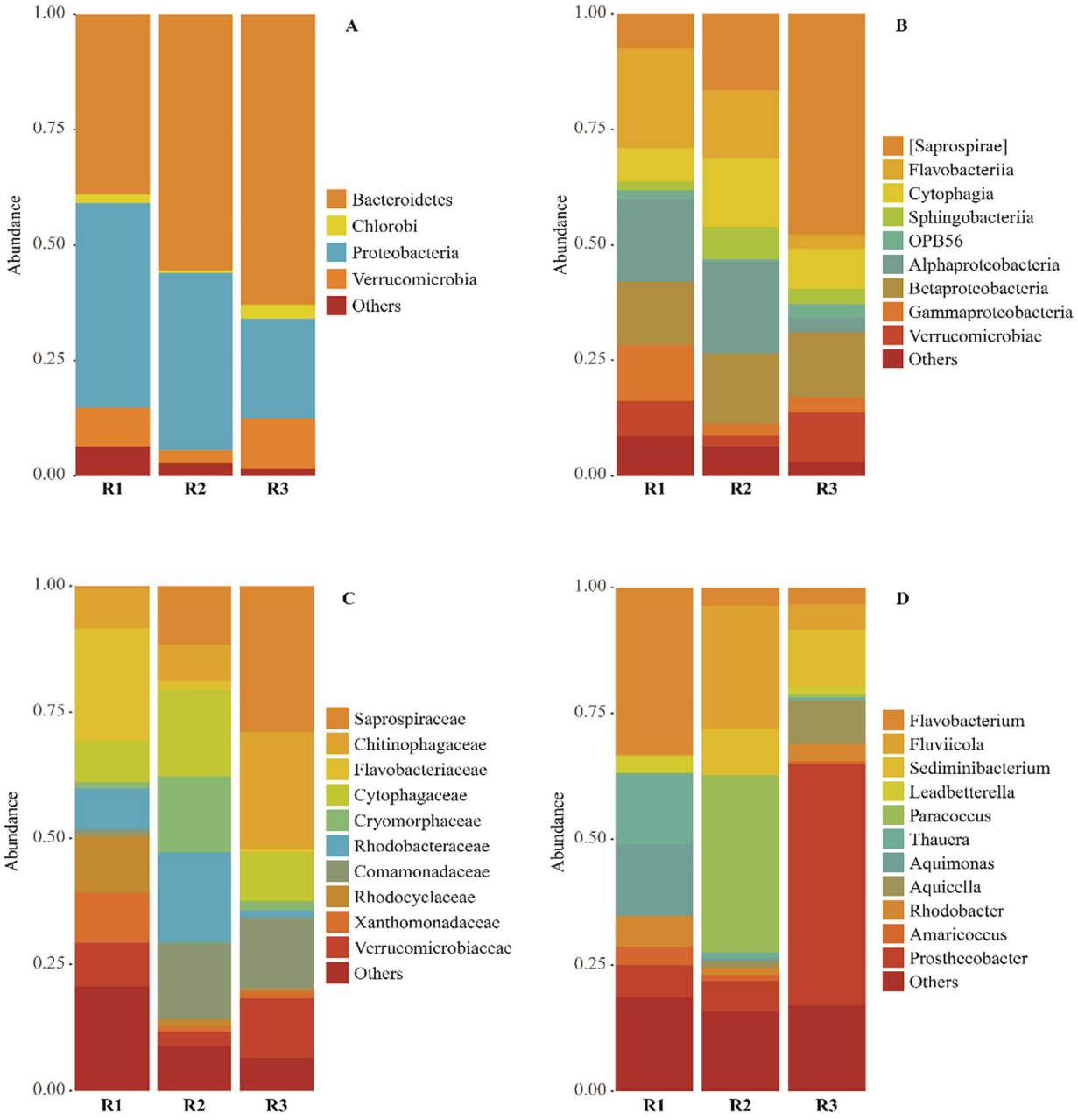

Fig. 3. Taxonomic affiliation of the aerobic granules of R1 (acetate), R2 (ethanol) and R3 (glucose) at different levels. (A) Phylum, (B) class, (C) family, (D) genus.

only electron donor and nitrite as the only electron acceptor. Hence, these two simultaneous conditions should lead to the washout of the GAO and, thus, to a PAO-enriched sludge (Kishida et al., 2006; He et al., 2018).

Some studies have observed that the use of glucose or similar substrates rich in energy favors filamentous bacteria growth (Tay et al., 2002). The degradation of these complex substrates (glucose and others) is a multi-step process, involving many types of intermediates and requiring the participation of different microbial groups. On the other hand, the use of acetate and other simple substrates requires the participation of specific groups related to granules formation, such as PAOs/denitrifying PAOs (DPAOs), GAOs/denitrifying GAOs (DGAOs) and EPS producers. Acetate is suggested as the preferred substrate for poly-P bacteria, and ethanol is reported as one of the preferred substrates of heterotrophic denitrifying bacteria, but can also be used by EPS-producing bacteria (Puig et al., 2008).

3.8. Granules overview

Carbon source had an impact on the formation and characteristics of the granules formed. In general, acetate provided rapid formation of granules, which presented the best results of performance, sedimentability, resistance and larger diameters. On the other hand, ethanol formed granules with better stability, although they had an inferior performance compared to those grown on acetate. Finally, glucose was the substrate for which granulation took the longest time to occur. In addition, granules grown on glucose had the lowest microbiological diversity and performance. The comparison of the granules formed in R1 (acetate), R2 (ethanol) and R3 (glucose) is shown in Table 5.

4. Conclusion

Considering the overall results, acetate seems to be an ideal substrate for the cultivation of AGS that target SNDPR. However, it is

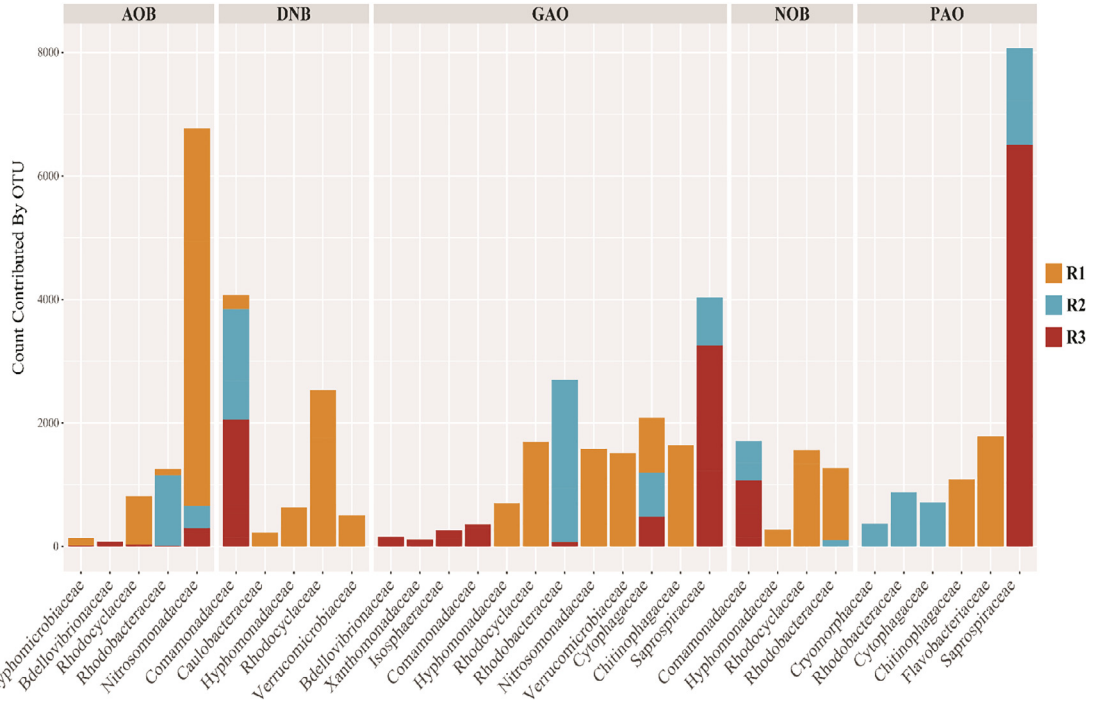

Fig. 4. PICRUSt prediction of key functional groups involved in SNDPR at family level in R1 (acetate), R2 (ethanol) and R3 (glucose).

Table 5
Comparison of the granules formed in the AGS systems fed with the substrates acetate (R1), ethanol (R2) and glucose (R3).

Characteristics	R1	R2	R3
Filamentous structure	Medium	Low	High
Disintegration	Partial	NO	NO
Stability	+ +	+ + +	NO
Size	+ + +	+ +	+
Dense and compact	+ + +	+ +	+ +
Surface porosity	+ + +	+ + +	+
Rapid formation	+ + +	+ +	+
Solids concentration	+ +	+ +	+
Settling velocity	+ + +	+ +	+
Amount of EPS	+ + +	+ +	+
COD removal	+ + +	+ + +	+ + +
Thickness of anoxic layer	+ + +	+ + +	+
Phosphorus removal	+ +	+	+
Physical resistance	+	+	+
Organic shock load resistance	+ + +	+ + +	+ + +
Salt shock load resistance	+ + +	+ +	+
Microbial diversity	+ + +	+ +	+

+, good; + +, very good; + + +, excellent; NO, not observed.

necessary to monitor the size of the granules formed and the stabilization strategies to avoid disintegration, such as sludge disposal and aeration intensity control as means of inhibiting the growth of filaments in the granules. The granules formed from ethanol also presented interesting results, mainly concerning the stability. Among the substrates evaluated, glucose was the carbon source that less favored the formation of aerobic granules.

Acknowledgements

The authors would like to acknowledge the financial support obtained from the following Brazilian institutions: Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq; Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES; Fundação de Amparo à Pesquisa do Estado de Minas Gerais – FAPEMIG; Instituto Nacional de Ciência e Tecnologia em Estações Sustentáveis de Tratamento de Esgoto – INCT ETEs Sustentáveis (INCT Sustainable Sewage Treatment Plants). We also would like to acknowledge the Central Analítica-UFC/CT-INFRA/MCTI-SISANO/Pró-Equipamentos CAPES and Central de Genômica e Bioinformática (CeGenBio) do Núcleo de Pesquisa (NPDM) for the analysis performed.

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