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Effects of the antibiotics trimethoprim (TMP) and sulfamethoxazole (SMX) on granulation, microbiology, and performance of aerobic granular sludge systems

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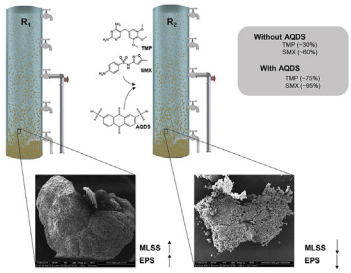
HIGHLIGHTS

- The AGS grown with TMP and SMX showed good settleability, but irregular structure.
- TMP and SMX did not impair the removal of C, N, and P in the AGS system.
- Adsorption on sludge played a significant role only in the removal of SMX.
- AQDS significantly improved the removal of TMP and SMX in the AGS system.
- The AGS grown with SMX and TMP was microbiologically very similar to the inoculum.

GRAPHICAL ABSTRACT



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ABSTRACT

This work assessed the effect of the antibiotics trimethoprim (TMP) and sulfamethoxazole (SMX) on the granulation process, microbiology, and organic matter and nutrient removal of an aerobic granular sludge (AGS) system. In addition, after the maturation stage, the impact of the redox mediator anthraquinone-2,6-disulfonate (AQDS) (25 μM) on the biotransformation of the antibiotics was evaluated. The reactor R1 was maintained as a control, and the reactor R2 was supplemented with TMP and SMX (200 μg L^{-1}). The ability to remove C, N, and P was similar between the reactors. However, the structural integrity of the AGS was impaired by the antibiotics. Low TMP (~30%) and SMX (~60%) removals were achieved when compared to anaerobic or floccular biomass aerobic systems. However, when the system was supplemented with AQDS, an increase in the removal of TMP (~75%) and SMX (~95%) was observed, possibly due to the catalytic action of the redox mediator on cometabolic processes. Regarding the microbial groups, whereas Proteobacteria and Bacterioidetes increased, Planctomycetes decreased in both reactors. However, TMP and SMX presence seemed to inhibit or favor some genera during the formation of the granules, possibly due to their bactericidal action.

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1. Introduction

The antibiotics trimethoprim (TMP) and sulfamethoxazole (SMX) belong to the emerging micropollutant (EM) class, are generally used together in human and veterinary medicine, and are usually present in wastewater and even surface water. For example, in rivers, TMP is usually found in concentrations in the order of tens to hundreds of ng·L^{-1}, while SMX is detected in concentrations below 100 ng L^{-1} (Carvalho and Santos, 2016).

This presence deserves attention since there is evidence that these drugs can have an impact on the environment. Regarding TMP, there are records of its negative effect on mussel cells (Binelli et al., 2009; Matozzo et al., 2015), mammals, and fish (Papis et al., 2011). SMX is reported to affect the population growth of algae and crustaceans (Isidori et al., 2005), and may even cause a transgenerational impact on nematodes (Yu et al., 2017).

Among the various sewage treatment technologies used to remove TMP and SMX, the anaerobic treatment appears to be more efficient, with removals higher than 90% (Alvarino et al., 2014; Carballa et al., 2007). On the other hand, aerobic floccular sludge (AFS) systems registered lower efficiencies, but with wider variation ranges. Removals are between 0 and 83% for TMP and between 40 and 75% for SMX (Alvarino et al., 2014; Jewell et al., 2016; Kang et al., 2018b), except for AFS rich in AOB (ammonia-oxidizing bacteria), which could reach SMX removals higher than 95% (Kassotaki et al., 2016).

Some studies on granulation in the presence of SMX have been performed. Regarding organic matter removal, sulfate reduction, and SMX removal, Qiu et al. (2019) investigated the effects of SMX on the long-term performance of sulfate-reducing upflow sludge bed (SRUSB) reactors inoculated with sulfate-reducing bacteria (SRB) granules and flocs. They concluded that SMX had no significant effect on chemical oxygen demand (COD) and sulfate removals in the two reactors, and the SMX removal by the SRB granules (49%–61%) was higher than that by SRB flocs (39%–41%). On the other hand, Chen et al. (2017), cultivating denitrifying granules in the presence of SMX (0–100 mg L^{-1}) in an upflow anaerobic sludge blanket (UASB) reactor, showed that SMX affected the performance of the denitrifying granules since the increasing concentration of SMX reduced the specific denitrifying activity (SDA). Another conclusion of this study was that the content of extracellular polymeric substances (EPS) increased because the denitrifying granules presented a better resistance to SMX stress. Besides, they did not observe any influence on the UASB performance in the presence of SMX.

Concerning aerobic granular sludge (AGS) systems, Du et al. (2019) indicated that, under long-term exposure to SMX (1 mg L^{-1}), removals of COD and total nitrogen were inhibited, more filamentous bacteria were observed, and more EPS was secreted. Regarding efficiency removal, there are only long-term studies for SMX, and their removal range is between 60 and 91% (Liu et al., 2019; Zhao et al., 2015). Comparatively, although these values have a wider range of variation than those reported for anaerobic systems, they are better than most of the values obtained with floccular biomass, which indicates AGS as a promising technology for the treatment of EMs.

Associated with this, the use of redox mediators also becomes a possibility to enhance the removal of EMs, since some cometabolic enzymes involved in EM removal can be activated in the presence of redox mediators. Moreover, literature reports the ability of redox mediators to accelerate electron transfer or to behave as final electron acceptors (dos Santos et al., 2007; Martinez et al., 2017; Van der Zee and Cervantes, 2009).

In this sense, several compounds that improve the degradation/biotransformation of many pollutants have been studied. Some of them are riboflavin, anthraquinone-2-sulfonate (AQS) and, especially, anthraquinone-2,6-disulfonate (AQDS), which were able to remove dyes (Silva et al., 2012), chromium (IV) (Meng et al., 2018), pentachlorophenol (Chen et al., 2016), pesticides (Liu et al., 2015), solvents, industrial detergents (Aulenta et al., 2010), among others. Some compounds can also immobilize these redox mediators to promote enhanced conversion rates of contaminants (Colunga et al., 2015). Accordingly, Toral-Sánchez et al. (2017) used graphene oxide to improve the removal of ioprimide in an UASB system. Alvarez et al. (2010) worked with metal-oxides nanoparticles to immobilize AQDS, which accelerated the reductive decolorization of an azo dye.

Regarding the antibiotics, Zhou et al. (2018) report that the degradation of SMX by *Shewanella oneidensis* MR-1, under Fe (III) reducing conditions, could be improved in the presence of AQDS and riboflavin. However, there are some inconsistencies and scarcity of studies investigating in detail the granule formation process, its microbial ecology, and the efficiency and operational stability of the system concerning the removal of TMP and SMX in AGS systems, especially when they are associated. Thus, the present study assessed the effect of TMP and SMX on the granulation process, microbiology, and on organic matter and nutrient removal of an AGS system. In addition, the impact of AQDS on the biotransformation of the antibiotics was evaluated.

2. Materials and methods

2.1. Configuration of systems and operational conditions

The experiment was carried out in two sequential batch reactors (SBRs) (diameter: 100 mm, height: 1 m, working volume: 7.2 L), inoculated with aerobic sludge (2.8 g VSS·L^{-1}) from a carousel AS system of a domestic wastewater treatment plant (WWTP) located in Fortaleza, Ceará, Brazil. The reactors were operated with settling times that were progressively decreased, from Stage I to III, to obtain granular biomass (Table 1). A 50% volumetric exchange rate and an air velocity of 2 cm s^{-1} were adopted. The reactors were wrapped with aluminum foil to prevent the photodegradation of the micropollutants.

Both reactors (R1 and R2) were fed with the same synthetic wastewater containing acetic acid (500 mg COD·L^{-1}), ammonia (~50 mg NH_4^+-N·L^{-1}), phosphate (~7 mg PO_4^{3-}-P·L^{-1}), magnesium (~75 mg $MgSO_4 \cdot 7H_2O \cdot L^{-1}$), calcium (~7,5 mg $CaCl_2 \cdot 2H_2O \cdot L^{-1}$), micronutrients (1 mL L^{-1}), whose solution was prepared according to Rollemberg et al. (2019a), and sodium bicarbonate (1 g L^{-1}), used as a buffer to keep the pH close to 7.0. The R2 feeding solution also contained the compounds TMP and SMX (~200 μg L^{-1} each),

Table 1
Time distribution among the phases of the 6-h operation cycles of the AGS reactors R1 (control) and R2 (supplemented with antibiotics) throughout the Stages I, II, III, and IV.

Phase (min)	Stage I	Stage II	Stage III	Stage IV
Filling	30	30	30	30
Anaerobic reaction	60	60	60	60
Aerobic reaction	250	260	265	265
Settling	18	8	3	3
Decanting	1	1	1	1
Idle	1	1	1	1
Total time of the cycle (h)	6			
Stage duration (days)	47	57	94	22

R2 was supplemented with the antibiotics sulfamethoxazole and trimethoprim at 200 μg L^{-1} at all stages.
At stage IV, 25 μM of the redox mediator anthraquinone-2,6-disulfonate were added only to R2.

obtained from Sigma-Aldrich (Milwaukee, WI, USA) and prepared using acetate as a solvent. R1 was maintained as a control, in which there was no exposure to the drugs mentioned above. The feeding solutions were kept at 4 °C, and the R2 feeding container was also wrapped with aluminum foil to prevent micropollutants' photodegradation. At Stage IV, R2 was also supplemented with AQDS (Sigma-Aldrich, USA), a model redox mediator compound at a concentration of 25 μM, to assess its impact on TMP and SMX biotransformations.

2.2. System monitoring

COD, ammonium, total suspended solids (TSS), and volatile suspended solids (VSS) were determined according to the Standard Methods for the Examination of Water and Wastewater (APHA, 2012).

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

These analyses were performed in duplicate for the influent and effluent samples three times a week. The dynamic sludge volumetric index (SVI) measurement for 5 and 30 min (Schwarzenbeck et al., 2005) was also performed at the same frequency.

The determination of the granulometric distribution of the biomass was carried out by passing the mixed liquor through sieves with 0.2 and 1 mm openings, and the dry weights of the total sample and the aliquots that passed through each of the sieves were recorded. This check was also carried out every fifteen days.

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

The microorganisms present in the mature granules (samples collected at Stage IV) were identified as described by Rollemberg et al. (2019). The DNA extraction was done in triplicate for each sludge sample.

2.3. Quantification of TMP and SMX

For the quantification of the influent and effluent concentrations of TMP and SMX in R2, the samples (500 mL) were previously filtered (0.45 μm) and acidified with HCl (pH 2.5–3). Then, they were percolated through Strata-X® cartridges (500 mg, 6 mL) (Phenomenex®, USA) for the solid phase extraction (SPE) of antibiotics, which were eluted with HPLC/UV grade methanol (4 mL) (99.8%, Neon, Brazil). The eluate (20 μL) was then analyzed by an LC-20 A Prominence high-performance liquid chromatograph (HPLC) equipped with a Shim-pack CLC-ODS (M) ® C18 column (4.6 × 150 mm, 5 μm) and a UV–Vis SPD-20 A detector (258 nm) (Shimadzu Corporation, Japan). The elution was performed by mobile phase composed of HPLC/UV grade acetonitrile (99.9%, Sigma-Aldrich, Germany) and 0.1% HCl solution with the following gradient: 10–80% increase in acetonitrile in 10 min, returning to 10% in 4 min. The flow rate was initially 1.0 mL min^{-1} and, after 5 min of run, it was increased to 2.0 mL min^{-1}. The oven temperature was maintained at 35 °C throughout the run. The detection limits of TMP and SMX were 3.2 and 2.4 μg L^{-1}, respectively.

At the end of Stage III (after maturation of the granules), the antibiotic removal profile throughout the R2 cycle was evaluated in duplicate (two cycles). During each cycle, dissolved oxygen (OD) was measured continuously (YSI 5000, YSI Incorporated, USA).

2.4. Quantification of TMP and SMX in the sludge

The quantification of TMP and SMX adsorbed on the sludge was carried out at the end of Stage III, with approximately 200 days of exposure to antibiotics, according to a methodology adapted from López-Serna et al. (2018). A volume of 100 mL of fresh sludge sample was lyophilized (Lyophilizer L101, Liobras, Brazil). After lyophilization, the dried sludge was weighed in a 20-mL glass vial, together with 5 mL of acetone (99%, Sigma-Aldrich, Germany). The mixture was completely vortexed and left for 12 h to allow the solvent to evaporate. A 12-mL volume of MilliQ® water at pH 9 was then added to the flask, which was vortexed vigorously to obtain a homogeneous suspension. The flask was then subjected to ultrasound-assisted extraction (UAE) for 30 min at room temperature and 60 Hz (Cristófoli Biossegurança, Brazil). Subsequently, the suspension was centrifuged for 5 min at 3600 rpm (Excelsa II 206 BL, Fanem, Brazil). The resulting supernatant was then collected with a glass pipette and transferred to a 25-mL volumetric flask. The extraction process was repeated once more with the addition of MilliQ® water. The next steps were the same as described before. The drugs were extracted from the resulting solution and quantified according to item 2.3. The recoveries of TMP and SMX were 73.4% and 80.3%, respectively.

3. Results and discussion

3.1. Granulation

The appearance of aerobic granules was observed from the 30th day of operation. Concomitantly, the SVI values decreased. At the beginning of the second stage of operation, the SVI_5/SVI_{30} ratio was already around 0.9 for both reactors (Fig. 1), marking the granulation achievement (Liu and Tay, 2007). After granulation (Stages II and III), the SVI_5 was between 30 and 50 mL g^{-1} for both reactors, which is consistent with the 50 mL g^{-1} SVI_5 reported by Kang et al. (2018b), who worked with a GSBR (*granular sludge batch reactor*) supplemented with 2 μg L^{-1} of SMX.

In the same period, the proportion of granules with a diameter greater than 1 mm became, on average, greater than 90% in both reactors. Biomass retention was greater in the control reactor (1.75–3 g MLVSS·L^{-1} from Stage II) than in the pharmaceuticals-added reactor (1–2 g MLVSS·L^{-1} from Stage II). The recorded values are lower than those reported by Kang et al. (2018b) and by Zhao et al. (2015) (GSBR supplemented with 50 μg L^{-1} of SMX), who recorded biomass retention close to 3 g MLVSS·L^{-1}.

During the operation with AQDS (Stage IV), the R2 granules showed no difference in terms of settleability and EPS composition (Table 2). The SVI_5/SVI_{30} ratio remained around 0.9, with a concentration of 1.5 ± 0.3 g MLVSS·L^{-1}.

Regarding the EPS values, in general, the control reactor showed higher PS and PN values throughout the experiment (Fig. 1). Theoretically, the addition of pollutants, such as TMP and SMX, should stimulate EPS production, which would act as a protective barrier against antibiotics (Shi et al., 2013). However, it was observed in the SEM analysis a granule structure quite different from that verified in the control reactor (Fig. S1), with bacterial

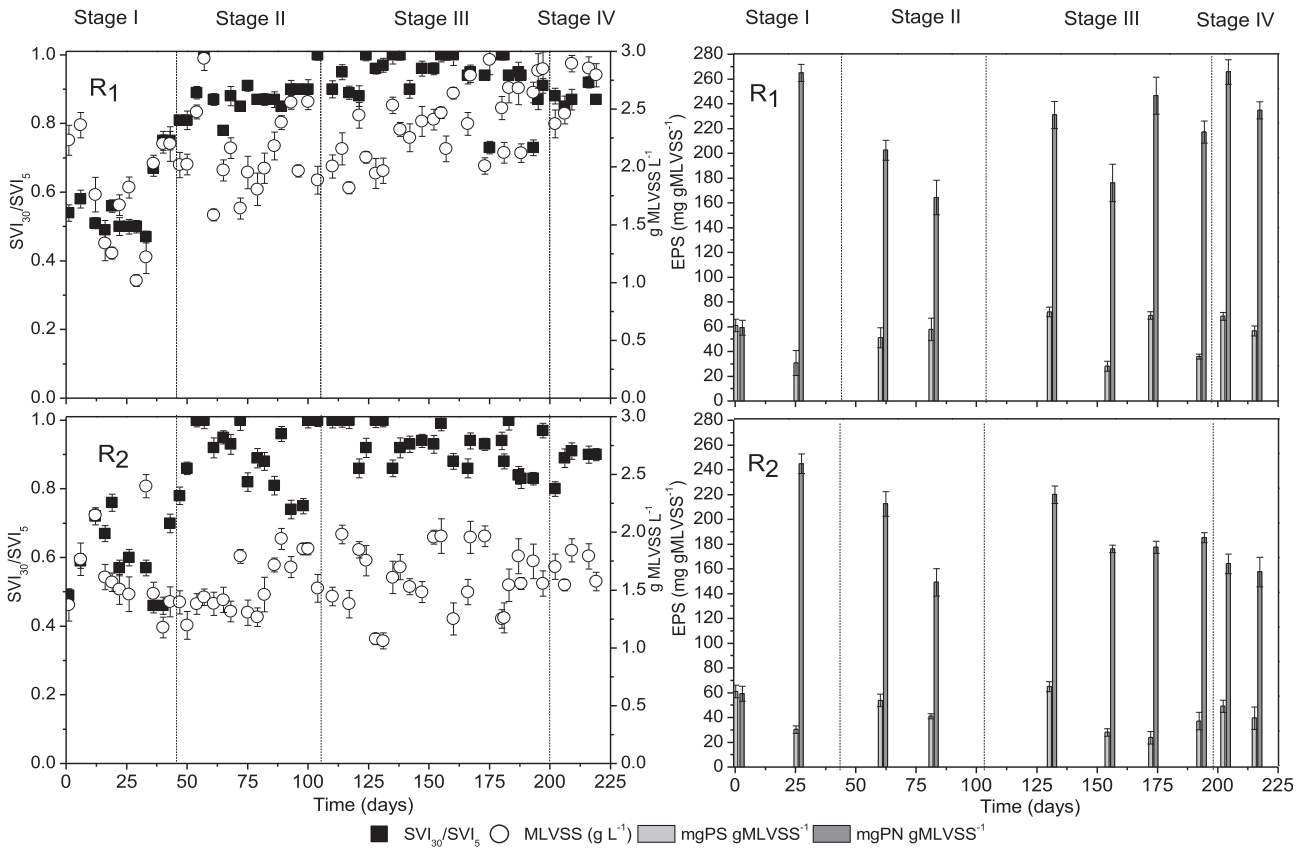

Fig. 1. Evolution of sludge settleability, biomass retention and EPS matrix in the reactors R1 (control) and R2 (supplemented with antibiotics) throughout the operation stages.

Table 2
Removals of COD, nitrogen and phosphorous in the reactors R1 (control) and R2 (supplemented with antibiotics).

Reactor		R1				R2			
Stage		I	II	III	IV	I	II	III	IV
COD	Inf. ($mg \cdot L^{-1}$)	578 (97)	602 (65)	571 (70)	581 (40)	565 (46)	532 (28)	569 (72)	589 (67)
	Eff. ($mg \cdot L^{-1}$)	53 (20)	28 (10)	42 (17)	46 (20)	28 (8)	31 (7)	38 (10)	46 (14)
	Removal (%)	91.5 (9.8)	95.5 (3.4)	92.7 (3.1)	93.6 (5.3)	94.2 (1.7)	94.3 (3.2)	93.4 (1.3)	92.2 (4.9)
N	NH_4^+-N inf. ($mg \cdot L^{-1}$)	64.9 (10.9)	54.9 (5.1)	50.1 (5.5)	55.7 (3.3)	53.6 (5.1)	50.5 (2.0)	50.6 (3.8)	57.1 (3.7)
	NH_4^+-N eff. ($mg \cdot L^{-1}$)	5.1 (2.2)	1.9 (0.5)	3.5 (1.5)	2.3 (0.8)	3.4 (1.8)	2.1 (0.6)	3.7 (1.7)	2.3 (0.9)
	NO_2^--N eff. ($mg \cdot L^{-1}$)	10.3 (4.5)	2.9 (1.5)	2.2 (0.7)	2.6 (1.0)	10.3 (6.8)	2.1 (0.9)	2.7 (1.3)	1.1 (0.3)
	NO_3^--N eff. ($mg \cdot L^{-1}$)	6. (2.6)	7.1 (1.6)	6.5 (1.4)	7.5 (1.4)	5.9 (2.0)	6.9 (1.3)	7.0 (1.4)	4.6 (0.5)
	Removal of NH_4^+-N (%)	92.5 (6.4)	96.3 (1.1)	92.9 (3.1)	96.1 (1.3)	93.4 (5.3)	95.8 (1.0)	92 (3)	97.5 (3.0)
	Removal of TN (%)	68.3 (9.0)	77.9 (2.6)	75.4 (3.2)	77.6 (2.9)	64.0 (14)	77.8 (2.8)	73.6 (5.3)	88.0 (1.7)
P	PO_4^{3-}-P inf. ($mg \cdot L^{-1}$)	7.5 (0.9)	7.7 (0.7)	7.4 (0.7)	7.5 (0.6)	7.7 (0.4)	7.5 (0.8)	7.4 (1.1)	7.7 (0.5)
	PO_4^{3-}-P eff. ($mg \cdot L^{-1}$)	3.3 (0.7)	2.1 (0.4)	1.9 (0.4)	2.1 (0.3)	3.5 (1.1)	2.5 (0.3)	2.3 (0.5)	2.1 (0.4)
	Removal (%)	55.0 (10.0)	71.9 (5.3)	73.1 (5.8)	70.0 (5.9)	53.4 (12.4)	66.5 (5.0)	69.6 (5.8)	73.6 (4.2)

The standard deviations are shown in parentheses.

colonies fully exposed, probably due to the reduction of EPS, agreeing with the verifications of Zhao et al. (2015).

3.2. Removals of carbon, nitrogen, and phosphorous

The average carbon removal, expressed in terms of COD, was above 90% during all stages of operation for both reactors (Table 2). These results are consistent with the COD removals found by Kang et al. (2018b), which were above 95% for both SBR with floccular biomass and SBR with granular biomass, both exposed to 2 μg L^{-1} of SMX. Liu et al. (2019), working with a GSBR supplemented with 50 μg L^{-1} of SMX, also found COD removals higher than 90%. Regarding nitrogen removal, the authors reported removals of around 50% and 30%, respectively, which were significantly lower than those found in the present study (between 60 and 80% for both reactors).

The phosphorus removal was between 60 and 70%, being lower than that presented by Kang et al. (2018b) (around 90% for a P/COD ratio of 0.02) and higher than that reported by Liu et al. (2019) (around 30% for a P/COD ratio of 0.0175). The lower removal obtained by Liu et al. (2019) can be explained by the fact that the authors did not include an anaerobic/anoxic period in the operation of the GSBR. However, Kang et al. (2018b) employed a cycle very similar to that used in the present study: filling (15 min), anaerobic/anoxic reaction (75 min), aerobic reaction (375 min for granular and 349 min for flocculent sludge), settling (4 min for granular

sludge and 30 min for flocculent sludge), decanting (10 min) and idle (1 min). Interestingly, while the present study achieved high nitrogen removal and low phosphorus removal, Kang et al. (2018b) obtained the opposite result. This seems to indicate that these two processes compete with each other. In fact, in an anoxic environment, ordinary heterotrophic denitrifying organisms (OHDOs) and polyphosphate-accumulating organisms (PAOs) compete for substrate and, due to kinetic reasons, OHDOs generally win the competition, thereby hindering phosphorus removal (Chuang et al., 1996).

When operated with AQDS (Stage IV), the average COD and phosphorus removals of R2 were the same as those found before the redox mediator (Table 2). Silva et al. (2012), evaluating the impact of AQDS on azo dye reduction (Reactive Red 2, RR2) in anaerobic systems (one-stage - UASB and two stages - acidogenic reactor followed by a methanogenic UASB) reported that redox mediator supplementation did not affect the COD removal of the studied systems. Since this type of mediator is not consumed by microorganisms (Cervantes and Santos, 2011; dos Santos et al., 2007; Mook et al., 2013), the processes tend to occur in the same way.

However, there was an increase in both the removal of ammoniacal nitrogen and total nitrogen (Table 2), the latter indicating that the denitrification process has been increased. Li et al. (2013) observed that the biocatalyst function of the denitrifying organism *Paracoccus versutus* sp. GW1 was improved in the presence of AQDS.

3.3. Removals of TMP and SMX

3.3.1. Removals of TMP and SMX in the absence of AQDS

TMP and SMX removals are shown throughout the operational stages (Fig. 2) and over an operation cycle (Fig. 3). Based on Fig. 2, there was a low TMP removal in the AGS reactor, remaining around 20% at Stages I and II and slightly increasing at Stage III. Such removal appears to occur during the anoxic period, with no additional removals during the aerobic phase of the SBR (Fig. 3). This compound was not found adsorbed on the sludge.

The literature reports that higher efficiencies are achieved in anaerobic conditions. For example, TMP removals higher than 90% were found in a UASB reactor fed with 10 ng TMP·L^{-1} (Alvarino et al., 2014). The authors attributed this high performance to TMP's chemical structure since heterocyclic compounds, such as pyridines, uracils or furans, resist degradation under anaerobic conditions, except pyrimidine (Adrian and Suflita, 1994). Thus, the presence of a pyrimidine substituted with the amine group could explain TMP's biotransformation under these conditions.

Jewell et al. (2016), working with an SBR reactor (cycle of 1 h under anoxic conditions followed by 2 h under aerobic conditions) with floccular aerobic sludge fed with sewage, whose TMP concentration was between 20 and 225 ng L^{-1}, found removals greater than 83%. However, it was not clear why the removal obtained in the present experiment was low, since the anoxic period employed (1.5 h) was longer than that of the referred work. A possible explanation is the TMP concentration used in the current experiment, which is around 1000-fold higher.

Another possibility is that the carbon source used can influence the removal of the antibiotics tested. Mery-Araya et al. (2019) performed tests with AGS from two different SBRs, one fed with acetate and the other one with glycerol, with COD of 800 mg L^{-1}. For a TMP concentration of 750 μg L^{-1}, they found that the biomass grown on acetate removed less TMP (12%) than that cultivated with glycerol (16%). According to the authors, more complex carbon sources would generate more diverse communities, which would have a greater capacity to degrade pollutants. Although the difference was relatively small for TMP, there was an increase of 73% for ibuprofen and 86% for naproxen in the presence of glycerol compared to acetate. Given that Jewell et al. (2016) operated a reactor fed with sewage, therefore a more complex substrate than acetate (used in the present study), this factor may have contributed to the difference in terms of TMP removal between both studies.

The TMP/biomass ratio used could also have motivated the low TMP removal. Jewell et al. (2016) exposed different dilutions (1:1 and 1:20) of a floccular sludge grown in an SBR to different concentrations of TMP (5 or 500 μg L^{-1}). They realized that the increase in the TMP/biomass ratio, both by increasing TMP's initial concentration and by diluting the sludge, decreased the TMP removal.

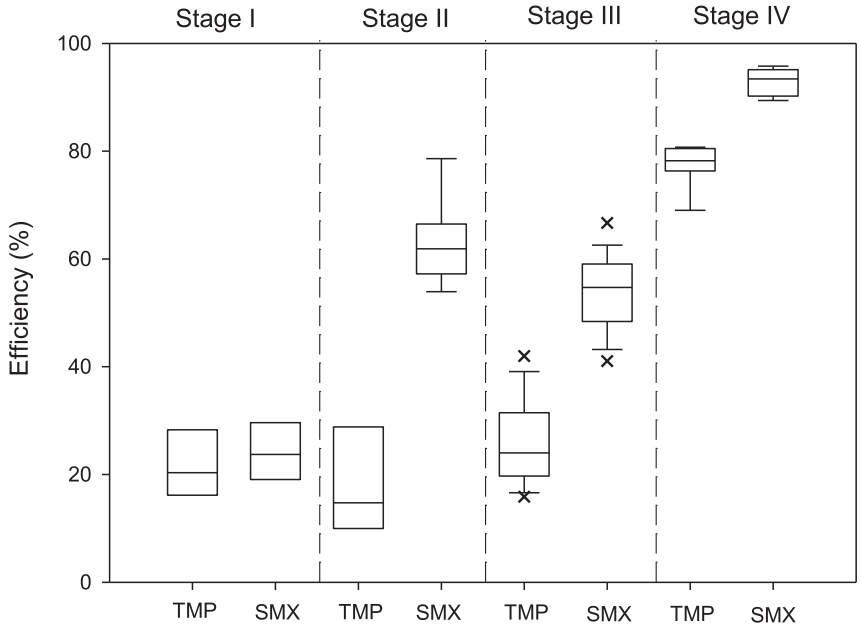

Fig. 2. Removal of TMP and SMX in the reactor R2 (supplemented with antibiotics) throughout the operational stages.

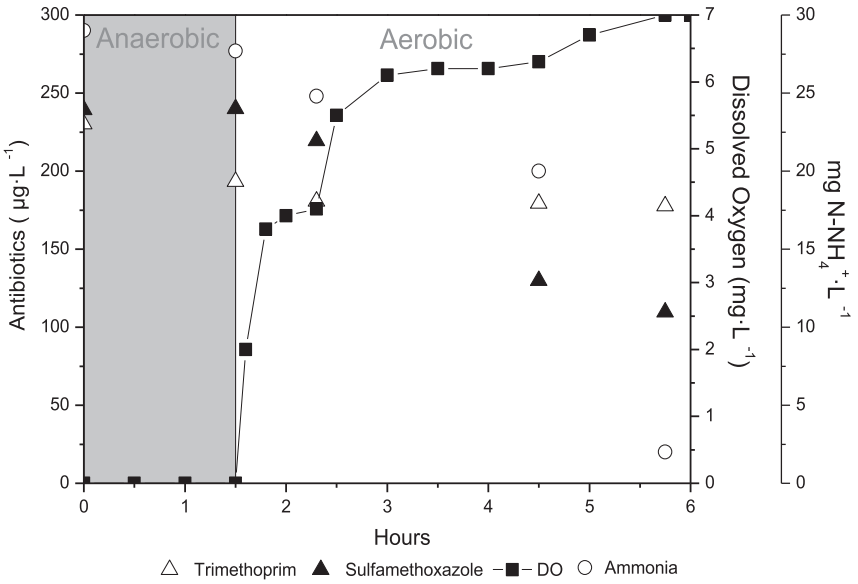

Fig. 3. Monitoring of one operating cycle in the reactor R2 (supplemented with antibiotics) at Stage III (190th day of operation).

However, the initial concentration of solids used in the dilutions was not provided. Besides, the lack of detection in the sludge suggests that the removal of the antibiotic is related to its biotransformation, although the removal of TMP was low.

For the SMX compound, during Stage I, it seems that the removal efficiencies were very close to those achieved with the TMP (about 20%), but they increased to values close to 60% at Stages II and III (Fig. 2). In the cycle profile, the removal occurred mostly in the aerobic phase of the SBR (Fig. 3). This result is consistent with the removals between 60 and 72% reported by Zhao et al. (2015), who worked with an SBR added with 50 μg L^{-1} of SMX. Nonetheless, it is much smaller than that found in an SBR used for the cultivation of floccular biomass with a high concentration of AOB, in which a removal of around 98% was obtained for SMX concentrations of 10 and 100 μg L^{-1} (Kassotaki et al., 2016).

Regarding the aerobic removal of SMX, Kassotaki et al. (2016) carried out a series of experiments with AOB-rich floccular biomass and concluded that SMX removal was clearly related to the rate of ammonia oxidation by sludge. According to the authors, if the rate (mg NH_4^+-N·g^{-1} SSV min^{-1}) was: between 0 and 0.4, the observed removal would be limited (16–33%); between 0.5 and 1.3, the removal would be moderate (53–83%); and, between 1.6 and 2.1, the removal would be high (79–91%).

Considering that the ammonia removals observed in the present study were above 90%, it is possible to safely assume that the cultivated sludge was rich in AOB, similarly to that used by Kassotaki et al. (2016). However, by analyzing Fig. 3, the observed effect appears contrary to that described by those authors.

Between the start of aeration (1.5 h) and hour 4.5, there is almost no ammonia removal, i.e., ammonia concentration drops from 27.2 to 20 mg NH_4^+-N·L^{-1}, likely due to competition for oxygen between the AOB and the OHOs (ordinary heterotrophic organisms). In this period, the rate of ammonia oxidation was approximately 0.05 mg NH_4^+-N·g^{-1} SSV min^{-1}, which would be compatible with a limited removal of SMX (16–33%). However, the removal of SMX is high, so that about 46% of the pharmaceutical is removed during this period. Then, after the decrease in COD, OHOs activity drops, while AOB activity increases. During this period, the ammonia concentration drops from 20 to 2 mg NH_4^+-N·L^{-1}, and the ammonia oxidation rate is 0.54 mg NH_4^+-N·g^{-1} SSV min^{-1}. This value would be consistent with a moderate removal (53–83%), but the SMX removal observed in the period is low (9%).

Kassotaki et al. (2016) also performed tests with the addition of acetate (100 mg L^{-1}) and found that, in the presence and absence of ammonia, SMX removal was 54% and 48%, respectively. This seems to indicate that, in the presence of acetate, OHOs would also be able to degrade SMX. The highest removal in the present study between hours 1.5 and 4.5 of the cycle corroborates this hypothesis.

Differently from TMP, a considerable fraction of SMX was detected adsorbed on the sludge (collected at Stage III). Thus, adsorption was the main removal mechanism of SMX in the AGS system, accounting for about 70% of its overall removal.

3.3.2. *Removals of TMP and SMX in the presence of AQDS*

Fig. 2 shows that TMP and SMX removals increased during Stage IV, reaching 75% for TMP and 95% for SMX, thus indicating that the AQDS helped in the biotransformation of the studied compounds. AQDS property in facilitating or enabling biotransformations in wastewater is due to its catalytic properties (because of the presence of quinones in its molecular structure) both to increase electron transport (redox mediator) and to be the final electron acceptor (Li et al., 2013; Meng et al., 2018; Zhou et al., 2018). Probably, in this work, AQDS acted as a redox mediator, facilitating the transfer of electrons through cometabolic processes involved in the biotransformation of antibiotics, both in aerobic and anaerobic/anoxic conditions.

The difficulty in removing antibiotics by AGS, especially TMP, could be related to the type of biomass developed and the main microbiological groups. Additionally, Alvarino et al. (2014) suggest that TMP removal could be related to biotransformation in an anaerobic/anoxic environment. Aerobic granules are very compact and have anaerobic/anoxic populations whose diversity and location usually vary according to the type of substrate and size of the granule developed (Nancharaiah and Reddy, 2018). In this sense, it was likely that this obstacle was related to the penetration of the compound into the inner layers of the granules, where its biotransformation would occur, thus being related to the cometabolism of these microorganisms.

However, the high removals obtained in the presence of AQDS indicated that possibly the main problem with the removal of

antibiotics was the difficulty in activating the oxidation reaction of the compounds associated with a co-substrate and not with a mass transfer limitation. According to the literature, the biotransformation of pollutants with the redox mediator occurs by a reduction in two different stages. The first one is the biological reduction of this compound during the oxidation of organic substrates (e.g., alcohols, sugars). Then, in the second stage, the substrate is chemically reoxidized during the reduction of the target pollutant (electron acceptor) (Van der Zee and Cervantes, 2009). Thus, in this study, after the biologically reduced AQDS probably transferred the electrons to TMP and SMX through a chemical reaction (Field et al., 2000). In fact, it is reported that TMP and SMX can be biotransformed through abiotic reductive reactions: *o*-demethylation for TMP and cleavage of the isoxazole ring for SMX (Bradley et al., 2006; Mohatt et al., 2011).

Some studies have already observed that antibiotics can be cometabolized, and this type of evidence reinforces the hypothesis raised in the present study. Feng et al. (2019) explored the effects of external carbon sources on the cometabolic degradation of ciprofloxacin by an enriched bacterial consortium XG. It was observed that supplementation with co-substrate stimulated the cell growth of pollutant degrading microorganisms and resulted in rapid degradation of the studied antibiotic. The same trend has already been observed for the degradation of SMX. Müller et al. (2013) observed that under aerobic and mesophilic conditions in the dark with a semi-continuous dosage of SMX, the biomass of an activated sludge system was able to use SMX as a source of carbon and/or nitrogen. However, the biodegradation of SMX was enhanced when a readily degradable energy supply (acetate) was provided, which promoted metabolic activity.

3.4. Microbial population dynamics

The granulation process provided a very significant change in populations (Fig. 4a). In the inoculum sludge, there was a distribution of several phyla, such as bacteria Proteobacteria (41%) Actinobacteria (22%), Planctomycetes (19%), Firmicutes (7%), Chloroflexi (4%) and archaea Thaumarchaeota (3%). There was a growth of the Proteobacteria group in the two reactors, with values of 61% and 69% in R1 and R2, respectively. The Proteobacteria phylum had a very important metabolism diversity for granulation, such as EPS producers, AOB, NOB, OHDOs, PAOs, DPAOs, glycogen-accumulating organisms (GAOs), and denitrifying glycogen-accumulating organisms (DGAOs). Some studies with emerging micropollutants have also identified the predominance of this phylum (Kang et al., 2018a; Zhao et al., 2015).

There was also an expressive growth of bacteria of the phylum Bacteroidetes, presenting a very low relative abundance to around 16% in both reactors. These are generally fermentative gram-negative bacteria, responsible for the degradation of polysaccharides (Birg et al., 2019). On the other hand, there was a decrease in Planctomycetes' presence, reaching values of 11% and 6%, for reactors R1 and R2, respectively. Although these bacteria are not reported frequently in aerobic granulation studies, they have an ovoid shape (Jeske et al., 2015). This morphology is typical of organisms favored in the process of AGS formation.

The results at family level showed that the permanence of Rhodocyclaceae and Xanthomonadaceae after granulation indicated that they were possibly the main responsible for the production of EPS, since several studies have reported this function in granular sludge (Mery-Araya et al., 2019; Szabó et al., 2017; Weissbrodt et al., 2013). As for the resistance to TMP and SMX exposure, the two families appeared in a higher percentage in the reactor with antibiotics (5.5% and 2.2%, respectively), while in the control reactor it was 3.2% and 1.3% (Fig. 4b).

Fig. 5 shows the population dynamics between the reactors and the inoculum sludge at the genus level. For similarity, the sludge from R2 is more similar to the sludge from the inoculum than the sludge from the control reactor R1, showing that the presence of antibiotics inhibited or favored some genera during the formation of granules, possibly provided by the bactericidal action. It was observed that the use of acetate as a carbon source provided a selection that favored the predominance of genera, such as *Defluviicoccus*, *Candidatus Competibacter*, and *Amaricoccus*, with an affinity for short-chain substrates. However, these groups are known to assimilate soluble organic matter in an anaerobic environment (Blackall et al., 1998; Wong et al., 2004) and compete directly for the substrate with PAOs, which may justify incomplete phosphorus removal. The main microorganisms responsible for phosphate removal belonged to the genus *Acinetobacter* (Tandoi et al., 1998), showing percentages of 4% in R1 and 5% in R2.

The nitrifying agents, *Nitrosomonas* (AOB) and *Candidatus Nitrotoga* (NOB), were favored by granulation. In the inoculum sludge, < 0.1% of both genera were found. However, greater abundance was observed on the sludge exposed to TMP and SMX, 2.8% of AOB and 18% of NOB, whereas on the sludge of the control reactor R1, the concentrations were 1.5% of AOB and 0.7% of NOB. For the granulation process, the favoring of these groups is already widely reported in the literature (Kong et al., 2017; Liang et al., 2015; Rosman et al., 2013). As for the presence of antibiotics, the relationship between the activity of groups that remove nitrogen, TMP, and SMX has already been reported by several studies (Eichhorn et al., 2005; Kang et al., 2018b; Kassotaki et al., 2016). This relationship is directly linked to the nonspecific action of the monooxygenase enzyme to degrade a wide variety of organic chemicals by cometabolic biodegradation, such as hydrocarbons, methane, alkenes, halogenated, and aromatic hydrocarbons (Nsenga Kumwimba and Meng, 2019).

Nonetheless, the difference observed in this study, in which the NOB group showed greater abundance than AOB, may be related to metabolic activities. According to the tests that revealed the growth rate of the nitrifying groups, a slightly higher growth is observed for the NOB of the sludge exposed to antibiotics (data not shown). One possibility that cannot be excluded either is that, as aerobic heterotrophs were more affected than nitrifying agents, possibly because they were more exposed to pharmaceuticals, there was less oxygen consumption and, consequently, more oxygen available for nitrifying agents that were located a little more internally in the AGS of reactor R2.

This relationship can also be identified for the main denitrifying microorganisms. The most abundant genera were *Thauera*, *Zoogloea*, and *Pseudomonas* with values below 0.1% in the inoculum and reached 3.3%, 0.5%, and 0.3% in the control reactor R1 and 5.3%, 1.0%, and 1.7% in R2, respectively. As with denitrification rates, abundances were also higher in R2. It should be noted that *Pseudomonas* have already been reported as consumers of SMX (Herzog et al., 2013); however, its increase in abundance cannot be exclusively related to its degradation because groups with similar metabolism and higher abundance were found in the reactor sludge with antibiotics.

4. Conclusion

The AGS produced in the presence of high doses of TMP and SMX (200 $\mu g\ L^{-1}$ each) showed good settleability, but its structural integrity was impaired, interfering with the amount of sludge in the reactor. The ability to remove C, N, and P was similar to that developed in the control reactor.

However, low TMP (~30%) and SMX (~60%) removals were

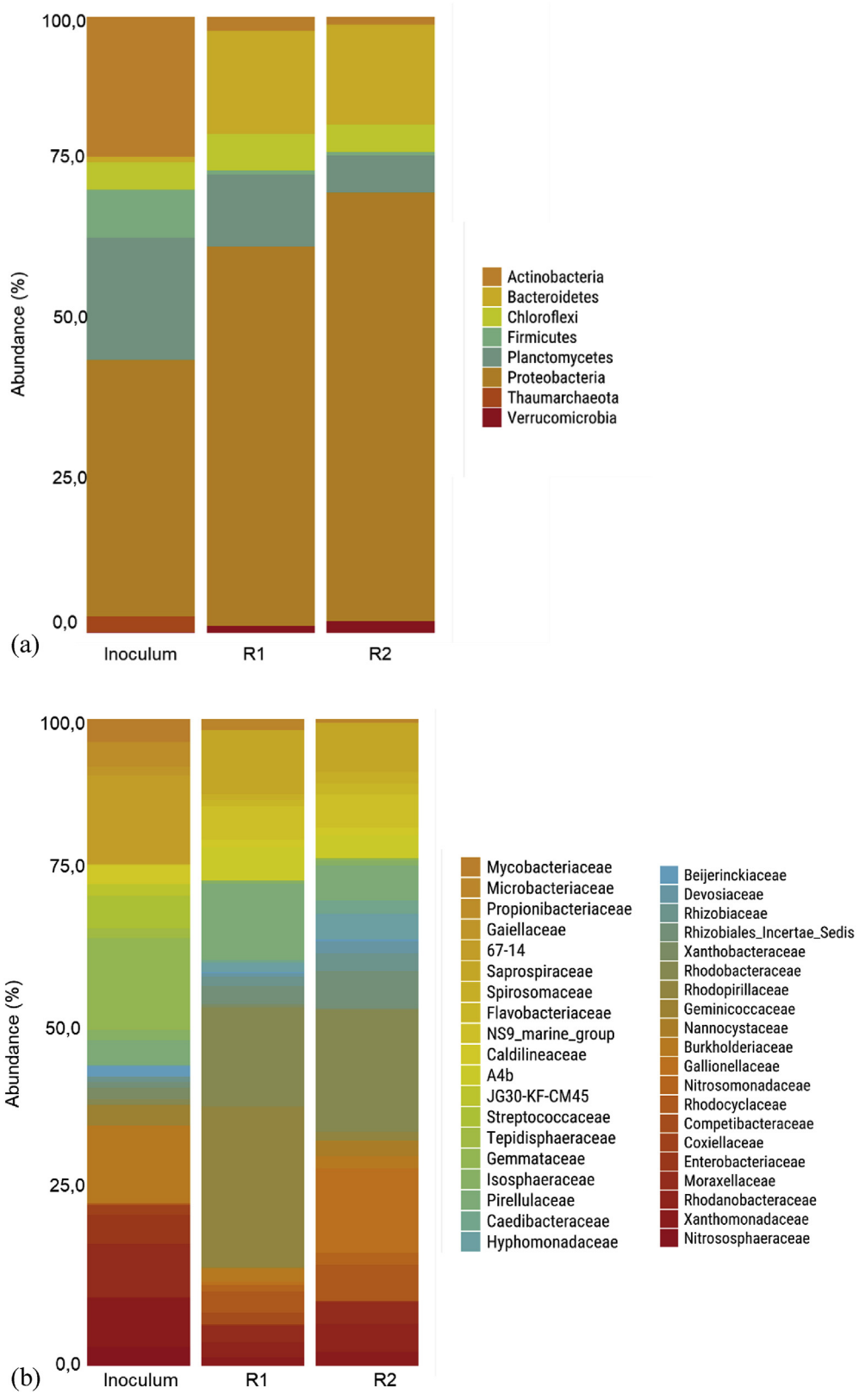

Fig. 4. Diversity of microorganisms at the levels of phylum (a) and family (b) in the inoculum and the reactors R1 (control) and R2 (supplemented with antibiotics).

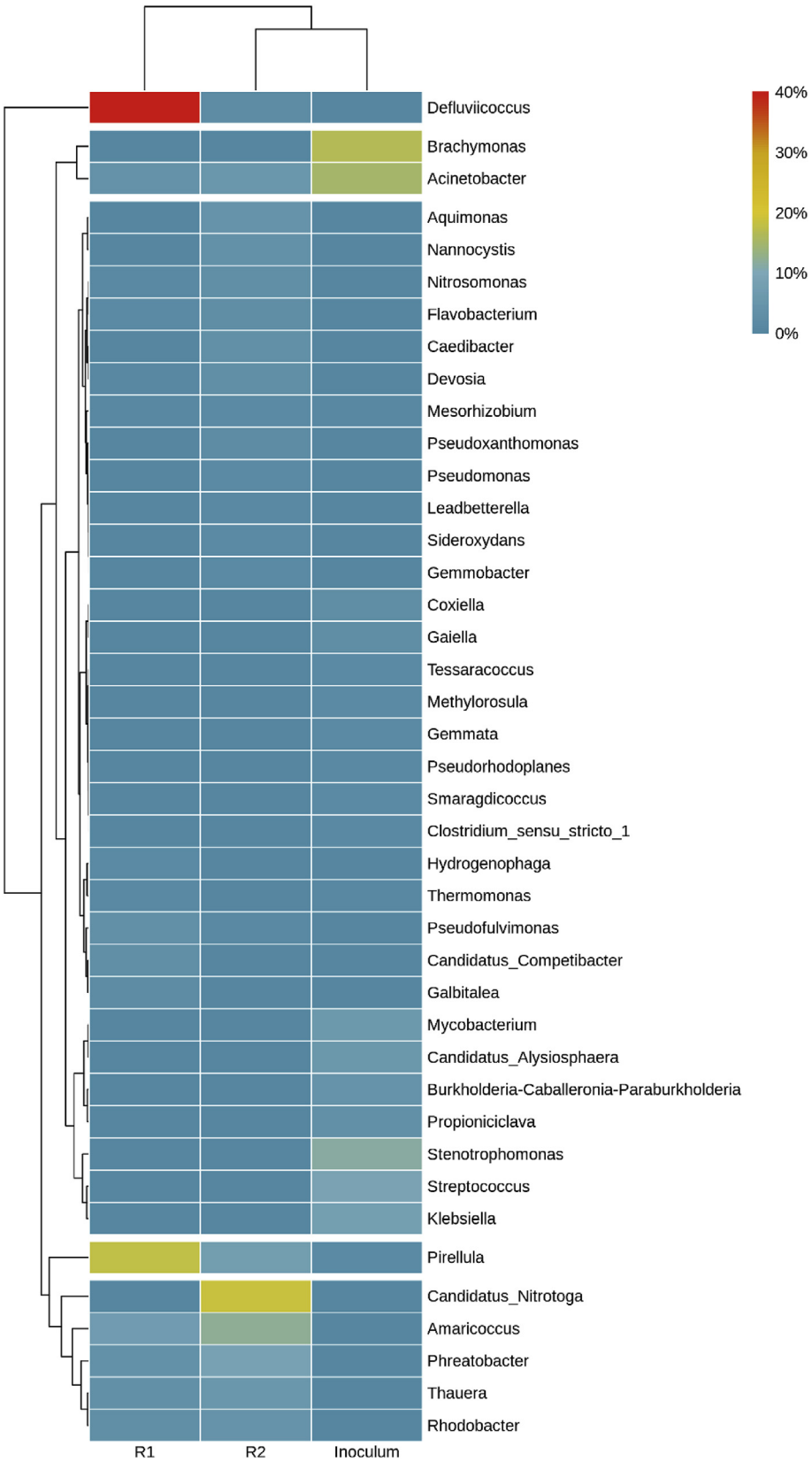

Fig. 5. Genus distribution of the microbiol community (abundance > 1%) in the reactors R1 (control) and R2 (supplemented with antibiotics).

observed when compared to anaerobic or floccular biomass aerobic systems. Significant concentrations of SMX were found adsorbed on the sludge, which seems to be the initial mechanism of removal in AGS systems. TMP showed to have very low capacity to be adsorbed on the sludge.

When the system was supplemented with AQDS, TMP (~75%) and SMX (~95%) removals were increased, possibly due to the catalytic action of the redox mediator in cometabolic processes.

In the two reactors, Proteobacteria and Bacterioidetes increased, whereas Planctomycetes decreased. It was found that the sludge exposed to antibiotics is more similar to the inoculum sludge than the sludge from the control reactor, showing that the TMP and SMX presence inhibited or favored some genera during the formation of the granules, possibly due to the bactericidal action. Among these, it was found that nitrifying bacteria, *Nitrosomonas* (AOB) and *Candidatus Nitrotoga* (NOB), were favored by granulation and had a much higher relative abundance of reactor R2 than in R1.

Credit author statement

Antônio Ricardo Mendes Barros: experimental planning, development and paper writing. Thaís Salvador Argenta: experimental planning, development and paper writing. Clara de Amorim de Carvalho: experimental development. Francisca Andréa da Silva Oliveira: molecular biology analysis. Paulo Igor Milen Firmino: Supervision, experimental planning, development and paper writing. André Bezerra dos Santos: Supervision, experimental planning, development and paper writing.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2020.127840.

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