



Impact assessment of carbamazepine on aerobic granular sludge: Monitoring morphology changes, extracellular polymeric substances production, microbial dynamics and toxicity

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ABSTRACT

Aerobic granular sludge (AGS) technology for treating pharmaceutical-contaminated wastewater has sparked a great deal of interest in recent decades. However, the prolonged effect of these pollutants in AGS systems still requires further studies. In this study, an aerobic granular sludge sequencing batch reactor (AGS-SBR) was used to treat synthetic wastewater containing carbamazepine (CBZ) at varying concentrations. The purpose of this study was to evaluate the system performance, monitor the stability of the granular biomass, as well as to assess the system ability to detoxify the contaminated wastewater and to identify potential key-bacterial genera.

Results: show that the AGS-SBR quickly adapted to the presence of CBZ as the removal of the main nutrients was not negatively affected. Regardless of the observed accumulation of CBZ, wastewater toxicity was reduced by up to 70 %. The structure and stability of the AGS were not affected, with larger aggregates prevailing throughout the treatment. Sequencing of the 16S rRNA gene revealed that *Thauera*, *Pseudomonas* and *Flavobacterium* were the most abundant genera and may have played a crucial role in the stability of the system through the production of extracellular polymeric substances (EPS). These genera were also found to have several species that contain CBZ-degrading enzymes.

In summary, this study underscores the resilience of AGS-SBR systems in treating CBZ-contaminated wastewater, highlighting the critical role of certain microbial groups for the developed AGS system. The authors suggest further studies aimed at validating the ability of *Thauera* and *Flavobacterium* to biodegrade CBZ, as well as optimizing AGS systems through bioaugmentation with the aforementioned genera.

1. Introduction

The widespread occurrence of pharmacologically active compounds (PhACs) in aquatic environments has become an increasing environmental and public health concern. These compounds, including analgesics, antibiotics, and anticonvulsants, are often recalcitrant to conventional wastewater treatment plants (WWTP) due to their low biodegradability, high persistence, and complex biotransformation pathways [1]. Even at trace levels, PhACs can exert chronic toxic effects on aquatic organisms, including neurotoxicity, oxidative stress, and disturbances in the nervous system and reproduction [2–6].

Among PhACs, carbamazepine (CBZ) stands out as one of the most

frequently detected and environmentally persistent compounds. Used extensively for the treatment of epilepsy and bipolar disorder [7], CBZ resists photodegradation, biodegradation, and adsorption onto sludge [8]. As a result, its removal efficiency in WWTPs is often below 10 % [9] with negative removal also reported due to deconjugation reactions and desorption [10,11]. Consequently, CBZ has been detected in WWTP effluents worldwide at concentrations ranging from hundreds of ng/L to several µg/L. For instance, Onchoke et al. [12] found concentrations between 1.29 and 2.12 µg/L in East Texas (USA), Son et al. [13] identified CBZ among the top five most abundant drugs in WWTP effluents in South Korea, with average levels of 265 ng/L. Other authors reported up to 7 µg/L in hospital wastewater [14] and 820 and 1059 ng/L in Leiria

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WTPP influent and effluent, respectively [15].

The environmental persistence of CBZ [7], combined with its ecotoxicological effects [16], underscores the urgent need for alternative treatment technologies capable of effectively removing this compound from wastewater. One such alternative is the use of AGS technology, which represent a promising and sustainable biological treatment technology. AGS consists of compact microbial aggregates with excellent settling properties, high biomass retention, and increased resistance to toxic compounds [17–19]. Several studies have demonstrated the capacity of AGS systems to remove a range of PhACs [20–27], including preliminary work involving CBZ [28–33]. However, key knowledge gaps remain regarding the long-term exposure of AGS systems to CBZ, particularly its effects on granule stability, nutrient removal, and microbial community dynamics.

Understanding the structure and function of the microbial community and its dynamics in the surrounding environment is crucial for improving AGS performance and for the development of targeted strategies such as bioaugmentation. In this regard, some microorganisms capable of degrading CBZ have already been identified [28,29], as well as some enzymes involved in CBZ-breakdown [29], including cytochrome P450 (CYP450) [10], laccase [30] and oxygen oxidoreductase [29].

While a few studies have addressed the treatment of CBZ-contaminated wastewater using AGS technology [27,31–35], the long-term behavior of AGS systems under sustained exposure to CBZ remains poorly understood. In particular, the effect of CBZ on nutrient removal, granule stability, and microbial community dynamics requires further investigation.

This study seeks to address these gaps by investigating the response of an AGS-SBR system to sustained CBZ exposure. Specifically, we assess: (i) the removal efficiency of CBZ, (ii) changes in granule morphology and extracellular polymeric substances (EPS) production, (iii) shifts in microbial community composition, and (iv) the ecotoxicity of treated effluent. Ultimately, the present study lays the groundwork for future advancements in AGS-SBR systems, particularly through bioaugmentation strategies that could further enhance the system ability to degrade pharmaceutical contaminants. In this context, *Thauera*, *Pseudomonas* and *Flavobacterium* could be strong candidates for the remediation of CBZ in wastewater. This approach has not been thoroughly investigated in previous research and provides a potential pathway for optimizing AGS-based treatment technologies and offer a scientific basis for the future optimisation of AGS technology for the treatment of pharmaceutical-laden wastewaters.

2. Material and methods

2.1. Reactor and operating conditions

A 5 L lab-scale SBR was continuously operated in successive cycles of 6 h. Each cycle consisted of 120 min of anaerobic feeding, 232 min of aeration, 3 min of settling and 5 min of effluent withdrawal. The AGS-SBR was operated with a volumetric exchange ratio of 50 % and a hydraulic retention time (HRT) of 4 h. Aeration was provided at the bottom of the reactor (~ 7.50 L/min; superficial air velocity of approximately 1.8 cm/s). The AGS-SBR was operated at room temperature, around 25 °C. The synthetic wastewater was composed of 1.45 g/L $\text{CH}_3\text{COONa} \cdot 3 \text{H}_2\text{O}$, 0.03 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.07 g/L $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$, 0.015 g/L KCl, 0.120 g/L NH_4Cl , 0.028 g/L K_2HPO_4 [36] and 3.5 mL/L of trace element solution (adapted from Kreuk et al. [37]).

Granular sludge from Lugo WWTP (Galicia, Spain) was used as inoculum with an initial concentration of ca. 3 g/L mixed liquor suspended solids (MLSS). The feeding and effluent withdrawal were provided by Watson Marlow 101 U/R and Watson Marlow 323 pumps (Watson Marlow, Wilmington, USA), respectively and the air supply by an Aalborg GCF17 mass flow controller (Aalborg, Aalborg, Denmark). The pumps and mass flow controller were controlled by automatic

timers (MasterClear).

Before starting the CBZ treatment, the granular sludge underwent a 2.5-month acclimation period. During this phase, only synthetic wastewater was fed into the SBR and the settling time was gradually reduced from 15 to 3 min to ensure that most of the flocculent sludge and smaller granules were washed out during withdrawal. Several important parameters were monitored to follow the progress of the system, including sludge volume index (SVI), nutrients removal, solids concentration and pH (Fig. SM1 — SM3). After acclimation, CBZ was intermittently added to the synthetic wastewater at different concentrations, as indicated in Table 1. CBZ treatment was divided into 3 treatment phases according to its initial concentration. The contribution of CBZ photodegradation was prevented by covering the entire SBR with a black sheet.

2.2. Analytical methods

2.2.1. SBR physicochemical analysis

The SVI was determined based on the height of a defined volume of biomass after 5 min (SVI_5) and 30 min (SVI_{30}), once a week. MLSS and mixed liquor volatile suspended solids (MLVSS) were determined three times per week, according to standard methods [38].

The pH of the synthetic wastewater, mixed liquor inside the reactor and effluent were monitored regularly using a HANNA HI83141 pH meter. Temperature and dissolved oxygen (DO) were also checked using a DO meter (HI2040–02, HANNA Instruments).

Chemical oxygen demand (COD), ammonium (N-NH_4^+), nitrate (N-NO_3), nitrite (N-NO_2) and phosphate ($\text{PO}_4\text{-P}$) were determined using Hach Lange cuvette test kits (Hach Lange, Dusseldorf, Germany) and a DR 2800 spectrophotometer (Hach-Lange GmbH). The removal performance of these nutrients was studied three times per week. Samples were collected in the synthetic wastewater, at the end of the anaerobic feeding and in the effluent. All samples were centrifuged and filtered through a 0.45 μm nylon membrane syringe filter prior to analysis.

2.2.2. CBZ quantification

The concentration of CBZ was determined by Ultra High-Performance Liquid Chromatography (UHPLC), as described by Quintelas et al. [39], three times per week. Samples were taken from the synthetic wastewater, at the end of the anaerobic feeding stage and from the effluent. All samples were filtered through a 0.2 μm nylon membrane syringe filter prior to analysis.

2.2.3. EPS extraction and quantification

The production of total EPS, loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS) were analyzed in the 1st week (with CBZ loading) and the 4th week (without CBZ loading) of each treatment phase. A detailed description of the method employed to extract and quantify EPS can be found in Melo et al. [36].

Table 1

Treatment phases of the AGS-SBR system with intermittent CBZ dosage.

Treatment phase	Operational days	Influent CBZ concentration (mg/L)
<i>I</i>	Week 1 (d1-d7)	2
	Week 2 (d8-d14)	0
	Week 3 (d15-d21)	2
	Week 4 (d22-d28)	0
<i>II</i>	Week 1 (d29-d35)	5
	Week 2 (d36-d42)	0
	Week 3 (d43-d49)	5
	Week 4 (d50-d56)	0
<i>III</i>	Week 1 (d57-d63)	8
	Week 2 (d64-d70)	0
	Week 3 (d71-d77)	8
	Week 4 (d79-d84)	0

2.2.4. Microbial community analysis

For the microbial assessment, 2 samples were collected upon receiving the granular sludge from Lugo WWTP (inoculum), after acclimation period (mature granules) and at the end of the CBZ treatment (4th week of phase III) (CBZ granules). The mature and CBZ granules were collected during the aeration stage of the SBR cycle. All samples were immediately centrifuged ($4\,000 \times g$ for 8 min) after being collected, the supernatant was discarded, and the pellet resuspended with phosphate-buffered saline (PBS). Samples were stored at $-20\text{ }^{\circ}\text{C}$ until extraction.

Genomic DNA was extracted using the commercial FastDNA SPIN kit for soil (MP Biomedicals, Solon, OH, USA), according to the manufacturer's instructions. The quantity and quality of DNA were assessed using a NanoDrop spectrophotometer (NanoDrop 1000, Thermo Scientific, Waltham, MA, USA). To check whether the 16S rRNA gene was amplifiable, polymerase chain reaction (PCR) was performed in a thermocycler (TC-96 LifeECO, Hangzhou Bioer Technology Co., China) using the primer set 968 F/1401 R [40]. The PCR program is described elsewhere [41]. PCR products were checked by agarose electrophoresis on a 1 % (w/v) agarose gel at 100 V for 15 min. The gel was then photographed and analyzed using a gel imaging system (ChemiDoc XRS+, Bio-Rad).

Clone libraries, 16S rRNA gene sequencing by Illumina MiSeq and data analysis were performed at the Research and Testing Laboratory (RTL, Lubbock, TX, USA). Details of the methodology can be found in Salvador et al. [41]. The primer set used for sequencing was 515 F (GTGCCAGCMGCCGCGGTAA) and 806 R (GGACTACHVGGGTWCTAAT) [42]. The FASTQ files were deposited in the European Nucleotide Archive (ENA) under the project accession number PRJEB76046. After identifying the most abundant genera in the CBZ granules, we investigated whether those genera possessed genes encoding enzymes capable of degrading CBZ using UniProt database.

2.2.5. Ecotoxicity test

An acute toxicity test of CBZ was performed by analyzing the changes in the bioluminescence of *Vibrio fischeri*, using freshly prepared bacteria [43]. *V. fischeri* strain NRRL-B-11177 was acquired by BioFix® Lumi (Macherey-Nagel, Düren, Germany) and grown in the laboratory under aerobic conditions, as described elsewhere [44].

Toxicity throughout the three treatment phases was assessed in the synthetic wastewater and in the treated effluent. Effluent toxicity was measured three times per week. Prior to the toxicity assay, samples were centrifuged (10 min at $10\,000\text{ rpm}$) and filtered through a $0.45\text{ }\mu\text{m}$ nylon membrane syringe filter. The toxicity procedure was adapted from ISO 11348-1 and ISO 11348-3, in a microplate reader (Biotek® Cytation3, Fisher Scientific, Seoul, Korea) following the methodology described by Silva et al. [45].

2.2.6. Image acquisition, processing and analysis

To assess the morphology and size of the granular biomass, 10 mL samples were taken at the mid-point depth of the reactor, at the beginning of the aeration stage, in order to obtain homogeneous and representative biomass samples. Three time points were selected to monitor the evolution of the AGS morphology: at the end of the acclimation period, in the middle of the CBZ treatment (phase II, 2nd week) and at the end of the CBZ treatment (phase III, 4th week).

After collecting the samples, the supernatant was removed to discard undesirable flocculent biomass and a 1:1 mixture of PBS and ethanol 99.8 % was then added. All samples were stored at $-20\text{ }^{\circ}\text{C}$ to preserve the biomass properties until the day of analysis.

To collect the AGS images, 4 mL of sample were transferred to a Petri dish, using a sectioned tip to allow larger aggregates to flow through. Images were acquired using an Olympus SZ61TR Zoom Stereo Microscope (Olympus, Shinjuku, Japan) under a total amplification of $8 \times$ coupled with an Olympus EP50 camera (Olympus, Tokyo, Japan). A specially developed program in MATLAB R2023b (MathWorks Inc.,

Natick, USA) was used to process the collected images and characterize the AGS samples in terms of relevant size and morphological parameters, including the equivalent diameter (Deq), area, solidity, eccentricity and convexity. A more detailed description of the QIA methodology can be found elsewhere [46]. Granules were categorized into three classes based on their equivalent diameters (Deq): small granules ($\text{Deq} < 250\text{ }\mu\text{m}$), intermediate granules ($250 < \text{Deq} < 1500\text{ }\mu\text{m}$) and large granules ($\text{Deq} > 1500\text{ }\mu\text{m}$).

3. Results

3.1. AGS-SBR performance

Figs. 1a and 1b show, respectively, the settling capacity of the granular biomass and the solids concentrations throughout the CBZ treatment. It can be seen that SVI_{15} gradually approached SVI_{30} until the $\text{SVI}_{30}/\text{SVI}_{15}$ ratio reached the unity after 50 days of treatment (Fig. 1a). MLVSS and MLSS concentration remained relatively constant throughout the entire treatment, with an average of $3.21 \pm 0.36\text{ g/L}$ and $3.56 \pm 0.36\text{ g/L}$, respectively (Fig. 1b).

The average pH of the synthetic wastewater was 7.69 ± 0.38 and the increment of CBZ concentration did not lead to a proportional increase in the influent pH (Fig. 1c). pH inside the reactor was 8.59 ± 0.41 and remained practically constant until the end of the SBR cycles (Fig. 1c). Although pH stayed at neutral levels, the slight increase observed inside the reactor and in the effluent was likely due to the release of CO_2 during the aeration stage [47].

The performance of the AGS-SBR in terms of organic matter and nutrient removal was monitored throughout the CBZ treatment. From Fig. 2a, it can be seen that CBZ shock loads did not affect the overall COD removal, with excellent removal performances being achieved during the entire time. Given the COD of CBZ itself (2.78 g COD/g CBZ), influent COD considerably increased from $682 \pm 15\text{ mg/L}$, (without CBZ) to $1015 \pm 53\text{ mg/L}$ (with CBZ). Despite these oscillations, effluent COD remained constant at $39 \pm 14\text{ mg/L}$ (Fig. 2a). This remaining COD could be the result of non-biodegradable matter, possibly from microbial degradation within the reactor [24].

Phosphate concentration in the influent fluctuated considerably, although no apparent correlation with the presence of CBZ was found, registering a mean of $4.09 \pm 0.46\text{ mg/L}$ (Fig. 2b). Its removal was affected since its concentration in the effluent varied greatly between the weeks with and without CBZ supply. For instance, in the presence of CBZ, $\text{PO}_4\text{-P}$ concentration dropped to $0.54 \pm 0.25\text{ mg/L}$ in the effluent, resulting in $87 \pm 6\%$ removal efficiency. On the other hand, during the weeks without CBZ, $\text{PO}_4\text{-P}$ concentration in the effluent was $1.61 \pm 0.33\text{ mg/L}$ ($58 \pm 9\%$ removal).

The evolution profiles of ammonium, nitrate and nitrite concentrations are shown in Fig. 3. Average $\text{NH}_4\text{-N}$ concentration in the synthetic wastewater was $29.14 \pm 11.17\text{ mg/L}$, being just slightly higher with the addition of CBZ. $\text{NH}_4\text{-N}$ removal efficiency was consistently negative in the presence of CBZ until the end of the 1st week of phase II (Fig. 3a). During that period, $\text{NH}_4\text{-N}$ concentration at the end of the anaerobic feeding stage ($66.30 \pm 33.30\text{ mg/L}$) and in the effluent ($47.83 \pm 19.32\text{ mg/L}$) was greater than in the influent. Nevertheless, from the 2nd week of phase II onwards, $\text{NH}_4\text{-N}$ removal spiked to $95 \pm 4\%$ until the end of the CBZ treatment, reaching only $2 \pm 1.76\text{ mg/L}$ in the effluent.

CBZ increment in the synthetic wastewater led to an increase of $\text{NO}_2\text{-N}$ concentration in the effluent (Fig. 3b), going from $7.41 \pm 4.37\text{ mg/L}$ (phase I) to $16.49 \pm 2.58\text{ mg/L}$ (phase II) and $18.27 \pm 1.51\text{ mg/L}$ (phase III). During the weeks when CBZ dosage was suspended, the average $\text{NO}_2\text{-N}$ concentration was fairly constant and equal to $3.55 \pm 0.94\text{ mg/L}$. Conversely, $\text{NO}_3\text{-N}$ concentration was substantially lower than $\text{NO}_2\text{-N}$ and was unaffected by the increase of CBZ concentration. In the presence of CBZ, $\text{NO}_3\text{-N}$ concentration was $2.97 \pm 1.57\text{ mg/L}$, while without CBZ it was only $0.83 \pm 0.43\text{ mg/L}$.

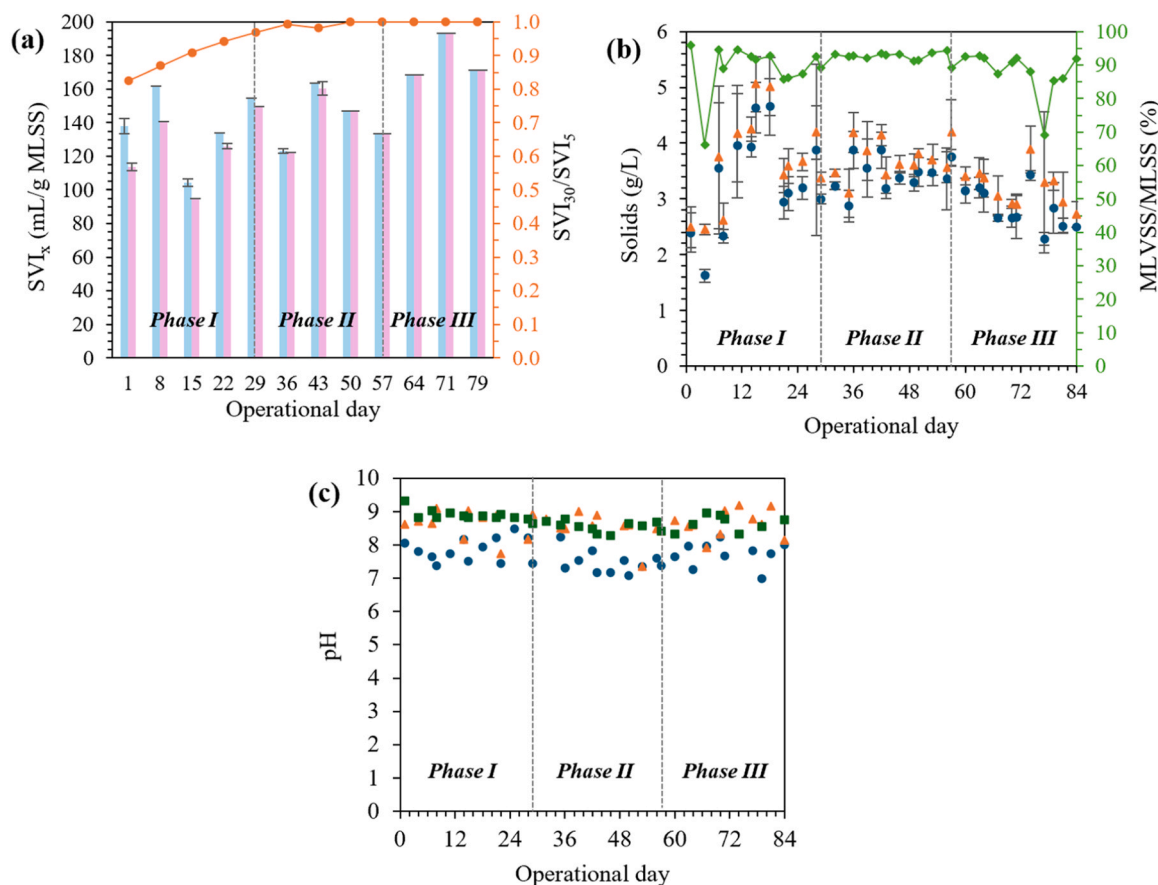


Fig. 1. Evolution of (a) SVI₅ (■) SVI₃₀ (■) and the corresponding SVI₃₀/SVI₅ ratio (●); (b) MLVSS (mg/L) (●) MLSS (mg/L) (▲) and MLVSS/MLSS ratio (◆) inside the reactor; (c) pH in the influent (●), during aeration (▲) and in the effluent (■) during phase I (2 mg/L CBZ), phase II (5 mg/L CBZ) and phase III (8 mg/L CBZ).

3.2. AGS morphology

The particle size distribution of the granular biomass reveals that the granular sludge was mostly composed of intermediate aggregates after acclimatization (86 %), with just a small fraction of small (5 %) and large (9 %) aggregates (Fig. 4a). However, during CBZ treatment, large aggregates became dominant until the end of the study, occupying an average of 93 % of the total area. The Deq remained constant throughout the study (Fig. 4b).

The evolution of the morphological characteristics (Fig. 4c) displays that the AGS remained smooth throughout the treatment time, with almost no irregularities (convexity $\approx 0.88 \pm 0.1$), compact (solidity $\approx 0.85 \pm 0.11$) and with fairly elongated shape (eccentricity $\approx 0.76 \pm 0.16$).

3.3. CBZ removal

CBZ removal profile is displayed in Fig. 5. The average influent CBZ concentration did not deviate much from the desired values, with 1.96 ± 0.06 , 4.85 ± 0.07 and 7.93 ± 0.15 mg/L in phase I, II and III, respectively. It can also be seen that CBZ removal fluctuated considerably and even reached negative efficiencies on some days. Average CBZ removal was 4.6, 5.6 and 16.4 % in phase I, II and III, respectively. When CBZ was no longer present in the synthetic wastewater, it was anticipated that the AGS would gradually release the accumulated CBZ. However, only negligible concentrations of CBZ were detected in the first few days.

3.4. EPS production during CBZ treatment

EPS are a complex mixture secreted by bacteria and are predominantly composed of proteins (PN), humic acids (HAS), polysaccharides (PS), nucleic acids, lipids and phospholipids [48]. The production of total EPS and the composition of LB-EPS and TB-EPS are represented in Fig. 6.

When CBZ was first introduced into the system (phase I, 1st week), LB-EPS increased drastically to 430 ± 10 mg/g MLVSS, whereas TB-EPS decreased to 179 ± 3 mg/g MLVSS. From the 4th week of phase I (day 25) until the 1st week of phase III (day 60), LB-EPS decreased sharply and remained somewhat constant with 167 ± 2 mg/g MLVSS. After 81 days of operation, in the last week of phase III, LB-EPS underwent once more a drastic reduction, reaching 47 ± 5 mg/g MLVSS at the end of the study. By contrast, TB-EPS concentration increased considerably in the last week to 256 ± 11 mg/g MLVSS. If we now consider the evolution of total EPS production, it can be seen that the first CBZ loading greatly stimulated its concentration as it went from 467 ± 6 (at the end of acclimatization) to 609 ± 11 mg/g MLVSS. From this point onwards, total EPS concentration remained virtually constant until the end of the study (302 ± 10 mg/g MLVSS).

LB-EPS content shows that the PS concentration was noticeably lower than PN and HAS, representing only 7 % of the total LB-EPS during the weeks when CBZ was present in the wastewater and just 3 % when it was not. LB-EPS were mainly composed of PN (< 70 %). In general, PN concentration was substantially higher when CBZ was present in the synthetic wastewater and the first exposure of CBZ led PN

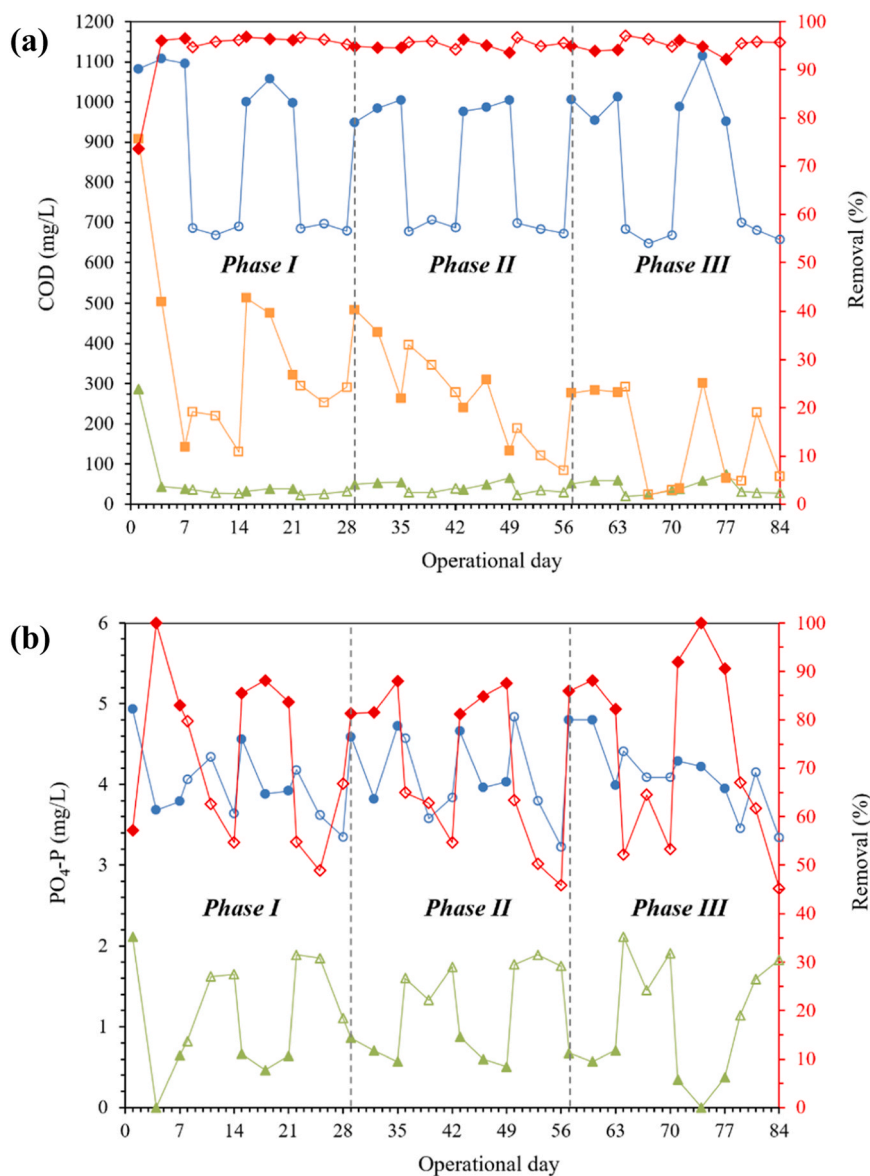


Fig. 2. Concentration profiles of (a) COD and (b) PO₄-P in the influent (●), after anaerobic feed (■) and effluent (▲), and overall removal efficiency (%) (◆) during the three treatment phases. The days on which CBZ was introduced into the synthetic wastewater are shown with the markers filled in.

production rising from 130 ± 6 (at the end of acclimatization) to 336 ± 9 – 110 ± 1 mg/g of MLVSS (1st week, phase I). HAS concentration was also higher in the presence of CBZ.

PS concentration in TB-EPS also remained the lowest compared to those of PN and HAS. Its concentration did not deviate much from the acclimation period, accounting just 6 ± 2 mg/g MLVSS (4 % TB-EPS). HAS content in TB-EPS was slightly higher than in the LB-EPS, particularly during the last two phases. During these latter phases, HAS concentration reached 37 ± 1 mg/g MLVSS (26 % TB-EPS). At the end of acclimatization, PN concentration registered 230 ± 3 mg/g MLVSS (86 % TB-EPS) and kept decreasing until reaching the lowest value of 78 ± 6 mg/g MLVSS in the 4th week of phase II. From this point onwards, it rose again until the end of the study, achieving nearly the same value as what was found before starting the CBZ treatment phases.

3.5. Ecotoxicity evaluation

Vibrio fischeri bioluminescence test presents high sensitivity, reproducibility and fast results, being widely used for toxicity assessments. The luminescence inhibition caused by the synthetic wastewater,

without CBZ, was on average 15.9 ± 0.5 % (Table 2). Although it did not confer toxicity to the samples, this slight luminescence inhibition may be associated with the adaptation of *V. fischeri* to the medium and to the conditions of the assay [45,49].

Meanwhile, the presence of CBZ in the synthetic wastewater caused an increase in the inhibitory effect on the bioluminescence of *V. fischeri*, reaching toxic levels in phase III (32.8 ± 1.1 %). It was observed that CBZ itself was only toxic when present at concentrations between 10 mg/L and at least 30 mg/L (Fig. SM4). This finding is in accordance with those of other studies [50,51].

After the biological treatment, *V. fischeri* showed negligible toxicity as the INH decreased considerably in all treatment phases [45,49]. From phase I to phase III, the effluent presented an average INH of 4.8 ± 1.0 , 12.8 ± 2.9 and 17.4 ± 2.3 %, respectively. Based on these results, it can be concluded that the AGS-SBR successfully detoxified the contaminated wastewater by up to 70 % (phase I), 44 % (phase II) and 47 % (phase III), reaching INH values in the treated effluent very close to those found in the weeks in which CBZ was not introduced into the system.

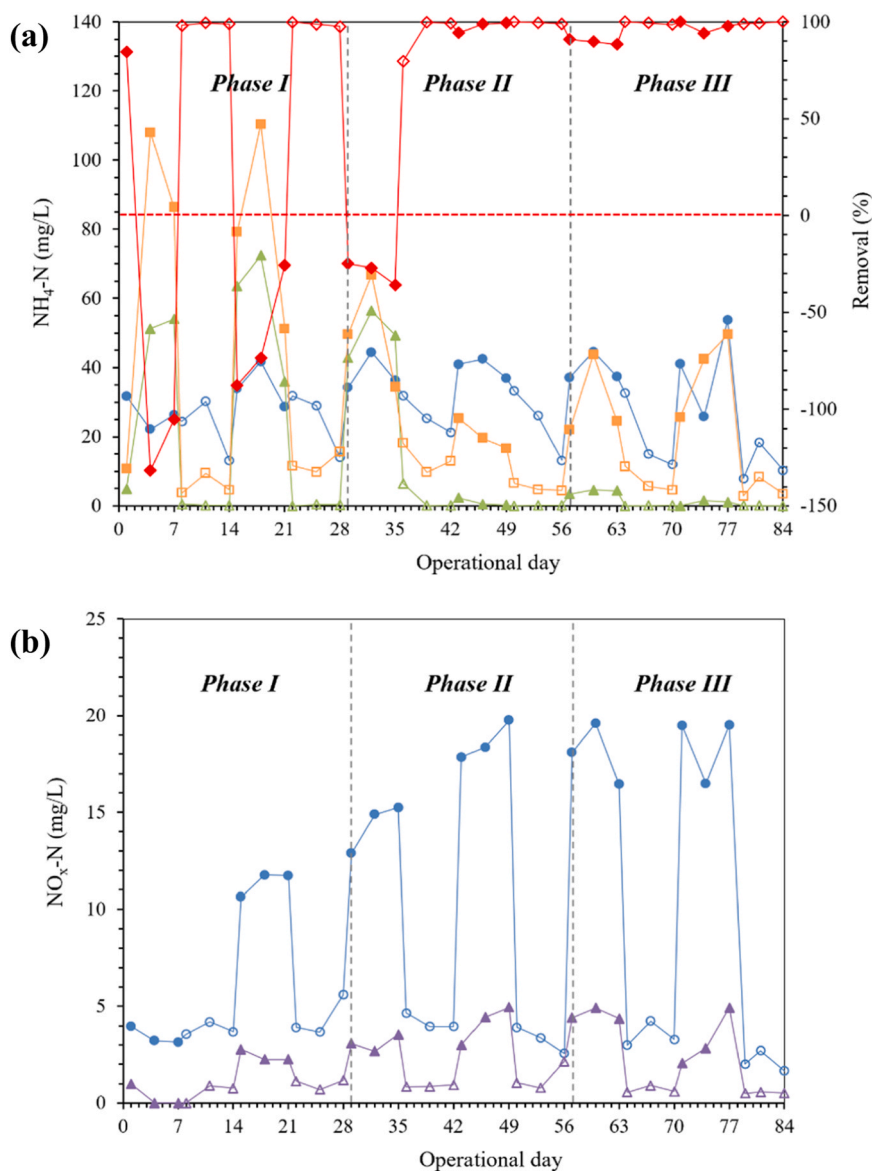


Fig. 3. Evolution of (a) $\text{NH}_4\text{-N}$ concentration in the influent (●), after anaerobic feed (■) and effluent (▲), and overall removal efficiency (%) (◆) and (b) $\text{NO}_2\text{-N}$ (●) and $\text{NO}_3\text{-N}$ (▲) concentration in the effluent during the three treatment phases. The days on which CBZ was introduced into the synthetic wastewater are shown with the markers filled in.

3.6. Microbial population dynamics and possible roles of abundant microorganisms

Fig. 7 shows the dynamic variations in microbial composition over time. The species that increased their relative abundance during acclimation are known to be acetate-degrading microorganisms, such as *Leucobacter* sp., *Cytophaga* sp., *Mesorhizobium* sp. and *Pseudoxanthomonas* sp.

The presence of CBZ in the AGS-SBR system also affected the microbial dynamics. At the phylum level (data not shown), *Proteobacteria* and *Bacteroidota* were the most enriched by the presence of CBZ, revealing their possible resistance to this drug. Conversely, *Actinobacteria* and *Firmicutes* may be more sensitive to CBZ, since microorganisms assigned to these phyla had their relative abundance reduced after CBZ treatment. *Thauera* sp. (37.9 %), *Pseudomonas* sp. (9.2 %), *Zoogloea* sp. (8.7 %) and *Flavobacterium* sp. (5.7 %) showed the highest relative abundance at the end of the study.

Table 3 displays the *in silico* proteome analysis results, where the tax ID (2) corresponds to the UniProt taxonomic code assigned to the genus

(1) and # species (4) corresponds to the total number of microorganisms belonging to that genus that possess the CBZ-degrading enzyme (3). According to UniProt database, several identified species from the three most abundant genera in CBZ granules contain enzymes responsible for CBZ-breakdown, namely CYP450, laccase, aldehyde oxidase and biphenyl dioxygenase.

4. Discussion

Being a promising technology for the biological treatment of wastewater contaminated with PhACs, assessing the long-term stability of AGS systems in the presence of such contaminants is vital. In this study, the health and stability of the granular biomass were not affected by the presence of CBZ. In fact, mature and larger granules prevailed throughout the treatment period (Fig. 4), with a high organic fraction ($\text{MLVSS/MLSS} > 85\%$, Fig. 1b) and excellent sedimentation properties ($\text{SVI}_{30}/\text{SVI}_5 = 1$, Fig. 1a). The values obtained are in line with the findings of other authors regarding the expected SVI of stable and mature AGS [52,53].

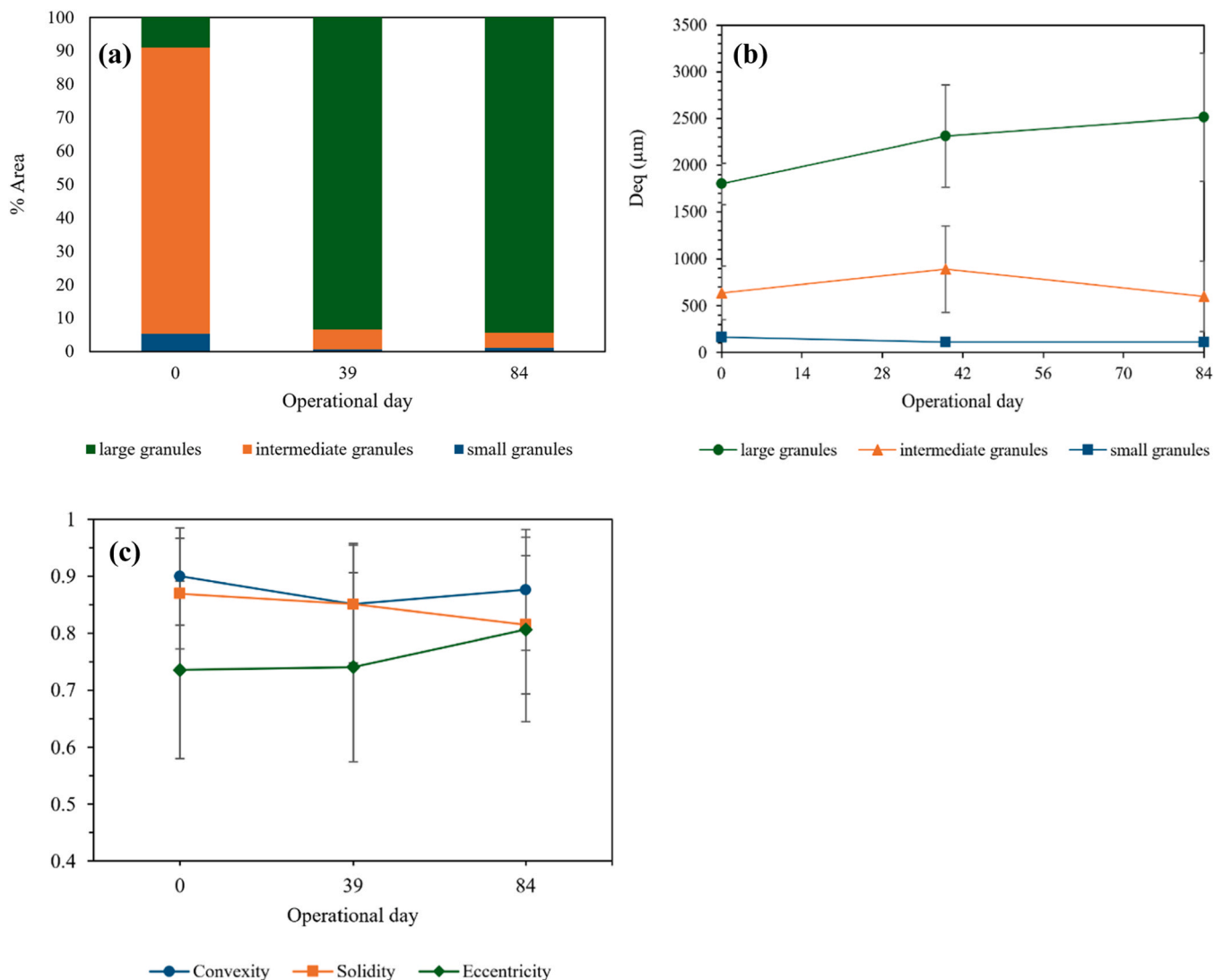


Fig. 4. Evolution of (a) area (%); (b) Deq (μm) according to the aggregates size: small ($\text{Deq} < 250 \mu\text{m}$) (■); intermediate ($250 \mu\text{m} \leq \text{Deq} \leq 1500 \mu\text{m}$) (▲) and large ($\text{Deq} > 1500 \mu\text{m}$) (●) and (c) convexity (●), solidity (■) and eccentricity (◆) of total aggregates. Operational days 0, 39 and 84 correspond to the day before starting CBZ treatment, halfway through (phase II, 2nd week) and at the end (phase III, 4th week), respectively.

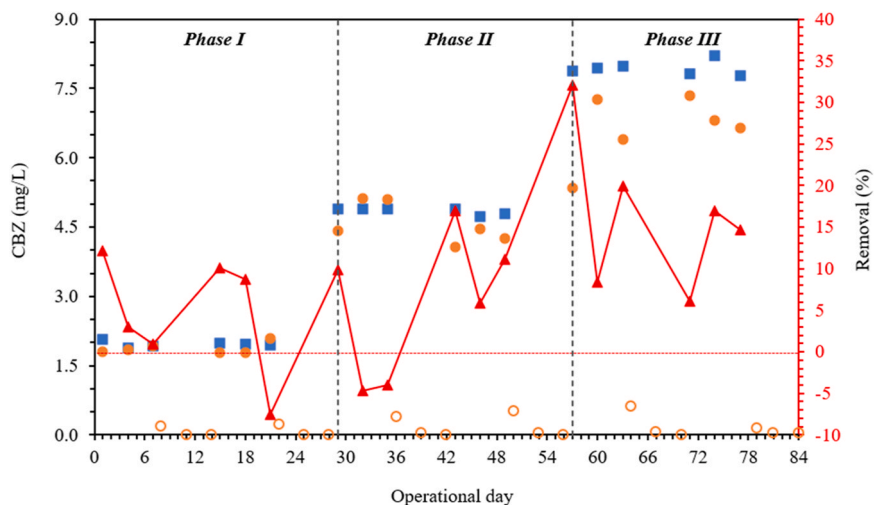


Fig. 5. CBZ concentration (mg/L) in the influent (■) and effluent (●) and the corresponding removal percentage (%) (▲) during phase I (2 mg/L CBZ), phase II (5 mg/L CBZ) and phase III (8 mg/L CBZ). Effluent concentration on the days in which CBZ was absent from the synthetic wastewater are marked with ○.

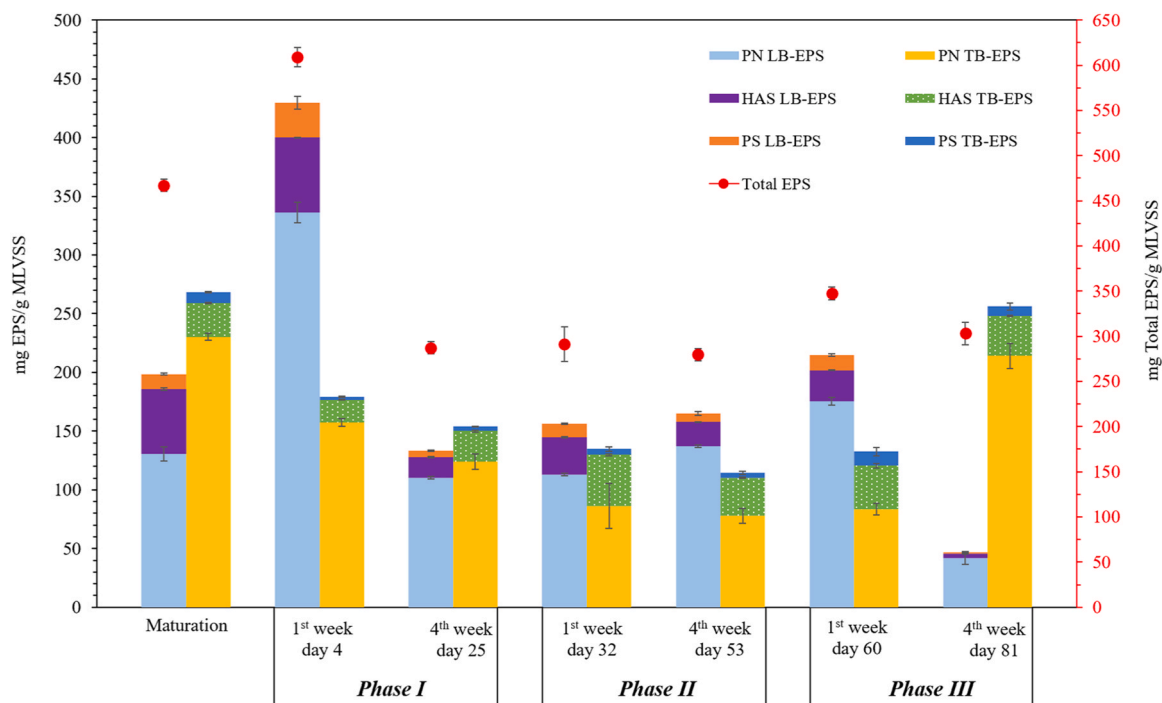


Fig. 6. Concentration profiles (mg/g MLVSS) of total EPS (●) and the contents of LB-EPS - PN (■), HAS (■), PS (■) - and TB-EPS - PN (■), HAS (■), PS (■) - at the end of the acclimation period and during the three treatment phases.

Table 2

Average *V. fischeri* luminescence inhibition (INH) caused by the synthetic wastewater (influent) and effluent during the three treatment phases, with and without the introduction of CBZ, after 30 min of exposure.

Phase		Influent CBZ (mg/L)	INH (%)
I	Influent	2	16.1 ± 1.2
	Effluent	2	4.8 ± 1.0
		0	5.5 ± 1.4
II	Influent	5	22.9 ± 2.4
	Effluent	5	12.8 ± 2.9
		0	12.6 ± 1.9
III	Influent	8	32.8 ± 1.1
	Effluent	8	17.4 ± 2.3
		0	15.3 ± 2.8
Synthetic wastewater		0	15.9 ± 0.5

To implement AGS technology as an alternative to conventional wastewater treatment for the removal of PhACs and other emerging contaminants, it is also essential to assess how the presence of these pollutants affects the performance of the AGS system in terms of nutrient and organic load removal. That said, the removal of COD, PO₄-P and NH₄-N was monitored during the 3 phases of the CBZ treatment. Considering the dilution factor after anaerobic feeding, it was observed that during the first half of the treatment COD removal was affected by the presence of CBZ (Fig. 2a). However, from then onwards, the microorganisms responsible for degrading the organic load adapted and most of the influent COD began to be removed during the anaerobic feeding stage. Polyphosphate-accumulating organisms (PAOs) and glycogen-accumulating organisms (GAOs) can convert organic substrates, such as acetate, into intracellular storage polymers (PHAs) in the absence of oxygen. Since microorganisms belonging to *Flavobacterium*, *Thauera*, *Zoogloea* and *Pseudomonas*, which are known to be PAOs and GAOS, were abundantly detected in the CBZ granules (Fig. 7), it is likely that they contributed to the removal of COD during the anaerobic feeding stage. The remaining COD served as an easily degradable carbon

source for the growth of other heterotrophic microbial populations, which, in turn, were responsible for removing the remaining COD during the aeration stage [24]. Overall, despite the fluctuations observed in the influent COD and the initial destabilization during the feeding stage, the system was able to maintain an excellent COD removal efficiency (Fig. 2a). This observation is in line with other studies involving the removal of different pharmaceuticals using AGS [31,34,54].

Phosphorus removal was greatly affected by the presence of CBZ, with higher efficiencies being achieved when CBZ was present in the synthetic wastewater (Fig. 2b). During the anaerobic phase, PAOs release orthophosphate as they convert organic matter into PHAs. These PHAs are used as an energy source for PAOs to grow and form polyphosphate bonds during the aerobic phase, thereby removing the soluble orthophosphate [55]. It seems that, in our case, the presence of CBZ, which constitutes an extra carbon source, favored the metabolism of these slow-growing microorganisms and, ultimately, its abundance in the granular sludge (Fig. 7). Consequently, PO₄-P removal was enhanced in the presence of CBZ.

Until the 1st week of phase II, ammonium concentration after anaerobic feeding and in the effluent was greater than in the influent during the weeks with CBZ supply (Fig. 3a). During this period, the presence of CBZ inhibited the activity of slow-growing microorganisms responsible for nitrification (nitrifying bacteria), resulting in NH₄-N accumulation from the previous cycles. However, from that week onwards, nitrifying bacteria were able to adapt to the presence of CBZ, as NH₄-N removal reached 95 ± 4 % and remained constant until the end of the treatment. Additionally, the fact that the system was always able to maintain excellent NH₄-N removal in the absence of CBZ also suggests that nitrifying bacteria were continuously able to resume their metabolism immediately after the cessation of CBZ supply. These results show that, although the presence of CBZ had an initial adverse effect on the nitrifying bacteria, their long-term exposure to CBZ resulted in a gradual adaptation and recovery of their activity, enabling NH₄-N removal to be re-established. The gradual recovery of nitrifiers activity has also been observed in other studies aimed at removing PhACs using

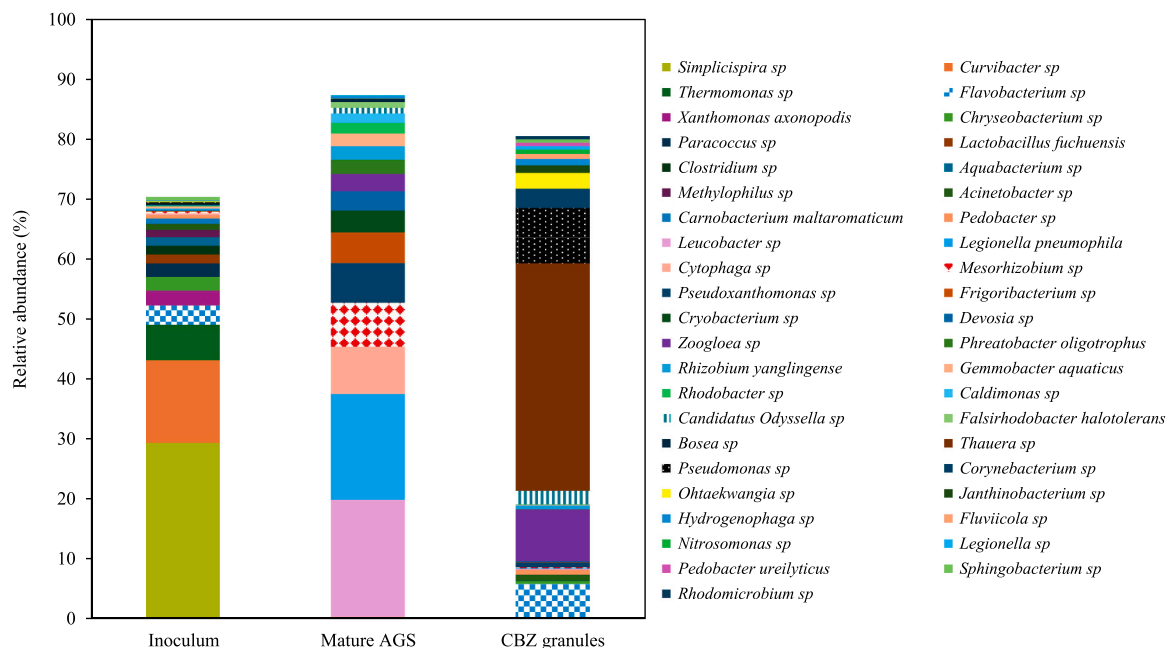


Fig. 7. Microorganisms with a relative abundance $\geq 0.5\%$ in the inoculum, mature granules (after acclimation period) and CBZ granules (after CBZ treatment).

Table 3

Number of species with CBZ-degrading enzymes of the three most abundant genera found in CBZ granules. Data were collected from UniProt database.

CBZ granules genus ⁽¹⁾	tax ID ⁽²⁾	Enzyme ⁽³⁾	# species ⁽⁴⁾
<i>Thauera</i>	33057	CYP450	40
		Laccase	1
		Aldehyde oxidase	2
<i>Pseudomonas</i>	286	CYP450	668
		Aldehyde oxidase	754
		Laccase	40
		Biphenyl dioxygenase	42
<i>Flavobacterium</i>	237	CYP450	128
		Aldehyde oxidase	44
		Laccase	1

AGS systems [24,56].

After nitrification, ammonium is oxidized to $\text{NO}_2\text{-N}$ by ammonia-oxidizing bacteria (AOB), which in turn is reduced to $\text{NO}_3\text{-N}$ by nitrite-oxidizing bacteria (NOB). Considering that $\text{NO}_2\text{-N}$ concentration in the effluent was greater than $\text{NO}_3\text{-N}$ (Fig. 3b), we can assume that the presence of CBZ favored the metabolism of AOB rather than NOB. The inhibitory effect on NOB was also observed by Amorim et al. [54] when treating simulated wastewater amended with a mixture of chiral pharmaceuticals in an AGS-SBR. Finally, since higher concentrations of $\text{NO}_3\text{-N}$ were obtained when CBZ was present in the system (Fig. 3b), it can be concluded that denitrifying bacteria were negatively affected by its presence. Denitrification inhibition in an AGS-SBR system under the presence of fluoxetine was also verified by Moreira et al. [56].

CBZ is known to be highly recalcitrant owing to its physicochemical properties [10,57,58]. On one hand, the presence of amides in the molecular structure makes CBZ resistant to biodegradation [59]. On the other hand, its removal through sorption onto activated sludge is quite low due to its moderate hydrophobicity [60,61]. In our study, it was expected that CBZ concentration inside the reactor after feeding would be half of what was found in the influent (considering the dilution factor). Instead, its concentration remained practically the same as what was found in the synthetic wastewater (data not shown), indicating that there was an accumulation of CBZ from the previous cycles. Consequently, CBZ concentration in the influent and effluent remained practically unchanged throughout the entire study, resulting in poor removal

efficiencies (Fig. 5). The accumulation of PhACs in AGS-SBR systems has already been reported by other authors [32,62], as well as low to negative CBZ removal efficiencies [27,34]. Muñoz-Palazon et al. [27] investigated the influence of temperature (6°C vs. 26°C) on the removal of various PhACs, namely diclofenac, naproxen, trimethoprim and CBZ at $25\ \mu\text{M}$. The authors found that while temperature progressively enhanced the removal capacity for all tested drugs, CBZ removal exhibited significant fluctuations and with negative efficiencies in both scenarios (-20% — 60%). They attributed these findings to the absorption-desorption process and the subsequent hydrolysis and conversion of CBZ's hydroxyl and carboxyl derivatives back into the original compound.

Bearing in mind this accumulative effect and the fact that CBZ was continuously supplied throughout the SBR cycles, it was also expected that, at the end of the same week, its concentration inside the reactor would be higher than at the beginning of the same week, which did not happen (data not shown). In addition, it was also expected that in the weeks in which CBZ dosage was suspended the accumulated CBZ would gradually be released in the effluent. Since this was not observed (Fig. 5), it is very likely that CBZ was partially attached to the biomass (bioadsorption) and/or biodegraded. The combined contributions of bioadsorption and biodegradation on CBZ removal using AGS have already been reported in previous studies [27,31]. For example, Kent et al. [31] reported that once the initial adsorption capacity of the AGS was saturated, CBZ removal was mostly through biodegradation (around 10%) during the anoxic feeding phase of the SBR and due to the layered nature of the granules.

The hypothesis that the AGS-SBR was able to remove CBZ to some extent is reinforced when we take into consideration the results obtained in the toxicity assays. Indeed, the developed system was able to reduce the INH of CBZ-contaminated wastewater to values similar to those found in the effluent when CBZ was not present in the influent (Table 2). For instance, in phase III, toxicity fell from 32.8% (contaminated influent) to just 17.4% (treated effluent), a value very close to that observed during the weeks without CBZ supply (15.3%). To the best of our knowledge, this is the first time the toxicity of the treated effluent using an AGS system to treat CBZ-contaminated wastewater has been reported. Given that no significant CBZ concentration was detected in the effluent during the weeks without CBZ supply (Fig. 5), it is possible that the metabolic products of the microorganisms contributed to the

slight toxicity observed during those weeks. Nevertheless, it should be emphasized that even these metabolic products did not present a relevant degree of toxicity, remaining below 20 % in all assays (Table 2).

EPS matrix plays a crucial role in AGS systems by maintaining the integrity of the granular structure and protecting microorganisms from toxic substances [63–65]. Therefore, monitoring its production is essential for ensuring a stable AGS process. Considering that LB-EPS are present in the outer layers of the granular sludge [66] and, consequently, have direct contact with toxic compounds, it is reasonable to have verified a sudden increase in LB-EPS production at the beginning of the CBZ treatment (phase I, 1st week), possible as a self-protection strategy by the microorganisms (Fig. 6). Several authors have observed the same response when studying the influence of toxic compounds on EPS production [63,67]. However, Fig. 6 also shows that increasing the concentration of CBZ did not lead to a proportional improvement in LB-EPS production. Melo et al. [48] pointed out that the decrease in LB-EPS could be attributed to the rise of larger aggregates. This may have been the case for our study since larger granules were predominant throughout the CBZ treatment (Fig. 4a). TB-EPS are located in the inner layers of the granules [66]. The fact that its concentration remained relatively constant until the beginning of phase III (Fig. 6) led us to believe that its content was somewhat protected from the toxic environment during this period. Only the 1st week of phase I showed a slightly negative impact on its production, most likely because it was the first shock from introducing CBZ into the reactor.

The composition of EPS (Fig. 6) demonstrates that they were mainly composed of proteins. It is known that PN content in EPS is substantially higher in systems under toxic conditions than under normal conditions [68] and is the primary EPS component that interacts with a number of drugs, including antibiotics, antiepileptics and anti-inflammatory agents [22,48,69]. PN has also been reported to stimulate microbial aggregation, thereby reinforcing the structural stability of granules by increasing cell surface hydrophobicity [70]. As illustrated in Fig. 4c, the solidity of the granules remained close to unity, indicating that the aggregates were well-compacted throughout the entire treatment. Therefore, we can conclude that enhanced production of PN allowed the integrity of the granular biomass to be maintained when facing the toxic environment caused by CBZ exposure. The slight oscillations observed in the PS content in the LB-EPS can be explained by the fact that PS is hydrophilic, which may limit water transport and, consequently, prevent cell dehydration. Hence, it is possible that microorganisms secreted PS to mitigate the damage caused by the presence of CBZ in the vicinity [71].

The presence of CBZ interfered immensely with the microbial dynamics of the granular biomass (Fig. 7). Some authors have observed similar results when treating CBZ under different biological processes [72–74]. *Thauera*, *Zoogloea* and *Flavobacterium* have been widely recognized for their ability to produce EPS and, thus, their role in the formation and stability of granular biomass [75–77]. The predominance of these bacteria genera at the end of the study indicates their tolerance to prolonged CBZ exposure, as well as their role in protecting the granular biomass and ensuring the system stability (Figs. 4 and 6).

Although *Thauera* and *Flavobacterium* have not yet been explicitly identified as significant contributors to CBZ removal, they have already been extensively studied in water treatment systems exposed to other drugs. For example, when assessing the potential impact of PhACs on the bacterial population within an AGS system, Amorim et al. [75] observed that *Flavobacterium* was the most abundant genus with a relative abundance of 50 % after pharmaceutical exposure. Tiwari et al. [78] also recorded the growth of *Flavobacterium* in the presence of several drugs, amongst which CBZ. *Thauera* spp. were significantly enriched after Yu et al. [79] added sulfamethoxazole to an AGS reactor with low C/N influent. They attributed this increase to the fact that *Thauera* spp. are capable of degrading complex aromatic compounds like sulfamethoxazole; note that CBZ is also an aromatic compound drug. Cui et al. [80] and Yan et al. [20] also got similar results when treating

sulfamethoxazole and tetracycline, respectively.

Pseudomonas have been recognized as a viable option for CBZ removal in biological systems. For instance, Li et al. [81] isolated *Pseudomonas* sp. strain CBZ-4 from municipal wastewater and achieved a 46.6 % removal rate after 144 h under optimal conditions (pH = 7.0 at 10 °C), using CBZ as the sole carbon source. Dwivedi et al. [82] also identified another strain, *Pseudomonas* sp. KSH-1, as a potential microorganism capable of degrading CBZ and Nguyen et al. [30] demonstrated that the integration of a peroxymonosulfate-based advanced oxidation process with a biological process involving *Pseudomonas jinjuensis* synergistically removed CBZ from groundwater. In their study, the biological process reduced CBZ from 10 µM to 1.3 µM under aerobic conditions and 1.7 µM under anoxic conditions (around 83.6 % removal).

In light of CBZ's recalcitrant nature, the engineering of a bacterial consortium that is resistant to CBZ toxicity and, at the same, is capable of degrading it, could significantly enhance the biological treatment of wastewater contaminated with this drug. To date, only a few studies have reported improved CBZ removal, yet all of them have used pure cultures, including *Starkeya* sp. C11 and *Rhizobium* sp. C12 [83], *Labrys portucalensis* F11 [84], *Gordonia polyophrenivorans* [85] and *Streptomyces* MIUG 4.89 [86]. Unlike the use of pure cultures, the incorporation of AGS systems into existing WWTPs is feasible. Although further studies are needed to prove and consolidate the involvement of *Thauera*, *Flavobacterium* and *Pseudomonas* in CBZ biodegradation, the findings of this study have demonstrated the potential and resilience of AGS technology in treating CBZ-contaminated water. Applying isolation strategies and confirming the metabolic activity of these species could thus be a starting point for the subsequent optimization of biological systems aimed at removing CBZ through the bioaugmentation of these strains

5. Conclusions

The biological treatment of pharmaceutical-contaminated wastewater remains a significant challenge, particularly in the case of recalcitrant compounds such as CBZ, which exhibit high resistance to biodegradation and limited bioadsorption. This study assessed the performance and stability of an AGS-SBR system under prolonged CBZ exposure and demonstrated its notable resilience, supported by the formation of stable and robust granular biomass. The structural integrity of the granules and EPS production played a central role in protecting the microbial community and maintaining essential nutrient removal processes.

Although CBZ removal efficiency remained limited, the developed AGS-SBR system was able to effectively detoxify the contaminated wastewater, evidencing for the first time the detoxification potential of this technology in the context of recalcitrant pharmaceuticals. The dominance of bacterial genera *Thauera*, *Pseudomonas*, and *Flavobacterium* at the end of the operational period contributed to biomass stability, likely through EPS synthesis. Moreover, in silico analysis confirmed the presence of CBZ-degrading enzymes within their predicted proteomes, reinforcing their potential role in system resilience.

It should be noted that the experimental design included a drug withdrawal phase (CBZ concentration = 0), which may have facilitated microbial recovery and thus influenced the observed system resilience. Therefore, while the results highlight the robustness of AGS system under CBZ stress, further studies under uninterrupted CBZ exposure are required to confirm long-term microbial adaptation and tolerance.

Given the inherent difficulties associated with the biological remediation of recalcitrant drugs, future research should focus on enhancing treatment efficiency through targeted bioaugmentation strategies, incorporating microbial strains with proven capabilities for biomass stabilization and CBZ degradation. In this regard, *Thauera*, *Pseudomonas*, and *Flavobacterium* emerge as promising candidates for the development of more effective AGS-based technologies for the treatment of CBZ-contaminated wastewater.

CRediT authorship contribution statement

Mariana Costa: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Cristina Quintelas:** Writing – review & editing, Supervision, Investigation, Formal analysis, Conceptualization. **Campos Ferreira Eugénio:** Writing – review & editing, Supervision, Funding acquisition. **Silva Ana Rita:** Writing – review & editing, Investigation, Formal analysis. **Salvador Andreia:** Writing – review & editing, Investigation, Formal analysis. **Mesquita Daniela:** Writing – review & editing, Investigation, Formal analysis.

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. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jece.2025.118202](https://doi.org/10.1016/j.jece.2025.118202).

Data Availability

No data was used for the research described in the article.

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