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Parabens in aerobic granular sludge systems: Impacts on granulation and insights into removal mechanisms

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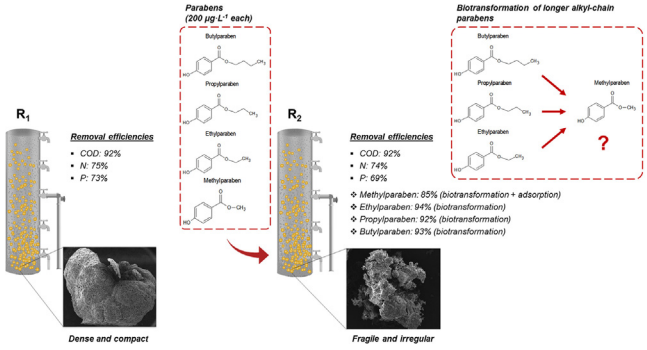
HIGHLIGHTS

- Granules with good settleability were grown even in the presence of parabens.
- Parabens did not affect organic matter and nitrogen removal.
- High paraben removal efficiencies were achieved (>85%) in the AGS system.
- Adsorption played a role only in methylparaben removal.
- Methylparaben might be a probable intermediate of paraben degradation.

GRAPHICAL ABSTRACT



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ABSTRACT

This work assessed the impact of methylparaben, ethylparaben, propylparaben, and butylparaben (200 μg L^{-1} each) on the granulation process as well as on the organic matter and nutrient removal of an aerobic granular sludge (AGS) system (6-h cycle). Additionally, some insights into the main paraben removal mechanisms were provided. In the presence of parabens, aerobic granules with good settleability, but with fragile and irregular structure, were grown. No significant effect of parabens on organic matter (>90%) and nitrogen (~70%) removal was evidenced. On the other hand, phosphorus removal was slightly impaired, although high removal efficiencies (~70%) were reached. High paraben removal efficiencies were achieved (>85%) in the AGS system, with methylparaben being the most recalcitrant compound. Concerning the removal mechanisms, biotransformation was the main mechanism in the removal of all parabens (85.5% for methylparaben and 100% for the others), whereas, apparently, adsorption played a role only in the removal of methylparaben. In addition, this compound was also suggested as a probable intermediate of the degradation of the larger alkyl-chain parabens. Lastly, regarding the microbial community, with the exception of *Mycobacterium*, the reactors shared the same genera, which may explain their comparable operational performances. Additionally, some genera that developed more in the presence of parabens may be related to their degradation. Therefore, although antimicrobial agents such as parabens compromised the granule structure, AGS system maintained a good operational performance and showed to be very efficient in paraben removal.

1. Introduction

The increasing presence of micropollutants, such as pharmaceuticals, personal care products, hormones, surfactants, industrial chemicals,

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pesticides, and others, in the environment is mainly due to the pollution with domestic, hospital, and industrial wastewater, agricultural runoff, and landfill leachate (Luo et al., 2014). Among these compounds, there are parabens, esters of p-hydroxybenzoic acid used as preservatives in cosmetics, pharmaceuticals, and some food and industrial products. Methylparaben (MeP), ethylparaben (EtP), propylparaben (PrP), and butylparaben (BuP) are the most used parabens and, therefore, the most commonly found in aquatic environments, in which, generally, MeP is the most concentrated (≤200 ng L^{-1}), followed by PrP (≤50 ng L^{-1}), while the others are at concentrations of few ng L^{-1} (Błedzka et al., 2014; Haman et al., 2015; X. Ma et al., 2018). Concerning raw domestic wastewater, MeP is also the most concentrated (51–4200 ng L^{-1}), followed by PrP (13–1400 ng L^{-1}), EtP (2–880 ng L^{-1}), and BuP (4–140 ng L^{-1}) (González-Mariño et al., 2011; Karthikraj et al., 2017; W.L. Ma et al., 2018; Wang and Kannan, 2016).

These compounds, however, have been associated with human health problems, such as breast cancer and hormonal changes, for acting as endocrine disruptors (Boberg et al., 2010; Darbre et al., 2004; Nowak et al., 2018). Thus, it is necessary to remove parabens from wastewater prior to its release into water bodies. The activated sludge (AS) system generally has good removal efficiencies of these micropollutants (>80%), mainly attributed to aerobic biodegradation, while adsorption plays a secondary role (Ashfaq et al., 2017; Li et al., 2015; Lu et al., 2018; Wang et al., 2017). However, it is relevant to investigate the paraben removal potential of more compact and cost-effective wastewater treatment systems with high organic matter and nutrient removal capacity, such as aerobic granular sludge (AGS) systems.

AGS is a variant of the AS process which has emerged in the late 1990s, and it has been grown mainly in sequential batch reactors (SBRs) (Morgenroth et al., 1997). Compared to AS, AGS has several advantages, such as higher settling velocity and biomass retention in the form of compact and dense granules (>0.2 mm), and simultaneous removal of carbon, nitrogen, and phosphorous due to the presence of aerobic, anoxic, and anaerobic zones in the granules, which provides greater metabolic cooperation among microorganisms (Ab Halim et al., 2015; Nancharaiah and Reddy, 2018; Rollemberg et al., 2018; Winkler et al., 2018).

Although aerobic granules have already been evaluated for the removal of aromatic compounds, such as phenol, o-cresol, p-nitrophenol (Ramos et al., 2015), and even some pharmaceuticals (ibuprofen, prednisone, sulfamethoxazole, among others) (Amorim et al., 2016; Moreira et al., 2015; Zhao et al., 2015), there are no studies on paraben removal in AGS systems. According to the literature, pharmaceuticals and personal care products negatively affect the quality of granules and may compromise their stability and activity (Zhao et al., 2015). Thus, it is important to evaluate the overall performance of AGS systems during the treatment of paraben-containing wastewater.

Based on the foregoing, the objectives of the present work were (1) to evaluate the impact of the parabens MeP, EtP, PrP, and BuP on the granulation process, granule stability, and removal of organic matter, N, and P of an AGS system treating synthetic wastewater, and (2) to identify the main paraben removal mechanisms in this system.

2. Material and methods

2.1. Experimental setup

The experiment was carried out in two sequential batch reactors (SBRs) (diameter: 100 mm, height: 1 m and 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.

Both reactors (R_1 and R_2) were fed with the same synthetic domestic wastewater containing acetic acid (500 mg COD L^{-1}), ammonium chloride (75 mg NH_4^+-N L^{-1}), potassium phosphate (10 mg PO_4^{3-}-P L^{-1}), calcium chloride dihydrate (10 mg Ca^{2+} L^{-1}), magnesium sulfate heptahydrate (5 mg Mg^{2+} L^{-1}), micronutrients (1 mL L^{-1}), and sodium bicarbonate (1 g L^{-1}), used as a buffer to keep the pH close to 7.0. The solution of micronutrients contained 50 mg L^{-1} of $ZnCl_2$, $MnSO_4 \cdot H_20$, H_3BO_3, $AlCl_3$, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, $CoCl_2 \cdot 6H_2O$, and $NiCl_2$ (each), and 30 mg L^{-1} of $CuCl_2$. However, while R_1 was maintained as a control, R_2 was supplemented with a mixture of parabens, namely MeP, EtP, PrP, and BuP (200 μg L^{-1} each) (Sigma Aldrich, USA), from the beginning of the experiment. The influent of each reactor was stored in a refrigerator at 4 °C to prevent the proliferation of microorganisms in the feed tank and, consequently, their premature degradation.

The reactors were operated over three stages at room temperature (27 °C) with 6-h cycles, 12-h hydraulic retention time (HRT), and 50% volumetric exchange rate. The cycles were divided into 30 min of filling, 60 min of anaerobic reaction, 248–263 min of aerobic reaction, 20–5 min of settling, 1 min of decanting, and 1 min of idle. The settling time was gradually decreased from 20 min (stage I: 1–46 days of operation) to 10 min (stage II: 47–104 days of operation) and then to 5 min (stage III: 104–210 days of operation) to act as a selection pressure on filamentous microorganisms and promote sludge granulation. To keep the cycle time constant (6 h) throughout the experiment, the time subtracted from the settling phase was added to the aerobic reaction phase. During this phase, aeration was performed by compressors (Aco-002, Sunsun, China), providing a bubble upflow velocity of approximately 1.5 cm s^{-1}.

At the end of stage III (after granule maturation), the removal of parabens, organic matter (COD), N (ammonium, nitrite, nitrate), and P (phosphate) over the R_2 cycle was evaluated in duplicate (two cycles). During each cycle, dissolved oxygen (DO) was measured continuously (YSI 5000, YSI Incorporated, USA).

As MeP showed to be more persistent than the other parabens in the aerobic period, there may have been some transient accumulation of this compound. Accordingly, MeP was evaluated as a possible intermediate for the biotransformation of the other parabens. For this, R_2 was initially fed with paraben-free synthetic wastewater for 15 days (approximately 60 cycles) for the complete elimination of these compounds from the mixed liquor (verified by their absence in the effluent). Then, the reactor was supplemented with only EtP, PrP, and BuP for 7 days (28 cycles). After that, another cycle test was performed concerning only paraben removal.

2.2. System monitoring

2.2.1. Physicochemical analyses

The influent and effluent samples were collected immediately before all the analyses. 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 precolumn (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 glass microfiber filter) (Millipore, USA) were 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.

MeP, EtP, PrP, and BuP were determined in both liquid and sludge. For this, they were previously extracted from the samples. For the extraction of the parabens from the liquid, 500-mL samples were prefiltered (0.45-μm glass microfiber filter) (Millipore, USA) and then percolated through Oasis HLB cartridges (3 cc, 60 mg, 30 μm) (Waters Corporation, USA) at a liquid-solid ratio of 8.3 mL mg^{-1}. Subsequently, the parabens adsorbed on the cartridges were eluted by 4 mL of HPLC/UV grade methanol (99.8%, Neon, Brazil), yielding recoveries of 90.2%, 89.8%, 94.0%, and 94.7% for MeP, EtP, PrP, and BuP, respectively.

For the extraction of the parabens from the sludge (only at the end of stage III), a method adapted from López-Serna et al. (2018) was used as follows: 100 mL of fresh sludge sample (without the sludge liquor) were lyophilized (L101 Freeze Drier, Liobras, Brazil), and then the dried sludge was weighed inside a 20-mL glass vial to which 5 mL of acetone (99%, Sigma-Aldrich, Germany) were added. After complete vortexing, the mixture was left to stand overnight to allow solvent to evaporate. Then, 12 mL of MilliQ® water at pH 9 were added to the vial, which was vortexed again to obtain a homogeneous suspension. The vial was then submitted to ultrasound-assisted extraction (UAE) for 30 min at 60 Hz and room temperature (Cristófoli Biosafety, Brazil). Subsequently, the suspension was centrifuged for 5 min at 3600 rpm (Excelsa II 206 BL, Fanem, Brazil), and the supernatant was transferred to a 25-mL volumetric flask. The MilliQ® water extraction process was repeated once more with the pellet, and the supernatant was added to that already present in the volumetric flask, which was completed with MilliQ® water. Finally, parabens were extracted from the resulting solution as described above for liquid samples.

After the extraction, 10 μL of the methanolic solution containing MeP, EtP, PrP, and BuP were injected in a high-performance liquid chromatograph (HPLC) LC-20A Prominence equipped with a Shim-pack CLC-ODS(M)® C18 column (4.6 × 150 mm, 5 μm) and a UV–Vis SPD-20A detector (Shimadzu Corporation, Japan). Then, they were eluted by a mobile phase composed of ultrapure water and HPLC/UV grade acetonitrile (99.9%, Sigma-Aldrich, Germany) at a constant flow of 1.2 mL min^{-1}, using the following gradient: increase from 10% to 60% of acetonitrile in 8 min, followed by 10% reduction in 4 min. The oven temperature was maintained at 35 °C throughout the run.

2.2.2. Granules morphology and settleability

Granule formation and development were evaluated by sieving and gravimetry every 15 days of operation, and the structure of the mature granules was analyzed by scanning electron microscopy (SEM) (Inspect S50, FEI Company, USA).

The settleability of the sludge was evaluated by the dynamic sludge volume index (SVI) – a modified version of the SVI proposed by Schwarzenbeck et al. (2004) which allows the determination of SVI at several times. In this work, SVI was determined for the settling times of 5 and 30 min (SVI_5 and SVI_{30}, respectively). The settling velocity of the sludge was determined, in triplicate, according to Q. Wang et al. (2018), using an acrylic column with a working height of 0.4 m and a diameter of 75 mm, filled with synthetic effluent.

2.2.3. EPS extraction and characterization

The extracellular polymeric substance (EPS) content of the granular biomass was measured in terms of protein (PN) and polysaccharides (PS). 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, and maintained in a 55-kHz ultrasonic bath for 5 min. After that, the mixture was filtered (0.45 μm) and diluted (dilution factor of 2) (Tay et al., 2001). To quantify the PN and PS contents, a modification of the Lowry method and the phenol-sulfuric acid method were used, respectively (Long et al., 2014).

2.2.4. AGS community microbial analysis

The sludge samples were collected during the aeration phase at the end of stage III. The DNA of the microorganisms present in the mature granules was extracted (in triplicate for each sludge sample) using the 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.

2.3. Statistical methods

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

3. Results and discussion

3.1. Formation, stability and characteristics of the AGS

The inoculum sludge had SVI_5 and SVI_{30} equal to 189 and 93 mL g^{-1}, respectively, and, therefore, SVI_{30}/SVI_5 ratio of 0.5. Throughout stage I, granule formation was observed along with the decrease of the SVI_{30} and SVI_5 values and the increase of the SVI_{30}/SVI_5 ratio in both reactors (Fig. 1). Therefore, as R_1 and R_2 presented granule formation at the same time, parabens did not cause a delay in the process. The VSS value, which initially was 2.8 g L^{-1}, also decreased at this stage, with mean values of 1.8 g L^{-1} in R_1 and 1.6 g L^{-1} in R_2. As the settling time decreased (stages II and III), the SVI_{30}/SVI_5 ratio of both reactors increased considerably to close to 1. However, regarding the biomass in stages II and III, while in R_1 the mean value of VSS increased to approximately 2.2 g L^{-1}, in R_2 it remained close to 1.5 g L^{-1} (Fig. 1). This initial decrease in VSS after inoculation occurs because, in AGS systems, most of the filamentous solids present in the inoculum sludge are washed out by the imposed selection pressures (short settling time, 50% volumetric exchange rate and decanting velocity). At the following stages, the tendency is for the system to adapt and biomass to grow, as observed in R_1 (Rollemberg et al., 2019; Wang et al., 2004). However, the biomass growth in R_2 was probably controlled by the presence of parabens, as they have antimicrobial action (Doron et al., 2001; Haman et al., 2015).

As for the granule size, both reactors showed high proportions of granules with diameter greater than 1 mm (>70 wt%) still in stage I. After AGS maturation (end of stage III), granules larger than 1 mm were about 97.4 wt% of sludge in R_1 and 92.7 wt% in R_2. Regarding the settling velocity, the granules cultivated in R_1 presented a mean velocity of 21.3 ± 1.2 m h^{-1}, and those cultivated in R_2 presented a mean velocity of 22.2 ± 2.3 m h^{-1}. Therefore, the parabens did not interfere in the settling process, which was also confirmed by the statistically similar SVI_{30} values of the two reactors (<40 mL g^{-1}) ($p = 0.13$).

The sludge age was also monitored throughout the operation period, remaining the same between 6 and 11 days in R_1 and between 5 and 9 days in R_2. These values favored the stability of the granules, since long sludge ages (>11 days) lead to deterioration of aerobic granules according to Zhu et al. (2013), who evaluated selective sludge discharge in an AGS SBR with a 75% volumetric exchange rate.

EPS are biopolymers, composed of PN, PS, and other substances, which act on granule formation and stability (Rollemberg et al., 2018), being a fundamental parameter to understand the structure of the formed granules, mainly in the presence of micropollutants. At the reactor startup, the contents were 61 mg PS g VSS^{-1} and 59 mg PN g VSS^{-1}. After 28 days of operation (stage I), there was a significant increase in

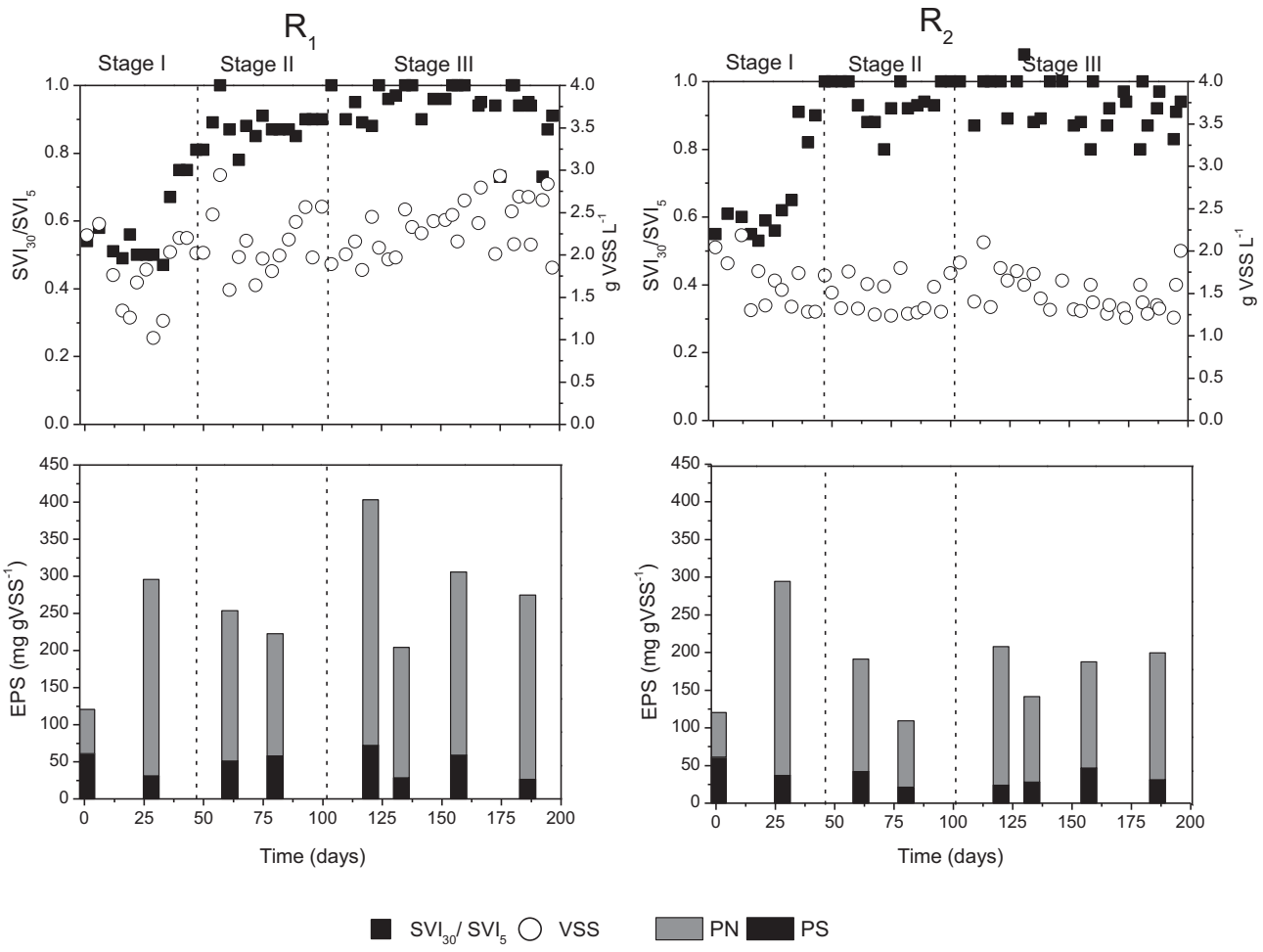

Fig. 1. AGS characterization of the reactors R_1 and R_2 throughout the experiment.

the EPS content in both reactors, with no significant difference between them. Therefore, the granulation process used in the systems stimulated effectively EPS production. However, at stages II and III, the EPS content in R_2 was remarkably lower than that in R_1, probably due to the continuous exposure to parabens. Regarding the contents of PN and PS, as AGS was being formed, the content of PN remained higher than that of PS in both reactors, being 9 times higher in R_1 and 7 times higher in R_2 at stage III (Fig. 1).

In fact, the granules of R_1 had a denser and more compact structure, while those of R_2 had a fragile and non-uniform structure (Fig. 2). Possibly, the microbicidal action of parabens (Darwish and Bloomfield, 1995; Doron et al., 2001) may have influenced the production of EPS and thus the formation of granules, justifying the lower VSS concentration of R_2.

Contrary to what was observed in the present study, Zhao et al. (2015) reported an EPS increase in an AGS system treating synthetic wastewater (1000 mg COD L^{-1}, 90–110 mg NH^{4+}-N L^{-1} and 4–7 mg

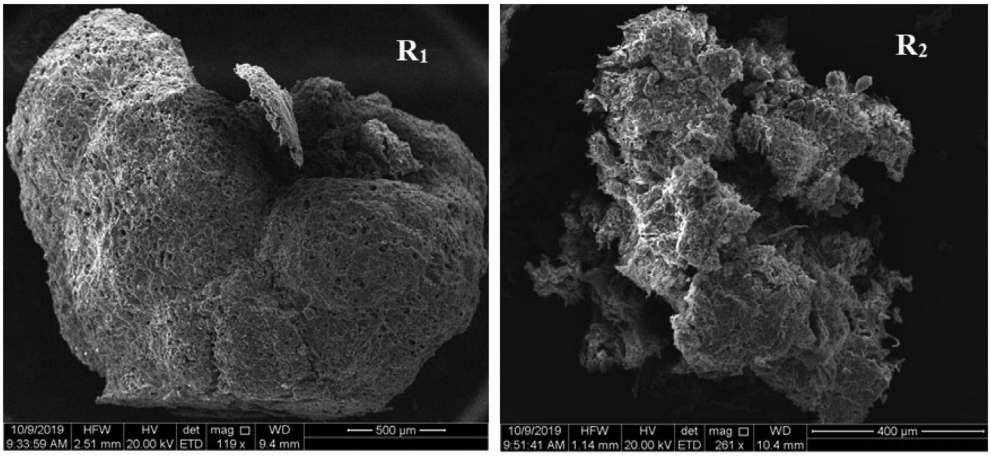

Fig. 2. Granule scanning electron micrograph of the reactors R_1 and R_2 at the end of stage III.

Table 1
Removal efficiencies of organic matter, N, and P of the reactors R_1 and R_2.

Reactor		R_1			R_2		
Stage		I	II	III	I	II	III
COD	Influent (mg L^{-1})	578 ± 97	602 ± 65	571 ± 70	611 ± 60	561 ± 23	590 ± 56
	Effluent (mg L^{-1})	53 ± 20	28 ± 10	42 ± 17	36 ± 16	20 ± 8	45 ± 20
	Removal efficiency (%)	91 ± 9	95 ± 3	92 ± 3	93 ± 3	98 ± 1	92 ± 3
N	Influent NH_4^+ (mg N L^{-1})	64.9 ± 10.9	54.9 ± 5.1	50.1 ± 5.5	60.4 ± 12.1	57.5 ± 10.6	52.2 ± 3.1
	Effluent NH_4^+ (mg N L^{-1})	5.1 ± 2.2	1.9 ± 0.5	3.5 ± 1.5	2.2 ± 1.7	2.4 ± 0.6	3.6 ± 1.5
	Effluent NO_2^- (mg N L^{-1})	10.3 ± 4.5	2.9 ± 1.5	2.2 ± 0.7	9.9 ± 5.7	2.1 ± 0.9	2.8 ± 0.9
	Effluent NO_3^- (mg N L^{-1})	6.0 ± 2.6	7.1 ± 1.6	6.5 ± 1.4	6.8 ± 2.0	6.7 ± 2.3	7.0 ± 0.9
	Nitrification efficiency (%)	92.5 ± 6.4	96.3 ± 1.1	92.9 ± 3.1	96.4 ± 7.7	95.6 ± 1.4	92.0 ± 3.0
	Removal efficiency (%)	68.3 ± 9.0	77.9 ± 2.6	75.0 ± 3.0	69.4 ± 7.0	79.1 ± 6.3	74.0 ± 4.5
P	Influent PO_4^{3-} (mg P L^{-1})	7.5 ± 0.9	7.7 ± 0.7	7.4 ± 0.7	7.1 ± 0.9	7.7 ± 0.7	7.1 ± 1.1
	Effluent PO_4^{3-} (mg P L^{-1})	3.3 ± 0.7	2.1 ± 0.4	1.9 ± 0.4	3.5 ± 1.1	2.3 ± 0.5	2.1 ± 0.4
	Removal efficiency (%)	55.0 ± 10.0	71.9 ± 5.3	73.1 ± 5.8	51.3 ± 14.2	69.5 ± 9.0	69.2 ± 8.1

PO_4^3-P L^{-1}) in 3-h cycles after the addition of five pharmaceuticals (ibuprofen, naproxen, prednisolone, norfloxacin, and sulfamethoxazole, at 50 μg L^{-1} each). According to the authors, this increase was the defense mechanism of microorganisms against the toxicity of the added micropollutants. However, it is worth mentioning that, differently from the present study, these authors performed the granulation process in the absence of the aforementioned pharmaceuticals.

3.2. Removal of organic matter, N, and P

The results of removal of organic matter, nitrogen, and phosphorus are presented in Table 1. Throughout the whole operation period, the COD removal efficiency was higher than 90% in both reactors, indicating that the sludge maintained great biological activity, even with the presence of parabens, since there was no statistical difference ($p = 0.36$). For the nitrogen removal, nitrification was quite satisfactory in both reactors, being above 92% since the beginning of the operation. However, between stage I and the subsequent stages, there was an increase in the denitrification process, which may be related to the granule growth, favoring the establishment of the anoxic/anaerobic zone in them and, consequently, the mechanism of simultaneous nitrification and denitrification. As a result, the nitrogen removal efficiency of both reactors also increased from stage I to the last two, remaining greater than 74% in the maturation and stabilization stage (stage III), with no statistical difference ($p = 0.59$). Therefore, parabens did not impair nitrogen removal either. For the phosphorus removal, the granulation process with phase distribution in the operating cycle, with an anaerobic phase of 1.5 h, favored the metabolism of the microorganisms, increasing the removal efficiency from stage I to stage III in both reactors (from 55 to 73% in R_1 and from 51 to 69% in R_2). Although the reactors had similar phosphorus removal efficiencies at stages I ($p = 0.84$) and II ($p = 0.72$), R_2 presented a slightly lower efficiency than R_1 at stage III ($p < 0.01$), which may be associated to the continuous exposure to parabens for more than 100 days. Nonetheless, the phosphorus removal in the paraben-supplemented reactor remained very efficient (~70%).

Although no works that report the effect of parabens on the activity of microorganisms responsible for the removal of organic matter, N, and P have been found, there are some studies with other types of micropollutants (Amorim et al., 2016; Moreira et al., 2015). For example, Amorim et al. (2016) evaluated the performance of an AGS SBR (6-h cycles, 40% volumetric exchange rate and 9.7-h HRT) after the addition of a mixture of eight chiral pharmaceuticals (alprenolol, bisoprolol, metoprolol, propranolol, venlafaxine, salbutamol, fluoxetine, and norfluoxetine, at 1.3 μg L^{-1} each). Their results showed that, after the anaerobic filling, both COD absorption and P release decreased because, probably, the activity of the polyphosphate-accumulating organisms (PAOs) was negatively affected. The authors reported that nitrification was also hindered by the presence of such micropollutants. After ceasing the addition of the chiral pharmaceuticals to the influent, the activity of PAOs was reestablished, but that of both nitrite-oxidizing bacteria and denitrifying bacteria remained partially inhibited. Moreira et al. (2015), when evaluating the influence of the addition of high concentrations of fluoxetine (0.9 and 1.2 mg L^{-1}) on an AGS SBR operated with 12-h cycles, observed that, while COD removal was not affected during the entire experiment, phosphate, ammonium, and nitrate removals were impaired only in the beginning due to the initial exposure to fluoxetine. After 64 days of operation, the microbial community adapted to the compound, and the removal efficiencies of the system were restored.

3.3. Paraben removal

At stage I, the system achieved high mean removal efficiencies for all parabens tested (>90%), and a small accumulation of MeP was found (Table 2). Nonetheless, at stage II, with the exception of BuP, these efficiencies were negatively affected, especially those of MeP and EtP (Table 2), whose minimum values were close to 50 and 65%, respectively (Fig. 3). Probably, the decrease of the settling time from 20 to 10 min (from stage I to II) was responsible for the initial instability observed (Fig. 3), as the biomass that was not able to settle was washed out, which may have transiently impaired paraben removal. Once the biomass adapted to new operational conditions, paraben removal recovered.

After the reactor reached stabilization at stage II, the settling time was decreased to 5 min (stage III). Again, with the exception of EtP, there was an initial variation in paraben removal efficiency, especially for MeP (Fig. 3). While PrP and BuP removal efficiencies stabilized slightly after the day 125 of operation (Fig. 3), ensuring mean values greater than 90% (Table 2), MeP removal efficiency remained quite unstable until approximately the day 155 of operation, from which showed an increasing trend, reaching values close to 90% only at the

Table 2
Mean influent and effluent paraben concentrations and removal efficiencies of the reactor R_2.

Stage		I	II	III
MeP	Influent (μg L^{-1})	205 ± 9	219 ± 17	210 ± 24
	Effluent (μg L^{-1})	17 ± 7	14 ± 7	31 ± 11
	Removal efficiency (%)	91.6 ± 3.3	84.5 ± 15.8	85.1 ± 5.6
EtP	Influent (μg L^{-1})	245 ± 16	247 ± 28	223 ± 24
	Effluent (μg L^{-1})	15 ± 14	18 ± 9	7 ± 4
	Removal efficiency (%)	94.0 ± 5.6	87.6 ± 9.7	93.7 ± 6.2
PrP	Influent (μg L^{-1})	235 ± 7	256 ± 10	212 ± 25
	Effluent (μg L^{-1})	7 ± 4	14 ± 9	11 ± 6
	Removal efficiency (%)	97.1 ± 1.7	91.6 ± 8.1	91.8 ± 7.9
BuP	Influent (μg L^{-1})	214 ± 12	212 ± 8	213 ± 38
	Effluent (μg L^{-1})	10 ± 4	11 ± 7	7 ± 5
	Removal efficiency (%)	95.1 ± 5.4	95.1 ± 3.5	93.1 ± 8.3

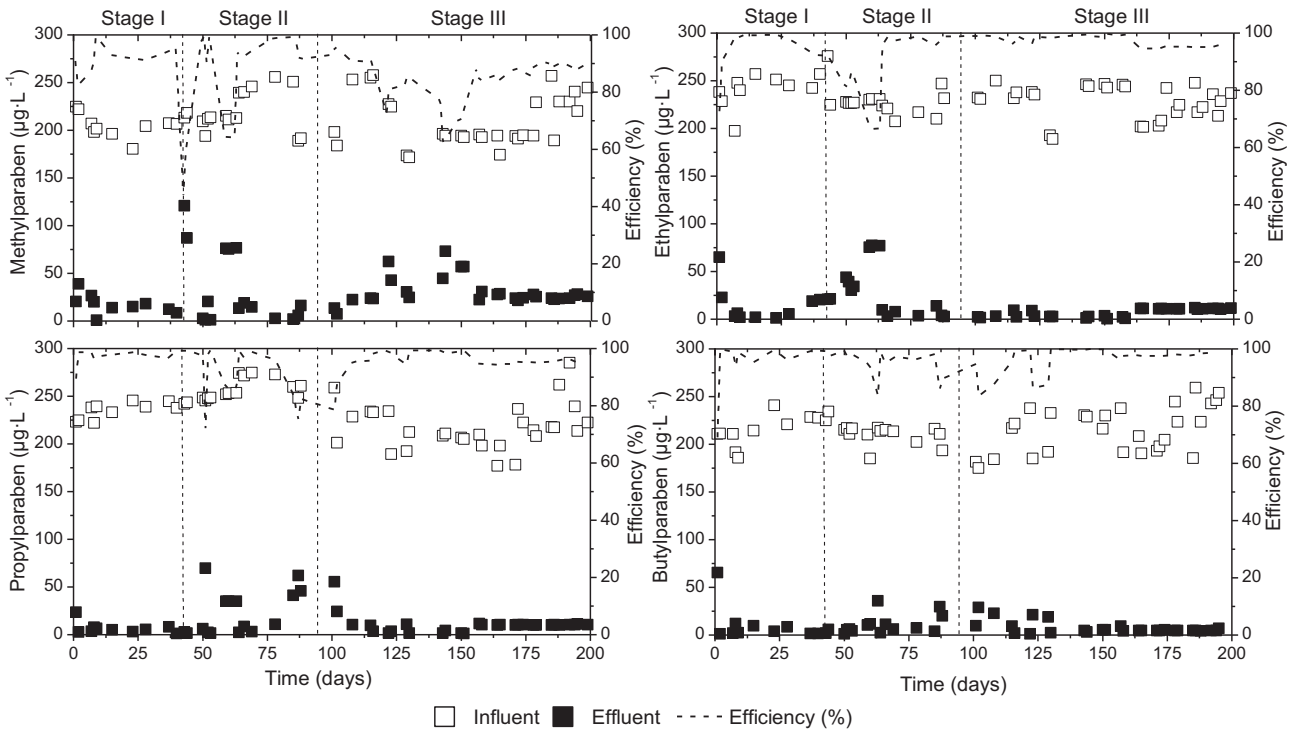

Fig. 3. Influent and effluent paraben concentrations and removal efficiencies of the reactor R_2.

end of the experiment (Fig. 3). Consequently, the mean MeP removal efficiency remained similar to that of the previous stage (85%) and was the lowest of the four compounds tested (Table 2).

The efficiencies obtained in the present work are in accordance with the overall removal efficiencies of parabens (60–100% depending on the compound) whose concentrations were less than 5 μg L^{-1} in some AS WWTPs (Butkovskyi et al., 2016; González-Mariño et al., 2011; Karthikraj et al., 2017; Li et al., 2015; Wang and Kannan, 2016). However, normally, it is not specified which treatment unit is primarily responsible for such removal. A study that monitored paraben removal in the different units of a full-scale WWTP, specifically a two-stage aerobic adsorption/biooxidation (AB) system designed for grey water treatment, was that by Butkovskyi et al. (2016). The authors observed a complete removal of MeP, EtP, PrP, and BuP in the AB system, in which the biooxidation stage (an AS system) was responsible for 30–100% (depending on the compound) of the overall paraben removal.

In AS systems, paraben removal may result from the combined action of the biodegradation and adsorption processes (Lu et al., 2018). Sun et al. (2014) observed high removal efficiencies of MeP (140–270 ng L^{-1}) and PrP (125–390 ng L^{-1}) in the primary treatment of an oxidation ditch followed by UV disinfection, reaching maximum values close to at 70% and 50% for MeP and PrP, respectively. Therefore, these authors suggested that adsorption plays an important role paraben removal. However, it is worth mentioning that, in primary treatment, there may also be active biomass, thus biodegradation cannot be disregarded. On the other hand, Lu et al. (2018), in aerobic batch assays with activated sludge (5.5 g MLVSS L^{-1}), did not observe significant removal of MeP, EtP, PrP, and BuP (1 mg L^{-1} each) by adsorption (1–15%).

Regarding the preference of removing compounds, although there is no clear tendency of which parabens are more easily removed in the above mentioned WWTPs, González-Mariño et al. (2011) and Lu et al. (2018) noticed that, under aerobic conditions, longer alkyl-chain parabens are degraded more rapidly. On the other hand, it is worth mentioning that Wu et al. (2017) found this same behavior only under anaerobic conditions, with no significant difference in the aerobic degradation kinetics of the tested parabens. Thus, in general, MeP seems to be the most recalcitrant of parabens, which may justify the lower removal efficiencies obtained in the present work for this compound (Table 2).

3.4. Removal of parabens, organic matter, N, and P over the cycle

The removal of parabens, organic matter (COD), N (ammonium, nitrite, nitrate), and P (phosphate) was evaluated over the R_2 cycle to have more insights into the process (Fig. 4). In the anaerobic phase, all parabens were removed concomitantly with the consumption of organic matter mainly by ordinary heterotrophic organisms (OHOs) as well as by PAOs and glycogen-accumulating organisms (GAOs) (Fig. 4). Thus, the presence of an easily biodegradable co-substrate (acetate) apparently did not hinder paraben removal.

In general, as micropollutants are present in wastewater at very low concentrations, they are hardly used as growth substrates. Therefore, the most likely hypothesis is that micropollutants are mainly degraded through cometabolic pathways by the action of non-specific enzymes (Fischer and Majewsky, 2014). In contrast, Fan and Wang (2017), studying paraben removal in an open horizontal filter, observed an effective removal of MeP, EtP, and PrP (50 mg L^{-1}) in the absence of an organic co-substrate. However, it is important to mention that they were in sufficient quantities to be used as carbon and energy source by the microbiota of the treatment system. Nonetheless, those authors also observed a cometabolic effect on the removal of such parabens when they were added to synthetic wastewater containing 50 mg COD L^{-1}. In the present study, although the parabens were supplemented at low concentrations compared to the primary carbon source (acetate), the hypothesis of metabolic degradation cannot be excluded, since some microbial groups may also have used them as growth substrates. Therefore, further investigation into the possibility of metabolic and cometabolic degradation of parabens, particularly at low concentrations, is recommended.

Paraben removals in the anaerobic phase were quite significant, particularly that of MeP (~70%) (Fig. 4). However, this was not expected, especially considering the short time of this phase (1.5 h), since paraben degradation is usually quite slow under anaerobic and anoxic conditions

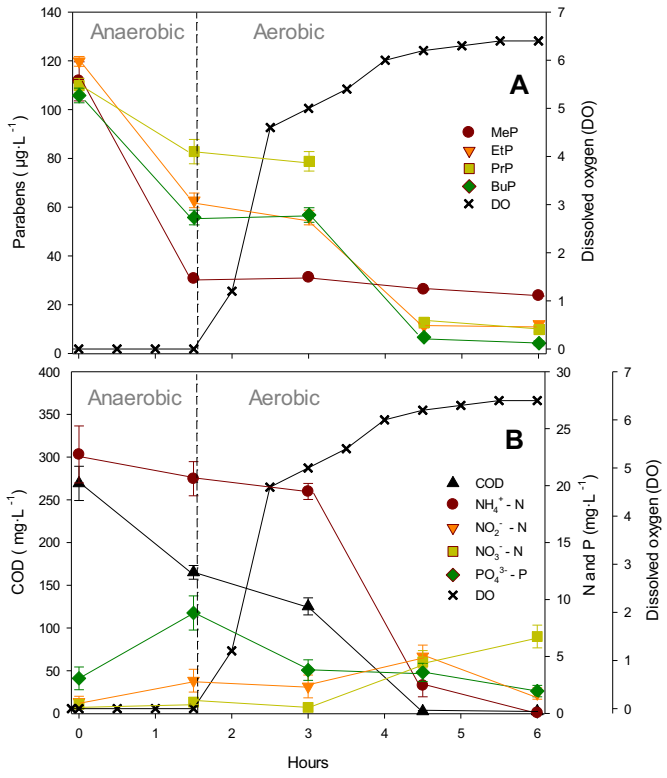

Fig. 4. Removal of parabens (a), organic matter, N, and P (b) throughout a cycle of the reactor R_2 at the end of stage III.

(Wu et al., 2017). Therefore, most likely, adsorption played an important role in the removal of these compounds. In contrast, Ashfaq et al. (2017) reported MeP (166 ng L^{-1}) and PrP (170 ng L^{-1}) removal efficiencies above 70% in the anaerobic tank of an A2/O treatment system (unspecified HRT), where the removal by adsorption was minimal. However, AGS has a much higher EPS content than AS and these are directly related to the removal of recalcitrant compounds in AGS systems (Nancharaiah and Reddy, 2018; Q. Wang et al., 2018). Therefore, the hypothesis of adsorption cannot be neglected. In fact, the aforementioned studies on the removal of chiral pharmaceuticals in AGS systems reported that adsorption played an important role in the process and was the main removal mechanism (Amorim et al., 2016; Moreira et al., 2015).

After 1.5 h of aeration (3 h of cycle), with the increase in DO and the beginning of nitrification, there was a reduction in the concentration of EtP, PrP, and BuP along with that of COD and ammonium, stabilizing when the co-substrate (acetate) was almost completely consumed and the ammoniacal nitrogen reached concentrations below 3 mg L^{-1} at 4.5 h of the cycle. Regarding the phosphorus accumulation metabolism, it apparently happened independently of paraben removal (Fig. 4).

Unexpectedly, MeP was practically constant throughout the aerobic phase (Fig. 4a), contrary to the results reported by Wu et al. (2017), in which parabens were rapidly degraded under aerobic conditions (half-life <20 min). One hypothesis that could justify the apparent persistence of MeP in the medium would be the conversion of the other parabens into the aforementioned compound during the degradation process. However, according to L. Wang et al. (2018), such conversion occurs only in the presence of methanol through the transesterification reaction (Fig. 5). In the absence of alcohols, paraben degradation occurs by hydrolysis of the ester bond, generating an alcohol and p-hydroxybenzoic acid (L. Wang et al., 2018), which can be subsequently biotransformed into phenol (Valkova et al., 2001) or benzoic acid (Wu et al., 2017) (Fig. 5). Thus, as the co-substrate used in the present work was acetate, the formation of MeP by transesterification is very unlikely.

To verify the hypothesis of conversion of the larger parabens into MeP, R_2 was initially fed with paraben-free synthetic wastewater until they were no longer detected in the effluent (~15 days). Then, it was supplemented with only EtP, PrP, and BuP, and, after 7 days of operation, a new cycle test was performed (Fig. 6).

During the anaerobic phase, the three compounds started to be removed (mainly EtP) (Fig. 6), but not as intensely as in the previous test with the four parabens (Fig. 4). Interestingly, MeP was detected at approximately 25 µg L^{-1} at the end of the anaerobic phase (Fig. 6). This reinforces the hypothesis that there is biotransformation of the tested parabens into MeP under these conditions and that the removal is not an exclusive result of adsorption. Nonetheless, it may still play a relevant role, since the expected MeP concentration, considering the above-mentioned reaction, was approximately 40 µg L^{-1}, although the unrecovered fraction of MeP (~15 µg L^{-1}) may have also been biotransformed.

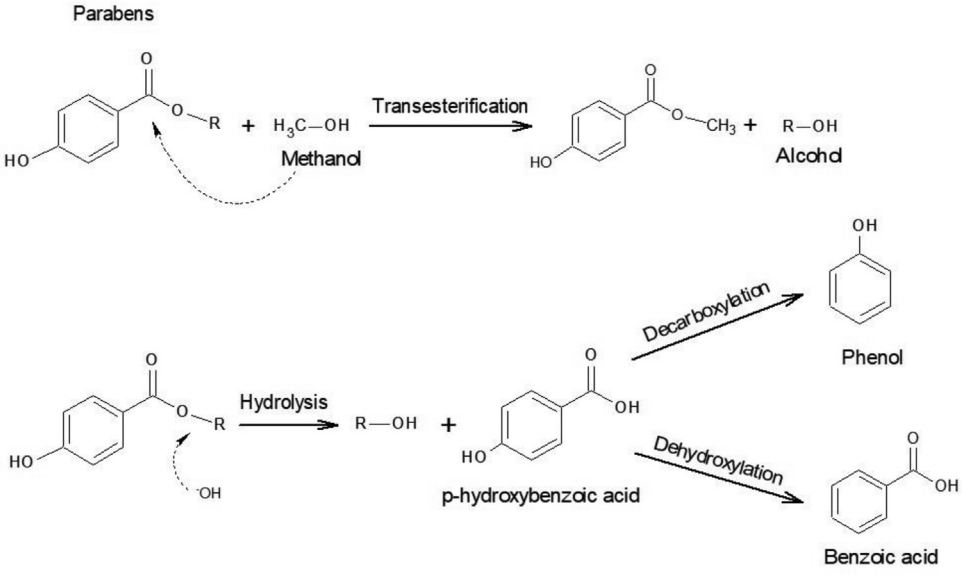

Fig. 5. Paraben degradation pathways under aerobic conditions. Adapted from Valkova et al. (2001), L. Wang et al. (2018), and Wu et al. (2017).

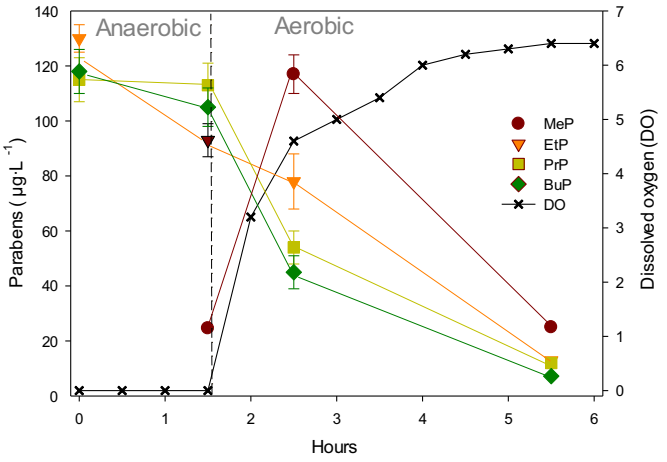

Fig. 6. Paraben removal throughout a cycle of the reactor R_2 when fed without MeP.

In the aerobic phase, EtP, PrP, and BuP were more significantly degraded (Fig. 6) as previously observed (Fig. 4). When the DO concentration was greater than 4 mg L^{-1} (2.5 h of cycle), MeP concentration was approximately 125 μg L^{-1} (Fig. 6), slightly lower than the expected (~150 μg L^{-1}). This condition reinforces the proposed hypothesis and indicates that the reaction may occur regardless of the redox conditions. Thereafter, all compounds were removed, and, at the end of this phase, EtP, PrP, and BuP concentrations were lower than 15 μg L^{-1}, while MeP concentrations were close to 25 μg L^{-1} (Fig. 6), similar to those found in the previous cycles (Fig. 4).

It is important to emphasize that, although MeP has been identified as a likely intermediate of the anaerobic and aerobic degradation of the longer alkyl-chain parabens, further investigations into paraben degradation pathways must be conducted, including the monitoring of other intermediates cited in the literature, such as p-hydroxybenzoic acid, phenol, and benzoic acid, to confirm the proposed hypothesis.

3.5. Paraben mass balance

Another reported mechanism of micropollutant removal in AGS systems, in addition to biodegradation, is adsorption on sludge (Nancharaiah and Reddy, 2018). However, regarding the parabens studied in the present work, MeP was the only one detected in the sludge at the end of stage III, at an approximate concentration of 300 μg g^{-1}. Therefore, the removal of the larger alkyl-chain parabens (EtP, PrP, and BuP) seemed to occur exclusively by biotransformation, which accounted for 94–97% of their inlet load (Fig. 7). As for MeP, considering the hypothesis that EtP, PrP, and BuP were biotransformed into

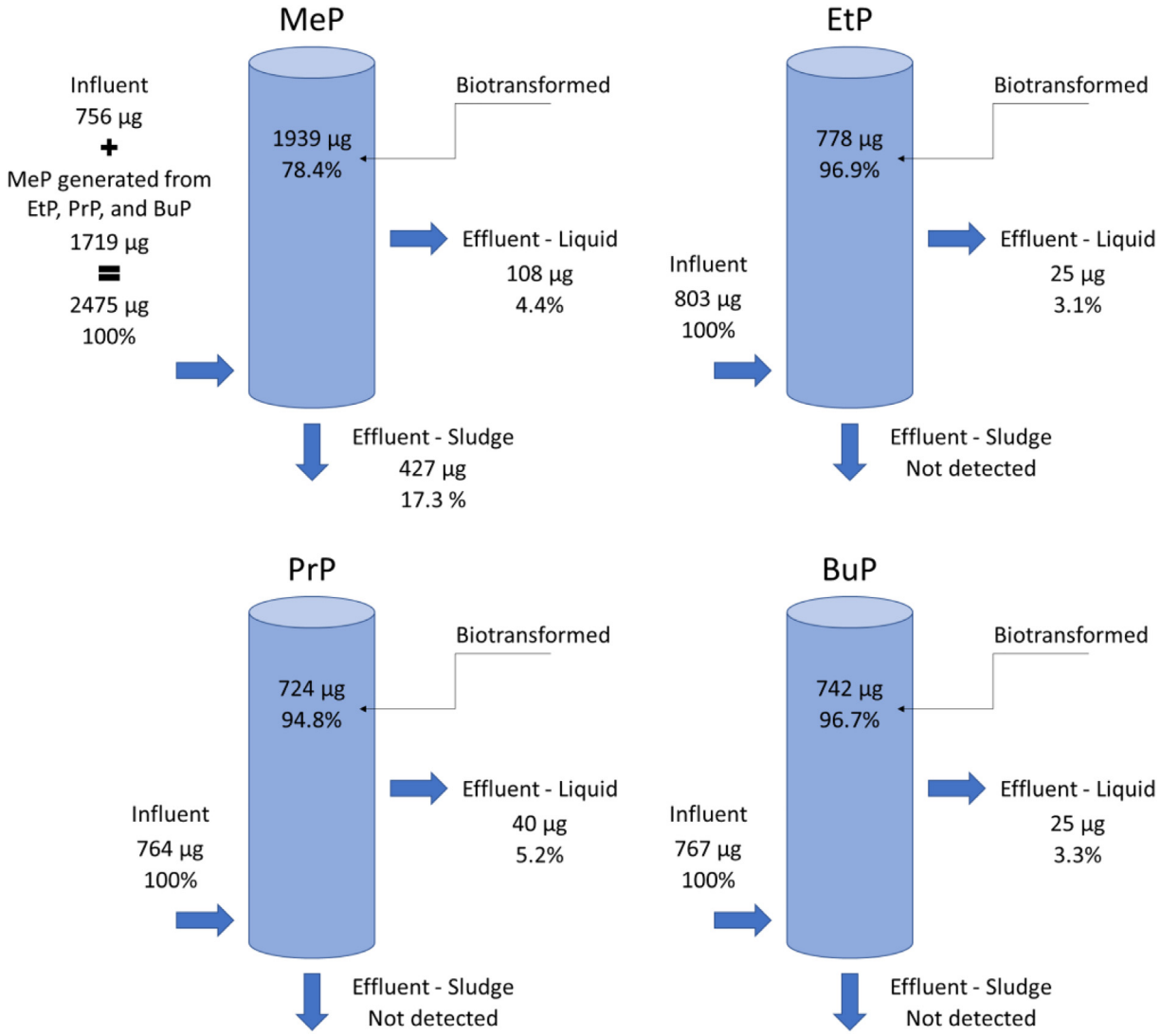

Fig. 7. Paraben mass balance in the reactor R_2 at the end of stage III.

this paraben, the fraction of MeP adsorbed on the sludge represented approximately 17% of its inlet load (influent MeP plus MeP generated from EtP, PrP, and BuP), whereas the biotransformed fraction accounted for approximately 78% (Fig. 7). Therefore, biotransformation was the main removal mechanism of MeP (85.5% of the total removal).

Ashfaq et al. (2017), evaluating an A2/O system during the treatment of wastewater containing 166 ng L^{-1} of MeP and 170 ng L^{-1} of PrP, found that 88.6% of the daily MeP load and 93.5% of that of PrP were removed by biotransformation, while adsorption on the sludge accounted for only 1.4% and 0.3% of the removal of MeP and PrP, respectively. Li et al. (2015), also in an A2/O system, observed that the longer alkyl-chain parabens (EtP, PrP, and BuP at the concentrations of 140, 438, and 28 ng L^{-1}, respectively) were almost completely removed (>99%), mostly by biotransformation (95–99%). On the other hand, similarly to the present work, although MeP (567 ng L^{-1}) had a significant portion removed by adsorption (in primary and excess sludge) (~22%), biotransformation was the main removal mechanism (Li et al., 2015).

3.6. Microbiological characterization

The microbiological analysis was performed with the sludge samples of the inoculum and the reactors R_1 and R_2 on day 180 of operation. After the granulation process, based on the Chao1 index, whereas R_1 maintained a microbial richness (423) similar to that of the inoculum (442), R_2 had a 20% reduction (353), i.e., there was a decrease in the number of species due to the continuous exposure to parabens. On the other hand, based on the inverse Simpson's index, the diversity decreased in both reactors (from 17.3 in the inoculum to 6.9 in R_1 and 11.9 in R_2), most likely due to the use of a simple primary carbon source (acetate) in the experiment. However, R_2 presented a diversity approximately 72% higher than that of R_1, i.e., the presence of parabens made the microbial community more evenly distributed, with a smaller number of dominant groups.

Concerning the microbial diversity at family level, the inoculum presented 28 different families, the most abundant being: Burkholderiaceae (20.7%), Moraxellaceae (14.8%), Xanthomonadaceae (13.5%), Streptococcaceae (9%), Enterobacteriaceae (7.9%), and Mycobacteriaceae (6.2%). In R_1, there were 32 families, the most abundant being: Rhodospirillaceae (39.3%), Pirellulaceae (17.4%), Rhodobacteraceae (9.8%), and Moraxellaceae (4%), whereas, in R_2, 29 families were identified, the most abundant being: Rhizobiales incertae sedis (25.8%), Rhodocyclaceae (14.7%), Pirellulaceae (11.5%), Microbacteriaceae (10.6%), and Moraxellaceae (6.1%). Therefore, the presence of parabens seemed to influence the population dynamics during the granulation process, affecting, consequently, the physicochemical characteristics of the granule (Section 3.1).

At genus level, it can be noticed that, after the granulation process, 19 of the 29 genera found in the inoculum remained in both reactors, while 9 other genera developed (Fig. 8). It is worth mentioning that, with the exception of *Mycobacterium*, R_1 and R_2 shared the same genera, which may explain why the systems showed a similar operational performance. Nonetheless, considering the relative abundances (as well as the presence of *Mycobacterium*), R_2 had a microbial community slightly more similar to that of the inoculum than that of R_1 (Fig. 8).

In the three samples, there was a predominance of heterotrophic bacteria. However, in R_1, the dominant genera were *Defluviicoccus* (39.3%) and *Pirellula* (17.4%), whereas, in R_2, the genera *Phreatobacter* (25.8%) and *Zoogloea* (14.1%) predominated. Among these, *Defluviicoccus* and *Zoogloea* are reported to produce EPS (Pronk et al., 2017; Tansel, 2018) as well as *Thauera* and *Thermomonas* (Shao et al., 2019; Zhang et al., 2020), which were less abundant in both reactors. Since the abundance of EPS-producing bacteria in R_1 were higher than in R_2, this can justify why the granules of R_1 had a denser and more compact structure, and it reinforces the negative effect of parabens on EPS production (Section 3.1). In addition, taking into account the detection level > 1% and the minimum prevalence of 66% (corresponding to the similar minimum presence between the two reactors), the most common genera in R_1 and R_2 were *Acinetobacter* and *Pirellula*, and those that most developed in R_2 were *Phreatobacter*, *Zoogloea*, *Galbitalea*, and *Thermomonas*.

Regarding the degradation of parabens, no studies that relate it to the genera identified in R_2 were found. However, some of them, which were indeed more abundant in R_2 than in R_1, are related to the degradation of similar compounds and may have participated in the degradation of parabens. For instance, Unz and Farrah (1972) reported that *Zoogloea* can degrade various aromatic compounds, such as benzoates, toluates, phenol, and cresols, and Li et al. (2019) pointed out that bacteria of the genera *Acinetobacter* and *Pseudomonas* are related to phenol degradation. Other genera that can also metabolize different aromatic compounds are *Thermomonas* (Godini et al., 2019), *Luteimonas* (Liu et al., 2014), *Pseudoxanthomonas* (Nayak et al., 2011), *Devosia* (Talwar et al., 2020), and *Rhizobium* (Chen et al., 1984), the last three being able to degrade p-hydroxybenzoic acid, one of the intermediates of paraben degradation. Therefore, although these genera were also present in the control reactor, they developed more in R_2, which may be related to the presence of parabens. Lastly, *Mycobacterium*, which was present only in R_2 (although with a very low relative abundance), is also reported to degrade polycyclic aromatic hydrocarbons (Guo et al., 2010).

4. Conclusions

The parabens did not prevent the formation of aerobic granules, which presented good settleability at the end of the maturation stage. However, these antimicrobial agents compromised the granule structure, which was fragile and uneven due to the lower production of EPS. The parabens did not affect the removal of organic matter (>90%) and N (>70%), but the removal of P was slightly impaired. Nonetheless, removal efficiencies of P close to 70% were reached.

High paraben removal efficiencies were achieved (>85%) in the AGS system, with MeP being the most recalcitrant compound. Concerning the removal mechanisms, biotransformation was the main mechanism in the removal of all parabens (85.5% for MeP and 100% for the others), whereas, apparently, adsorption played a role only in the removal of MeP. In addition, this compound was also suggested as a probable intermediate of degradation of the larger alkyl-chain parabens.

Lastly, regarding the microbial community, with the exception of *Mycobacterium*, the reactors shared the same genera, which may explain their comparable operational performances. Additionally, some genera that developed more in the presence of parabens may be related to their degradation.

CRediT authorship contribution statement

Thaís Salvador Argenta: Formal analysis, Investigation, Writing - original draft. **Antônio Ricardo Mendes Barros:** Conceptualization, Formal analysis, Investigation, Writing - original draft. **Clara de Amorim de Carvalho:** Investigation. **André Bezerra dos Santos:** Conceptualization, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Paulo Igor Milen Firmino:** Conceptualization, Writing - review & editing, Supervision.

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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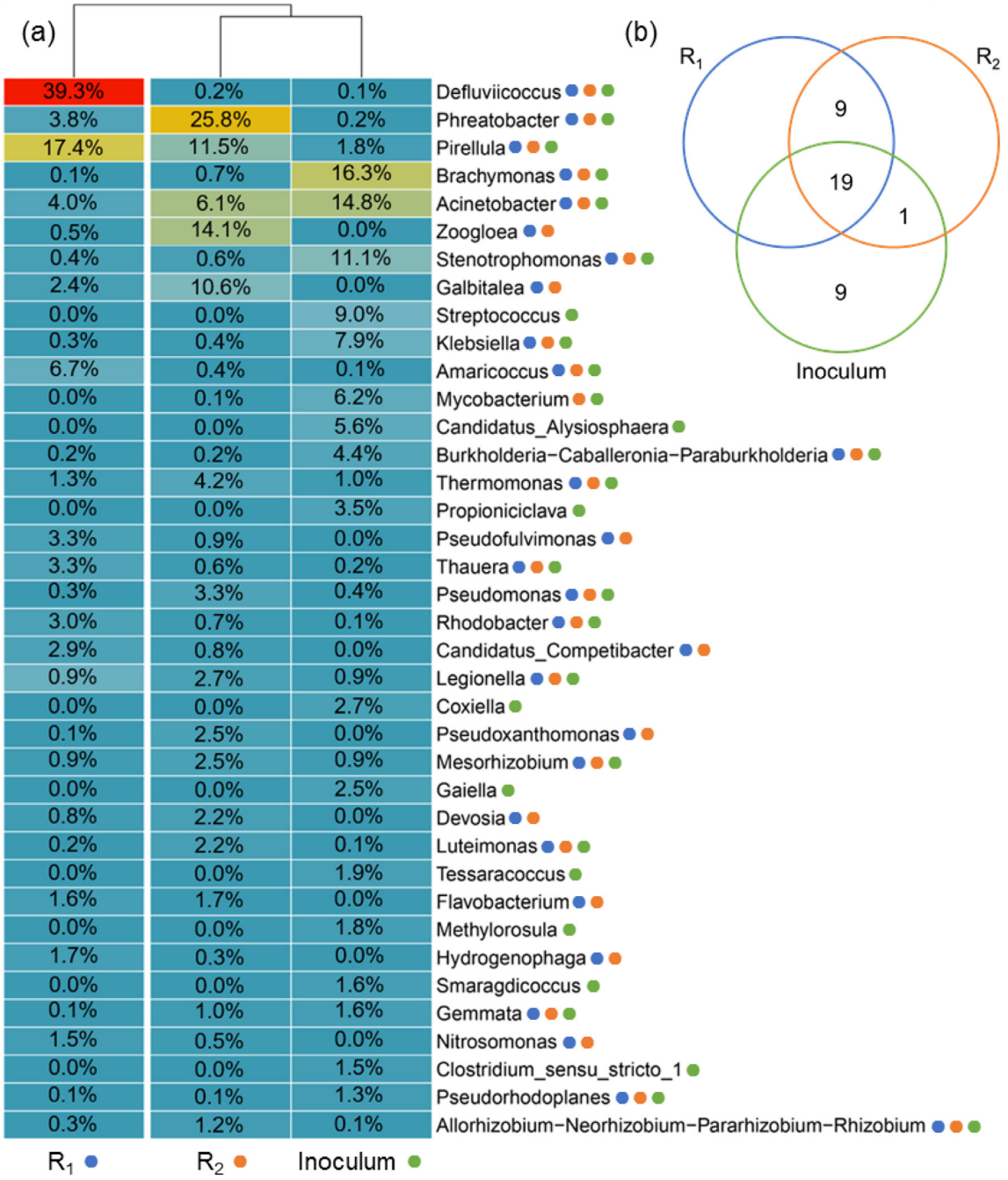

Fig. 8. Microbial diversity at genus level of the inoculum and the reactors R_1 and R_2. The heat map (a) shows the presence (colored circles) and relative abundance (percentage number) of each genus in the samples. The Venn diagram (b) shows the number of genera shared and not shared by the samples.

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