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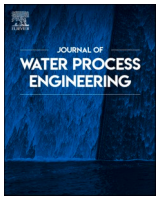

Treatment of hospital wastewater using aerobic granular sludge technology: Removal performance and microbial dynamics

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ABSTRACT

Hospital wastewater contains several contaminants of emerging concern that cannot be removed by conventional treatment processes. Many of these emerging contaminants are pharmaceutical compounds, which are found in hospital wastewater at high loads. The presence of these toxics affects to the performance of biological processes in receiving wastewater treatment plants. This research evaluated the capability of the aerobic granular sludge technology to remove pharmaceutical compounds from hospital wastewater in a single chamber, which to date has not been investigated with real hospital wastewater. Despite the high non-biodegradable organic matter content, COD and BOD_5 removal reached 75 % and 100 %, respectively. Nitrogen removal ranged from 70 %–90 %, and phosphate removal was maximum 50 %. The technology was able to efficiently remove antibiotics, antiepileptic and antidepressant drugs, whereas non-steroidal anti-inflammatory drugs were removed and released under oscillating patterns. The granular biomass increased in size, but it reduced the settling velocity. Bacterial and fungal communities were acclimated to pharmaceutical inlet, whereas the archaeal population had a progressive adaptation over time. The aerobic granular sludge technology is therefore a viable approach to enhance the disposal of real hospital wastewater prior to discharge into the urban wastewater network.

1. Introduction

Hospital wastewater (HWW) is a growing concern because it is constituted by toxic compounds such as pharmaceuticals (PhCs), heavy metals, and infectious organisms that, when released into the environment, pose serious environmental and human health risks [1]. HWW also contains high concentrations of ammonia, total nitrogen, total suspended solids and organic matter with a low biodegradability based on COD/BOD_5 ratio. HWW is characterized by the presence of hazardous materials for the prevention and diagnosis of diseases [2], such as antibiotics, antidepressants, analgesics, antiepileptics, antineoplastics, estrogens, radionuclides, metals, anaesthetics, lipid regulators, antipyretics and solvents [3]. Usually, HWW discharges into the urban wastewater (UWW) networks and both are co-treated in wastewater treatment plants (WWTPs), which could disturb the balance of physicochemical and microbial performance, due to the intrinsic toxicity of HWW is 10 to 100 times higher than UWW [4,5]. The conventional processes for UWW are not specially designed for removing pharmaceuticals, metals, or pathogens. Thus, hazardous material would be released into the environment through wastewater treatment discharges when HWW are co-treated with UWW in municipal WWTPs [6].

Some legal regulations have been established to define the management and treatment of hospital effluent before its disposal [7], because reducing the toxicity of HWW is an optimal consideration first to discharge to the urban sewage to meet the revised directive concerning to emerging contaminants in urban wastewater (2022).

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A deeper knowledge of the conventional parameters, chemical characterisation, microbiological load, and seasonal variation would allow the adoption and selection of the technical treatment train effective concerning raw water [5,7]. In this way, technologies for HWW treatment have been gaining attention [8]. Alternatives to conventional activated sludge (CAS) such as photocatalytic oxidation, electrochemical oxidation, advanced oxidation, and membrane technologies are suitable for achieving high-quality effluents [1,9]. Advanced oxidations are the leading studied technologies to reduce the PhCs in the HWW, for instance, ozonization can remove >90 % of PhCs [1,10]. Fenton and photo-Fenton systems, which could be applied to polishing post-treatment, reached to remove 90 % but this technology is scarcely explored for real HWW [9]. Only a few studies employed photocatalytic systems for treating real HWW, but Konstas et al. [11] pointed out removal ratios close to 80 % for longer exposition (simulating summer), and in the range of 42–57 % for shorter exposition (simulating winter). Other technologies such as membrane bioreactors are suitable alternatives for treating HWW with PhCs removal values higher than 80 %, but some of the most recalcitrant compounds remain in the effluent and the clogging of pores reduces the efficiency [12,13]. In most cases, these technologies are expensive, with high energy and cost requirements, which are not feasible to implement in the hospital vicinity prior to discharge into the UWW network [14], therefore it is difficult to achieve successful implementation [15].

Aerobic granular sludge (AGS) technology is postulated as a technology that enhances the robustness of a biological system for treating pollutants in comparison with CAS due to its overwhelming benefits [16]. AGS technology solves a few of these aspects with respect the advanced oxidations, such as the reduction of 20 % in space requirements compared with the CAS, a 20 %–25 % reduction in operation costs and 24 %–40 % lower electricity requirements [17]. This technology is based on large spheres of microorganisms embedded in a tridimensional matrix, which allows the co-existence of aerobic, anoxic and anaerobic niches and, consequently enhances the simultaneous removal of nitrogen, phosphorus and organic matter [14,18]. The high biomass retention and the long sludge retention time promote the growth of specialist microorganisms with metabolic pathways for the degradation of toxic compounds in raw wastewater [19–21]. Also, the strong biofilm conformed in the tridimensional matrix promotes resistance against the stress of toxic compounds, because toxicity does not affect the single cells, providing an attractive advantage facing changes in loads and the diversity of pollutants [22,23].

Here, we comprehensively profile the physicochemical performance of the AGS technology for the treatment of real HWW over a period of 150 days. We analysed the capability of the system to degrade pharmaceuticals and common pollutants (COD, N and P), describing the granular dynamics from a physical and microbial points of view. Overall, this study provides an economically viable and environmentally sustainable solution in one step, which could be implemented in the hospital vicinity before its disposal into the UWW network to reduce the effects on urban WWTPs and avoiding damage to humans, animals and ecosystems.

2. Material and methods

2.1. Start-up

An AGS system in a sequential batch reactor (SBR) was operated in a cylindrical column with a height of 90 cm, an inner diameter of 7 cm and an operational volume of 3 L. The air diffusor was at the bottom of the reactor, with an airflow of 4.0 $L{\cdot}min^{-1}$ using fine bubbles. The pH, temperature and oxygen probes were located at 0.35 m from the bottom. The pH was in the range of 7.6 to 7.9, temperature was 16 ± 0.7 °C and dissolved oxygen was 9.1 ± 0.4 mg $O_2{\cdot}L^{-1}$, keeping close to saturation. The effluent output exchanged 50 % of the water volume. The batch cycles were as follows: filling with raw water (4 min), aeration (170 min), decanting stage (3 min) and effluent discarding (3 min). Each cycle lasted for 3 h, and the hydraulic retention time (HRT) was 6 h. The feeding of the reactor was performed with a peristaltic pump from the top (Watson Marlow, United Kingdom). The influent was raw water from the sewerage network of pipes from a hospital in Granada (Spain), collected once a week during the same temporal window (every Wednesday from 8.00 to 9.00 a.m.). For the inoculation of the bioreactor, 1 L of granules cultivated were used, which were fed with synthetic wastewater simulating UWW following the solution described by Rosa-Masegosa et al. [24]. Granules were operated in the lab-scale reactor with synthetic wastewater until achieving high-performance removal for COD, BOD_5, N and P with values of 90 %, 94 %, 85 %, and 60 %, at 22 days. For 14 days the reactor was operated to ensure the stability of the biomass before feeding it with real HWW.

2.2. Physicochemical analysis

The granular sludge was analysed through mean size and settling time. The mean granular size was measured using a scalimeter with a representative number of samples ($n = 35 \pm 5$ pieces) taken during the aeration period [25]. The settling velocity of the granules was measured using a 50-cm glass column with a manual chronometer, following the protocol described by Hurtado-Martinez et al. [26]. Biomass concentration (MLSS) measurements in the bioreactor were performed according to the Standard Methods for the Examination of Water and Wastewater [27]. COD and BOD_5 were determined in duplicate in the influent and effluent [27]. Nitrogen and phosphorous ion concentrations (NH_4^+, NO_3^-, NO_2^-, PO_3^{4-}) were analysed by ion chromatography (Metrohm Ion Chromatograph, AG, Switzerland) [28].

2.3. Pharmaceutical compound extraction and quantification

Concentrations of PhCs were determined in triplicate [29]. Briefly, solid phase extraction (SPE) was employed as the pre-treatment method (HLB cartridges Oasis, 200 mg). Conditioning of the HLB cartridges was performed using 8 mL of methanol and 8 mL of Milli-Q water. Subsequently, the samples (100 mL) were passed through the cartridges, and the cartridges were washed with Milli-Q water (10 mL) and dried in air. The final extract elution was evaporated under nitrogen stream and reconstituted with 2 mL of methanol–water in v/v 10:90 [30]. Samples were kept at −20 °C until their processed within 2 months after.

The chromatographic separation was done with ultra-high performance liquid chromatography equipment (UHPLC 1260 Infinity II, Agilent), equipped with a Zorbax Eclipse plus C18 Column (3 × 50 mm - 1.8 μm particle size; Agilent) under positive electrospray ionization for all compounds, except for diclofenac (DCL), naproxen (NXP) and triclosan (TRC) that was under negative ionization [31]. The UHPLC was coupled in tandem with a triple quadrupole-QqQ 6470 LC/TQ (Agilent) with a quaternary UHPLC pump using gas flow of 0.4 mL/min at 350 °C. Data acquisition, qualitative analysis, quantitative analysis, mass spectral library management, and reports were calculated using MassHunter software (Agilent).

In previous studies, pharmaceuticals were selected due to their high-risk quotient, correlation to the study area or ecological risk [2,13,32]. For this study, seven drugs belonging to different therapeutic classes were selected based on their high risks: Antibiotic: trimethoprim (TMP); antidepressant: carbamazepine (CMP); antitumoral compounds: cyclophosphamide (CCLP) and TRC; anti-inflammatory compounds: ketoprofen (KTP), DCL and NPX [2,22]. Chemical solutions were also prepared as standard curves supplemented with 0.1 % EDTA using different concentrations range of TMP (0.05–15 μg mL^{-1}), CMP (0.05–50 μg mL^{-1}), CCLP (0.05–15 μg mL^{-1}), TRC (0.05–15 μg mL^{-1}), KTP (0.5–500 μg mL^{-1}) DLC (1–200 μg mL^{-1}) and NPX (3–500 μg mL^{-1}), which covered the concentrations measured in 10 random samples from the raw HWW and treated water measurements prior to proceed with analysis.

2.4. Nucleic acid extraction and massive parallel sequencing

The granular biomass samples were collected, once the reactor was stable, at Days 0, 3, 7, 15, 30, 60, 90, 120, 140 and 150. Representative granule samples were taken, submerged in a saline solution (0.9 % NaCl) and centrifuged at 5000 rpm for 20 min at 4 °C. Subsequently, the supernatants were discarded, and the pellets were stored at −20 °C. The nucleic acids were extracted in duplicate using the FastDNA SPIN Kit for Soil (MP Biomedicals, USA).

Next-generation sequencing (NGS) was carried out using the platform MiSeq Illumina of the duplicate DNA extracted. The duplicated DNA samples were amplified for the following primer pairs: Bacteria807F (5′-GGATTAGATACCCBRGTAGTC-3′) and Bacteria1050R (5′-TAGYTGDCGACRRCCRTGCA-3′) for the amplification of V5–V6 hypervariable regions of 16S rRNA of *Bacteria*, ITS1S (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS2S (5'-GCTGCGTTCTTCATCGATG-3') for *Fungi*. For the amplification of the 16S rRNA of *Archaea*, the primers 519SGF- (5'-CAGCMGCCGCGGTAA-3') and 907SGR (5'-CCCCGTCAATTCCTTTGAGTTT-3') were used [33].

2.5. Bioinformatic data curation

The next-generation sequences were subjected to a quality trimming analysis to keep the high-quality reads, using the open-access software MOTHUR [34]. For that, forward and reverse contigs were assembled with quality scores of a mismatched base equal to 0. Then, reads with more than eight homopolymers or any ambiguous base were removed, and the remaining reads of *Archaea* and *Bacteria* that did not align properly on the forward and reverse positions against the SILVA seed database were eliminated using the Needleman conditions. For all targets, chimerical reads were detected and removed using the VSEARCH algorithm, and reads that belonged to target genes different from the focus were discarded [22]. The clusters of sequences were calculated using the abundance-based greedy method with a similarity of 97 % for *Bacteria* and 95 % for *Fungi* and *Archaea*. The operational taxonomic units (OTUs) results of taxonomic consensus were affiliated with SILVA_nr data for *Archaea* and *Bacteria* and UNITE for *Fungi*. Finally, singleton OTUs were eliminated to build the OTU tables. For the construction of community maps, it was used the average abundance of duplicates for each sample day and target gene.

2.6. Real-time PCR

Real-time PCR (qPCR) assays were performed using extracted DNA from biological samples in Quant Studio™ 3 Real Time PCR Systems. *Bacteria*, *Archaea* and *Fungi* were quantified using diagnostic 16S rRNA genes and 18S rRNA with primers 341 F–534 R [35], ARCH915R-UNI-brevF [36] and FungiQuantF-FungiQuantR [37]. Genes involved in ammonia oxidation and nitrate denitrification were targeted with *amoA* and *nosZ* genes, respectively [38,39]. For phosphate-accumulating organisms, 16S rRNA of *Candidatus* Accumulibacter was used as a proxy for polyphosphate-accumulating organisms (PAOs) [40].

The reaction mixture of 25 μL was composed as follows: 19.36 μL DEPC sterile water, 2.5 μL buffer with $MgCl_2$, 0.5 μL dNTPs (8 mM), 0.15 μL of each primer, 0.125 μL Taq polymerase, 0.125 μL SYBR Green (x20) and 0.0625 μL BSA (BioLabs) [18]. The primer pairs and annealing conditions are described by Correa-Galeote et al. [40] and Muñoz-Palazon et al. [41]. Raw data were processed using the proprietary QS3 software (Applied Biosystems, Thermo Fisher Scientific). Gene quantification was normalised both by biomass and the volume eluted of nucleic acids.

2.7. Statistical analysis of diversity, similarity and multivariate

The α-diversity of archaeal, bacterial and fungal populations was calculated using the software PAST v. 4.09, applying the OTU table considering the indices of Chao-1, Shannon-Wiener, Simpson, Pielou's evenness and Berger-Parker, which were calculated with a 97 % confidence range by 999 bootstrap replications. The Whittaker index was performed to capture the β-diversity of all phylotypes among pairs of samples, using the OTU table in the PAST v. 4.09 software.

A PERMANOVA was performed to evaluate if the studied parameters were significantly related to the absolute quantification of genes, the physicochemical performance, the presence of pharmaceuticals and the dominant OTUs. The PERMANOVA was computed using PAST v. 3 with Bray–Curtis distance and under 9999 permutations.

The reads of the OTU table sequence samples were used for the calculation of the trees of *Archaea*, *Bacteria* and *Fungi* communities throughout the operation period. The phylogenetic tree was built by hierarchical clustering, using under 9999 bootstrap replications following the Bray–Curtis model and using the PAST software v. 3.4. [26].

Principal components analysis (PCA) was calculated using the R project v. 4.2.1 software and CoDaPack. Raw data were pre-treated as follows: a) correction to avoid zero values, b) transformation to the centred logarithm and clustering the compositional data of the OTU table of the microbial dynamics by distance distribution. To complement these results, a SIMPER analysis was done to select the phylotypes that contributed to dissimilarities between samples before and after steady-state (supported by PERMANOVA analysis), using the PAST software with dissimilarities with the Bray–Curtis distance.

Linkages among archaeal, bacterial and fungal dominant phylotypes and physicochemical characterisation were analysed using multivariate redundancy analysis (RDA). Linkage was determined using the centred logarithm of each parameter computed using 499 unconstrained Monte–Carlo simulations under a full permutation model, applying Canoco 4.5 for Windows [28].

3. Results and discussion

3.1. Effects of the treatment of hospital wastes in sewage on the performance of AGS

This experiment was carried out for 150 days, and the obtained results of the physicochemical analyses highlighted two removal phases (Fig. 1). The existence of two different phases was corroborated by one-way PERMANOVA (Table S1). Between these two stages, nitrogen, phosphate, COD and BOD_5 removal and the settling velocity of granules were statistically significant ($p < 0.05$), whereas mean granule size was not statistically significant ($p > 0.05$) . Based on these results, the experiment was studied into two phases: start-up and acclimatization (until Day 37) and steady-state (from Day 38 to Day 150).

The hospital wastewater characteristics were highly variable over operational time, as it is reported in other studies carried out with real HWW [5,13,42]. Organic matter, measured by BOD_5 and COD, was monitored throughout the operation period. The COD values oscillated, with an average value of 514 $mgO_2 \cdot L^{-1}$ and values of standard deviation of 391.83 mg $O_2 \cdot L^{-1}$, caused by the COD peak concentration of up to 2100 mg $O_2 \cdot L^{-1}$, but these trends are common values in the HWW inlet [8]. During the start-up phase, the COD removal ratio showed no obvious pattern, and the variability in the removal percentage was not strictly linked with the COD concentration in the influent. These results could be caused by the acclimatization process to the nature wastewater with a high load of pollutants and microbial loads, as well as by the biodegradability of wastewater. From Day 38, COD removal ratios were higher than 50 %, but during most operation the ratio was higher than 75 %. The COD removal ratio was strongly linked with the content of non-biodegradable matter, which corroborated with the mean COD/BOD_5 ratio of 2.77 ± 1.70, demonstrating the low degradability of HWW as reported Bhandari et al. [43]. Fig. 1 showed the BOD_5 (biodegradable organic matter) influent concentration, which was in the range of 100 to 300 mg $O_2 \cdot L^{-1}$; the effluent concentration in the steady-state was lower

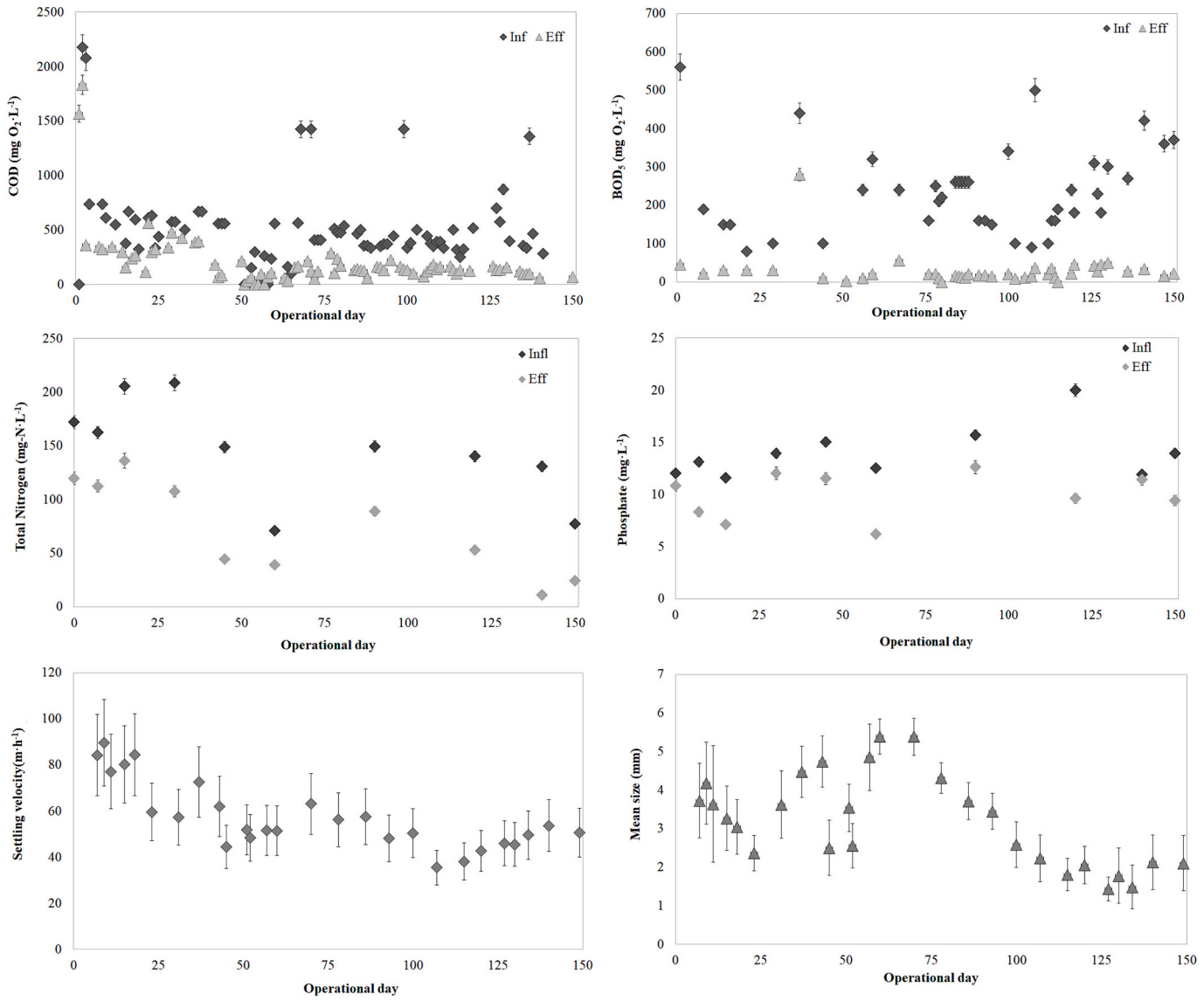

Fig. 1. Pollutant removal and AGS characterisation: COD (a); BOD_5 (b); total nitrogen (c); phosphate (d) in mg·L^{-1} in influent and effluent; mean size (e) and settling velocity (f) of granular biomass.

than 25 mgO_2·L^{-1} throughout the experimental period, achieving a removal rate close to 100 % (Supplementary Material).

The nitrogen influent values were in the range of 130 to 170 mg-N·L^{-1}, but values higher than 200 and lower than 70 mg-N·L^{-1} were also detected (Fig. 1). The removal ratio was close to 70 %, although it was imperative to note that ammonium oxidation was close to 90 % (Fig. S1). The mean phosphate concentration in the influent was 13.74 ± 2.69 mg PO_3^{4-}·L^{-1}, whereas that in the effluent ranged from 12 to 6 mg·L^{-1}. The removal ratios were not competitive during the start-up phase, and in the steady-state achieved maximum performance of 50 % (Fig. S1), obtaining higher efficiency under low N:P ratio. The N:P ratio largely oscillated throughout the experimental period, with values in the range of 3.52 to 17.70 and an average of 11.04 ± 4.44.

Regarding the COD:N ratio, the best performance was obtained with values close to 3.6:1, resulting in a COD removal close to 80 % and nitrogen removal in the range of 62 % to 70 %. This COD:N ratio was lower than that described by Hamza et al. [44], who used synthetic wastewater, but the performance met the requirements of the European legislation, even at high pharmaceutical and microbial loads. Nivedhita et al. [45] showed in their research as a single antibiotic had a negative impact on the removal performance in comparison with a control bioreactor operated without PhCs. To date, this is the first research that treats real HWW with AGS for testing the technology, and considering the obtained results, AGS system could reduce substantially nutrients load in presence of toxic compounds from HWW.

From the physical point of view of granules, HWW had impact on the mean size and settling velocity (Fig. 1). The mean granular size reached the maximum after the stabilization (Day 55). Nevertheless, from Day 75 onward, granular size progressively decreased with diameter close to 2 mm, slightly larger than the granules described by Nivedhita et al. [45] in the presence of oxytetracycline. In this case, smaller sizes of granules had advantages such as an increase in the specific surface area in contact with the pollutants, and the granules were more compact and smoother than those used as inoculum. Granule color changed from yellow to brown, probably because of the organoleptic characteristics of the real HWW, because previously granules were cultivated with synthetic wastewater. It is important to mention that despite the changes in mean size, the PERMANOVA results showed no statistically significant effect on the two phases ($p > 0.05$). The settling velocity decreased progressively from start-up until Day 40, whose values remained in the range of 40 to 60 m·h^{-1}.

It has been suggested that PhCs and personal care products (PPCPs)

could improve the settling capacity of granules [22,46]. However, in this study, the aerobic treatment of real HWW did not enhance the settling performance of the granular sludge, and the results observed in previous studies could be explained by the controlled conditions [22,46].

3.2. Occurrence of pharmaceutical compounds in urban wastewater and after AGS treatment

Seven pharmaceutical compounds were analysed in influent and effluent samples. The values were significantly higher than those observed for influent and effluent samples of urban WWTPs due to the hospital origin (Fig. 2).

Cyclophosphamide is a widely used drug in breast cancer therapy and one of the oldest and most widely prescribed alkylating cytostatic medicine [47]. It has previously been observed at a concentration range of 0.014–22 $\mu g \cdot L^{-1}$ in European HWW. In this study, the concentration of CCLP was even lower ranging from 0.02 to 0.25 $\mu g \cdot L^{-1}$ in the raw wastewater, and the removal rate was 100 % for the entire experimental period (Fig. S2).

TRC has been frequently detected in aquatic ecosystems and potentially damages various organisms; its removal has therefore attracted considerable interest. In municipal and HWW, TRC has been detected in the range of 0.07–14,000 $\mu g \cdot L^{-1}$ [48]. In this study the triclosan concentration in HWW was <0.2 $\mu g \cdot L^{1}$ throughout the experimental period. However, at Day 15, the removal performance was negative, it could be caused by bioaccumulation or release processes, whereas the average removal ratio was 60 %. These rates are comparable with those reported before, ranging from 35 % to 69 % [48–50]. The variations in the TRC concentration in the HWW discharged can be linked to seasonal changes as TRC is largely used in personal care and biocidal products [50] .

TMP is one of the most used antibiotics worldwide, and its concentration in UWW depends on several factors. In this study, the TMP variation varied from 0.21 to 1240 $\mu g \cdot L^{1}$ in the HWW discharged, but there was no seasonal pattern. The obtained results indicated that TMP was efficiently removed using the AGS technology, with a removal rate from 80 % to 99 %, but the results suggested possible release processes at Days 90 and 150. These results pointed out the ability of AGS to remove high loads of this pollutant, such as at Days 30 and 60, when the concentrations in the influent and effluent were 960–1240 $\mu g \cdot L^{1}$ and 0.74–1.11 $\mu g \cdot L^{1}$, respectively. The values of this matrix were higher than those usually reported, such as 6.6–7-7 $\mu g \cdot L^{1}$ [51,52]. Moreover, the AGS technology has previously been described as a potential technology for TMP degradation [22,32].

The reactor degraded 50 % of the CBP during the experimental period and in almost 100 % on the 1/4 of the experimental time. The CPB removal ranged from 52 %–74 %, although on Days 45 and 90, removal was negative. During these days, the CBP concentration of the influence was lowest (<1 $\mu g \cdot L^{1}$), and the concentration in the effluent did not exceed 2.5 $\mu g \cdot L^{1}$. These results demonstrate the ability of AGS to significantly reduce the high loads emitted by hospital wastewater.

The NPX removal had oscillated values because of influent concentration and possible removal-release processes. At the beginning, the removal rate ranged from 44 %–99 %, whereas the system released more NPX on Days 90 and 120, when the granules were long time exposed to PhCs, previously reported by Bessa et al. [53]. The same pattern followed the diclofenac discharged by the hospital, revealing oscillating concentrations from 0.29 to 1800 $\mu g \cdot L^{-1}$ over the experimental period. The removal performance was highly efficient under high influent loads,

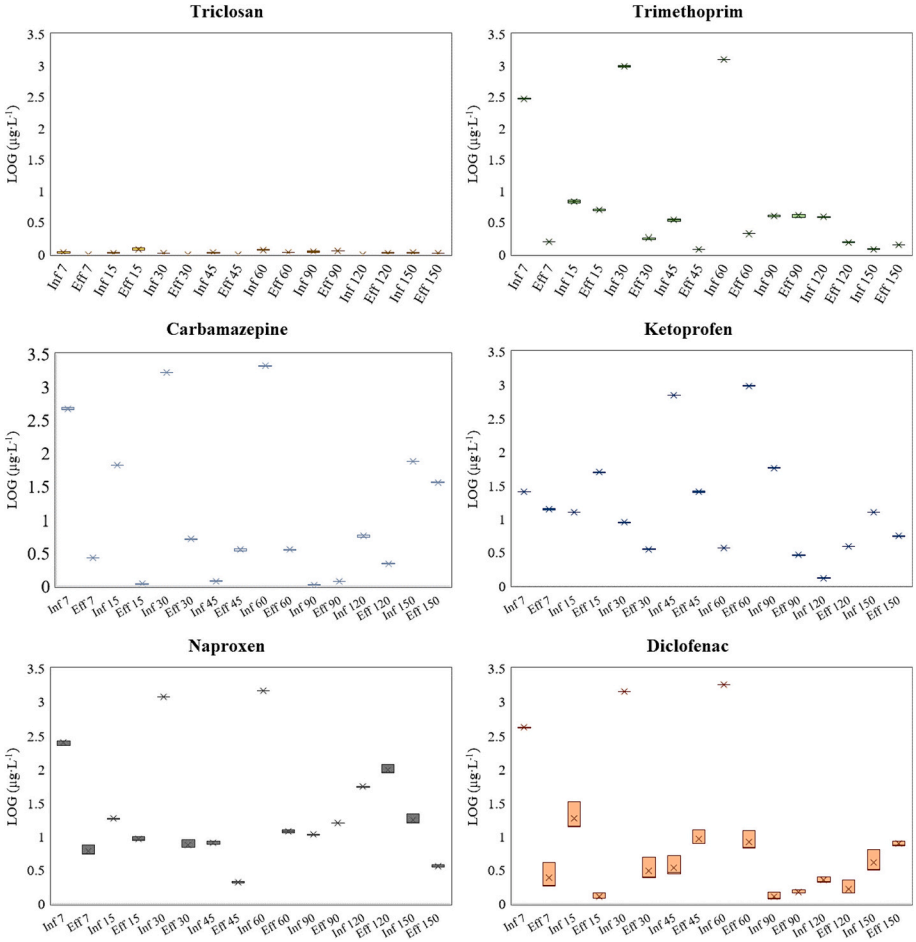

Fig. 2. Performance of treating the influent and effluent of hospital wastewater measured in triplicate in key days.

but the positive-negative rate of DCL removal could be linked to the desorption process as reported Bessa et al. [53].

KTP was the most recalcitrant compound, although the system was able to remove average values of 50 % of the KTP concentration following the results reported Arcanjo et al. [54]. In fact, the results of this research showed a negative removal rate of −320 %, could be caused by the accumulation over long time as pointed out Arcanjo et al. [54]. It is well known KTP can adsorb on suspended solids and sludge during wastewater treatment, and it has the highest tendency to attach onto particles among the studied compounds such as CMP or DCL as corroborated by many authors [55,56]. Revisions about the issues derived from KTP highlighted that full-model of the adsorption of this compound is still lacking, the best option to remove KTP from the aqueous phase is absorption processes [57].

Based on these results, the surface of granular sludge could promote the bioadsorption-desorption processes, especially in terms of nonsteroidal anti-inflammatory drugs (NSAIDs) such as NPX, DCL and KTP [2,22,55,56]. AGS could be postulated as a technology able to remove more efficiently PhCs than CAS in a single stage, which could be implemented as pre-treated to reduce the negative impact of the toxicity prior to disposal in the UWW networks [30].

3.3. Absolute quantification of microbial population dynamics

The absolute quantification of target genes showed a trend led by the drug concentration entering the system (Fig. 3). The 16S rRNA levels of *Bacteria* and *Archaea* were in the range of 10^{10} to 10^{12} and 10^{7} to 10^{8} magnitude orders, respectively, until Day 60. The highest loads of TMP, CPB, NPX and DCL were detected on Day 60; subsequently, the numbers of bacterial and archaeal 16S rRNA genes abruptly decreased by 2–3 magnitude orders, followed by a later recovery prior to the shock. These results suggest that the high loads affected the bacterial and archaeal populations, but the robustness of the microbial population in the granules allowed to retrieve the damage caused by the PhCs compounds due to the different mechanisms of resilience in the microbial community in a temporal line [58]. This effect was even stronger in communities of fungal and phosphate-accumulating organisms (PAO), with a total disappearance at Day 120. It is a novel picture of the most prolonged effect in time that has the high load of drugs in the PAO and fungal communities, as reported Huang et al. [59], which follows the trajectories of complete recovery because of ecosystem succession that promote return to the original state [58].

Granules are comparable to biofilms with several niches, and generally, PAO and fungal communities are contained in the interlayer zones and the core, respectively [18,21] . The spatial situation of PAO in the anoxic zone could exert a buffer effect in these communities since many authors describe how AGS systems have advantages since the toxic compounds do not reach individual cells but are distributed in all consecutive cells [40,60]. In the present study, the deeper localisation of these microorganisms facilitated their presence until Day 90. However, the PAO suffered in the later stages, when the PhCs had passed through the external layers of the granules to the interlayer, because the PAO organisms are sensible when they are directly exposed to PhCs [45,59]. The fungal population has been described as an essential part of the microbial community in biological wastewater treatment, with the ability to transform and degrade PhCs, but sometimes the number of fungal copies are below of detection limit using a specific primer set for *Fungi* [31,61,62]. The number of gene copies of PAO and fungi increased at Day 150 to values similar to those before the shock. Although denitrifiers, analysed by the *nosZ* gene, were also affected over long exposition, the copy number was only slightly affected. The *amoA* gene was not negatively impacted by the drug load in the influent, in contrast to the remaining functional genes. The number of copies trended to increase progressively over time, albeit with large fluctuations.

3.4. Dynamics of the microbial population in granules

3.4.1. Diversity of the archaeal population

Archaea are microorganisms with great ability to colonize the majority of environments in nature, but they have been less studied due to the the lack of knowledge about their metabolisms [63]. In biological wastewater, they play an essential role in the degradation of pollutants such as nitrogen, phosphate, aromatic hydrocarbons, chlorinated compounds, sulfur compounds, and heavy metals, among others. The archaeal population in the granular sludge treating real hospital wastewater was more diverse compared to previous findings obtained

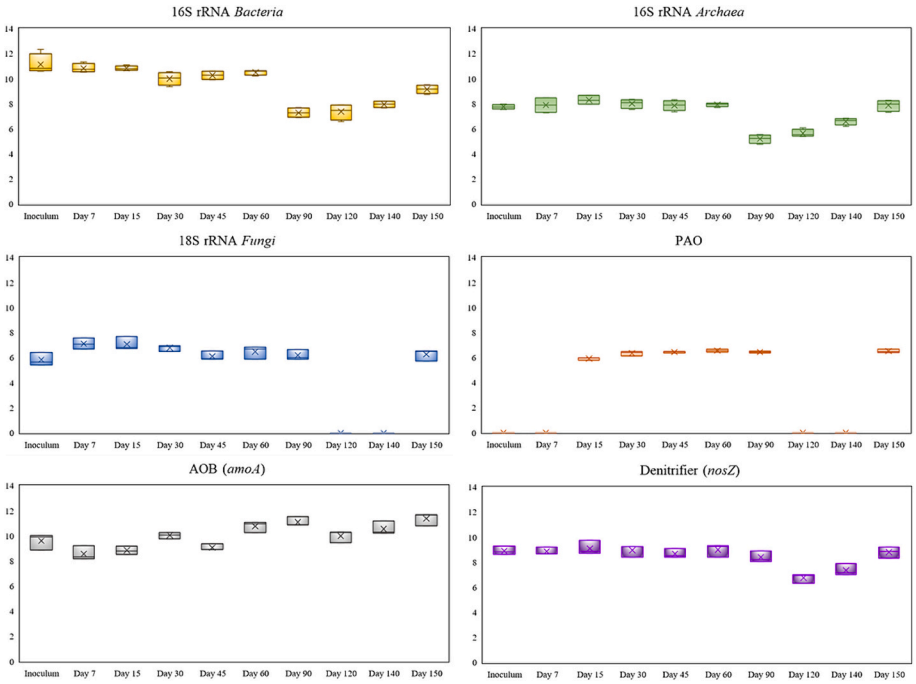

Fig. 3. Gene copy numbers (Log No. of copies/g of granules) determined by quantitative PCR in granular sludge treating hospital wastewater.

via NGS [25]. Overall, 21 OTUs were represented by a relative abundance of >1.5 % (Fig. 4). In the granules employed as inoculum, the most dominant phylotypes were *Methanocaldococcus* and an unclassified *Methanococcales* order, both accounting for >20 % of the total inoculum. Three OTUs belonged to unclassified *Bathyarchaeota*, which played an important role in the inoculum. The archaeal community, with great diversity and evenness, changed slightly until Day 15, with slight modifications related to the increase of an Otu01 belonging to *Bathyarchaeota* and Otu04 affiliated to *Methanospirillum*. However, a remarkable change in the population was observed via hierarchic clustering, which showed significative modifications before and after Day 30. From Day 30 to the end of the experimental period, the role played by *Methanocaldococcus* was greatly reinforced, reaching 50 % of the total relative abundance. Following the trend observed for the qPCR results, at Day 90, the community was dominated by three OTUs, two of which belonged to an unclassified *Bathyarchaeota* and *Methanocaldoccus*, possibly caused by the high drug loads in HWW. The proliferation of OTUs belonging to *Thermoprotei*, *Methanospirillum*, *Methanococcales* and *Halobacteriaceae* increased the diversity of phylotypes in the population dynamics. These results are interesting because the common perspective excludes the domain *Archaea* from biotechnological approaches except in anaerobic digestion, but these findings show that archaeon could play an unknown role in the removal of drugs from HWW. Few authors have reported the synergetic effects among *Archaea*, *Bacteria* and *Fungi* in the degradation of refractory organics [64]. Although few studies have incorporated the massive parallel sequencing of the archaeal 16S rRNA gene, to our knowledge, the is the first time *Archaea* were studied in a granular sludge (biotechnological wastewater treatment approach) treating real hospital wastewater.

3.4.2. Diversity of the bacterial population

The bacterial population dynamics revealed a great diversity of OTUs linked to processes depending on the raw wastewater. Fig. 5 shows the relative abundances of bacterial genera regardless of the clustering calculated for the OTUs. Fig. S3 shows the complete heat map with bacterial OTU clustering at 97 % similarity.

The population in the inoculum was represented by the *Dokdonella*, unclassified *Comamonadaceae* and *Hyphomicrobium*. *Dokdonella* is an aerobic denitrifying bacterial genus found in biofilms of AGS systems, even in the presence of pharmaceuticals, which had been described with a positive correlation with the existence of antibiotic-resistant genes (ARG) [23,65]. *Hyphomicrobium* is a denitrifier with the ability to accumulate phosphate, according to Yuan et al. [66] . Moreover, this genus plays an important role in granule formation and compactness because it excretes extracellular polymer substances (EPS) under high aeration pressure [67]. Nonetheless, the excessive proliferation of this genus could cause the destabilisation of the granules. *Comamonadaceae* is a family widely described in AGS systems, suggesting its function in granule stability, provided by its high ability to produce EPS [68].

In the present study, from Day 3, the granular community began to change, induced by the nature of HWW and its microbial load. Although *Dokdonella* and *Comamonadaceae* phylotypes were present, *Hyphomicrobium* abundance declined sharply, whereas several genera taxonomically affiliated with *Leadbetterella, Flavobacterium*, *Brevundimonas*, *Thauera* and *Rhodobacteraceae* unclassified proliferated. The influent composition encouraged the proliferation of several bacterial phylotypes competing for carbon and nutrient sources. This trend was observed until Day 7, when *Leadbetterella* acquired >30 % of the total relative abundance. This genus has also been observed in AGS treating pharmaceutical wastewater, and its relative abundance increased with increasing loads [69]. The changes mentioned in Section 3.2, given the real characteristics of HWW, modified abruptly the bacterial population because at Day 15, any phylotype exceeded 5 %, whereas at Day 30, new taxa started to play important roles, such as *Leucobacter*, *Xanthomonadaceae*, *Peptostreptococcaceae* and *Macellibacteroides*. In contrast, the phylotypes previously found in the system, such as *Comamonadaceae*, *Flavobacterium*, *Hyphomicrobium* and *Rhodobacteraceae*, reached 40 %, 18 %, 15 % and 12 %, respectively, of the total relative abundance. The oscillation in the drug loads between Days 15 and 30 could induce the changes in microbiome, highlighting the low drug loads at Day 45, with a subsequent increase at Day 60. The alternations resulted in vast modifications in the bacterial dynamics. The influent of Day 60 showed the highest drug loads, with obvious effects on the microbiota, which was corroborated by the proliferation of 16 OTUs with relative abundances of >7.5 %, some of which have not previously been described in this biological system, such as *Mycobacterium*, *Demequinaceae*, *Microbacteriaceae*, *Dysgonomonas*, *Cytophagaceae* (2), *Runella*, *Taibaiella* and *Fusibacter*. These phylotypes only were present at Day 60. The changes in the bacterial population, demonstrated by means of mean size, settling velocity and removal performance, are interesting because the granules represent robust biofilms that can resist considerably changes in influent characteristics, despite changes in the microbiome. Hierarchical clustering revealed as Day 60 act the frontier between the two stages for bacterial population. Possibly, the toxicity of the influent negatively impacted the bacterial community, which agrees with the decrease in the copy number of the 16S rRNA gene of *Bacteria* at Day 90. Consequently, any phylotype exceeded the 2 % of relative abundance, and only *Comamonadaceae* and *Dokdonella* showed abundances similar to

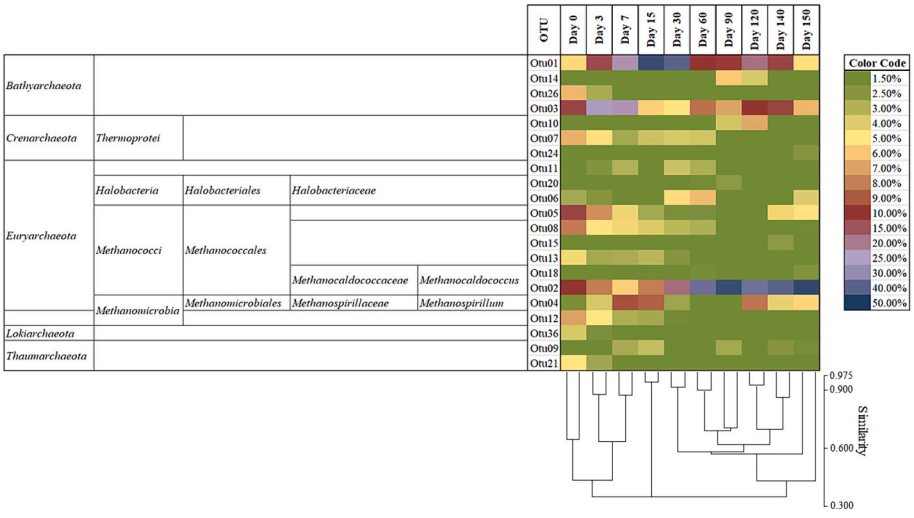

Fig. 4. Dynamics of the archaeal population in aerobic granular sludge treating real hospital wastewater for long-term operation linked with hierarchical clustering.

Fig. 5. Heat Map of the dominant bacterial taxa in the granular sludge system treating hospital wastewater for long-term operation, linked with hierarchical clustering.

those in the initial phase. Once the drug load remained stable, from Day 120 to Day 150, the microbial community denoted certain stability whose community was dominated by *Saprospiraceae*, *Comamonadaceae*, *Xanthomonadaceae* as well as the genera *Flavobacterium* and *Comamonas*. *Saprospiraceae* (*Bacteroidetes* class) preferentially degrades complex organic compounds as carbon sources [70]. Moreover, some interesting genera involved in nitrogen metabolism appeared during this period, such as *Diaphorobacter*, which simultaneously performs nitrification and denitrification [71], or the ammonia-oxidising genera *Acinetobacter* and *Comamonas* [72,73].

These taxa represent the core of the bacterial population, which promotes granular stability because they were present in both the inoculum and after recalcitrant bioremediation. The use of inoculum, regardless of their proceed, could promote the proliferation of specific genera able to remove recalcitrant compounds, remaining stable in its hard population core [18,74] . Finally, few potential human pathogens were present in the granules, such as *Enterococcus*, *Mycobacterium*, *Dysgonomonas* and *Clostridiaceae*, among others, bacterial pathogens commonly found in HWW.

3.4.3. Diversity of the fungal population

Throughout the experimental period, there were 43 representative OTUs with a relative abundance of >1.5 % (Fig. S4). but due to the huge fungal diversity were represented on Fig. 6 by its taxonomic affiliation. *Nectriaceae* was the most abundant family in the inoculum, with a relative abundance above 70 %. In addition, the *Hypocreales* order and the *Herpotrichiellaceae* family were represented with relative abundances of 15 % and 9.5 %, respectively. *Nectriaceae* played an essential role in granular biomass; during the first operation stage, its relative abundance peaked, reaching 96 % on Day 30. Some members of the family *Nectriaceae* are emerging and opportunistic human pathogens [75]; some species of this family have been reported in hospital water systems, posing a significant infection risk [76].

Similar to the archaeal and bacterial communities, the fungal population had a strong impact at Day 60 because the abundance of the *Nectriaceae* family was decreased from 96 % to 46 %. At this point, the *Trichosporonaceae* family acquired a significant role in the community, along with the *Nectriaceae* family. Subsequently, a progressive decrease in the abundances of *Nectriaceae* and *Hypocreales* was observed, whereas that of *Ascomycota* decreased. In this way, the population in the granular sludge was not dominated by one specific phylotype, and the families *Nectriaceae* and *Trichosporonaceae* as well as the phylum *Ascomycota* play functional roles in wastewater treatment [77]. The family *Trichosporonaceae* could act with syntrophic relationships with denitrifying bacteria, assisting them in the ring opening and chain breaking of organics [64]. Some members of this family have been described as promoters of sludge bulking [78], but filamentous growth could be crucial in granulation, with filamentous microbes acting as the core of granules and a later bridge union with the ability to immobilise other microorganisms, as reported by Gonzalez-Martinez et al. [79] . In this study, they were ubiquitous during the entire experimental period.

3.5. Alpha- and beta-diversity analysis

The α-diversity indices for the archaeal population showed similar values from inoculum to the end of the experiment in terms of diversity and evenness, but the species richness, as indicated by the Chao index, increased over time from 60 ± 14 to 173 ± 3 (Table S2). In general terms, the species richness of bacterial communities increased over time, which was also observed for evenness. The diversity measured by Simpson indices showed values in the same range. The species richness of the fungal community significantly increased during the initial operation phase and remained stable during the 30 days. However, a specialisation of granule-forming fungi was detected from Day 90 to the end of the operation, based on the decrease in the Chao index. This was corroborated by the trend observed in the fungal community studies. The opposite trend was observed for diversity and evenness indices, which were greatly decreased in the initial stage and started to increase from Day 60 onward, exceeding the values of the inoculum.

Based on these results, the bacterial and fungal communities play an important role in the formation of the granules and in the degradation of several pharmaceutical compounds, whereas the archaeal populations were not strongly modified.

The β-diversity, calculated using the Whittaker index, indicated an increase in the similarity between archaeal populations for pairs of samples throughout the experimental period, with higher dissimilarities between Days 140 and 150 (Fig. S5). The dynamics of the bacterial communities revealed a progressive tendency to differentiate between pairs of samples, demonstrating the change produced at Day 60, when the loading of pharmaceutical compounds was highest, with TMP > 1000 μg·L^{-1}, CMP > 2000 μg·L^{-1}, NPX > 1400 μg·L^{-1} or DCL > 1800 μg·L^{-1}; similar findings have been reported previously [46,80]. Although over time, the fungal communities showed values comparable to those of the bacterial populations, the trend was not as clear. In this case, the community at Day 140 played a significant role in the dissimilarity with the remaining samples, with the highest values for all

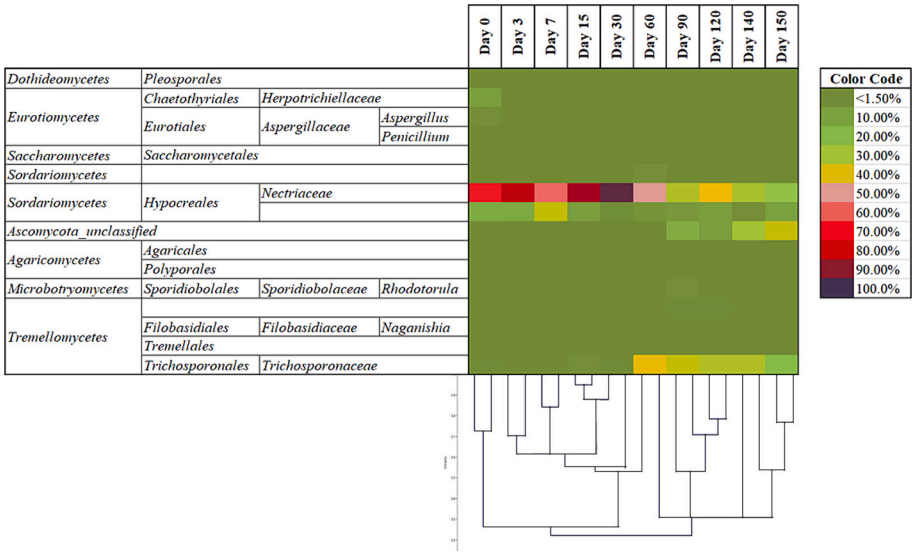

Fig. 6. Heat map of fungal population dynamics in a granular sludge system treating wastewater for long-term operation linked with hierarchical clustering.

domains.

3.6. Clustering distribution and phylotype dissimilarity contribution analysis

The results of the PCA indicated significant differences among the granular archaeal communities over time, with three clusters of sample pairs (Fig. S6a). The first cluster contains samples from start-up to Day 15 were clustered, the second cluster contains samples from Days 30 to 90, and the third cluster contains samples from Days 120 to 150. The PCA demonstrates the logical sequential changes suffered by de archaeal population, as shown in the heat map of the *Archaea* domain. Moreover, the distance found among archaeal samples over time was caused by specific phylotypes calculated by SIMPER analysis (Fig. S7). The taxa that contributed to this distance were *Methanocaldococcus* (33 %) and two OTUs belonging to *Bathyarchaeota* (27 %). This trend was observed in the archaeal population dynamics (Section 3.4.1), but both statistical analyses supported the hypothesis of the displacement of *Bathyarchaeota* by *Methanocaldococcus* in the steady-state period.

The PCA of the bacterial samples did not reveal any differences between the start-up and the steady-state stages, with a short distance between Days 0 and 150 (Fig. S6b). These findings can be linked to the high levels of diversity obtained in all samples, where the phylotypes had a high evenness. Based on the results of the SIMPER analysis, only 15 genera contributed to dissimilarities between both periods with >1.5 %, whereas communities with a lower diversity reported a major number of contributions in aerobic granular sludge systems [81]. The families *Saprospiraceae* and *Comamonadaceae* and the genera *Dokdonella* and *Leadbetterella* genera contributed >28 % to the dissimilarities; these changes were clearly observed in the dynamics population studies (Section 3.4.2).

Finally, the PCA performed for the fungal community highlights the unclear and progressive distance between samples over time, but the clustering did not define the operational period, in contrast to *Archaea*. For Days 3, 7 and 150, the distances were short, although the fungal phylotypes did not reveal high similarities. The phylotype that most contributed to the dissimilarities between the start-up and the steady-stable stages was *Nectriaceae* (with a contribution of 40 %), which was the most abundant phylotype in terms of relative abundance in granules across the entire experimental period. However, its relative abundance greatly decreased over time. *Trichosporonaceae* proliferated with the maturation of the granular sludge system, but it was not present in the inoculum; this is in agreement with the findings of Gonzalez-Martinez et al. [79]. This phylotype contributed with 30 % to the dissimilarities between the start-up and steady-state periods.

3.7. Multivariate redundancy analysis

Multivariate redundancy analysis was performed to link the copy numbers of genes and the archaeal, bacterial and fungal communities with the performance evolution of the system. The linkage of absolute abundance genes analysed by qPCR and the physico-chemical parameters is shown in Fig. 7. The RDA demonstrated that Triclosan and Ketoprofen were negatively correlated with the abundances of *Bacteria*, *Archaea*, *Fungi* and, more specifically, with denitrifier, while phosphate accumulating bacteria and ammonia oxidating bacteria had not correlation. The *amoA* gene demonstrated a strong positive correlation with high cyclophosphamide removal, which was only detected during the first 15 days. Interestingly, TMP showed no correlation with the number of 16S rRNA genes of *Bacteria*, although it is an antimicrobial compound with bactericidal action.

Antibiotics could change the microbial community structure, functioning or composition, and bactericides could promote the disappearance of some phylotypes and alter the bacterial community [82]. In another study, phosphate removal from raw wastewater was not linked to the abundance of PAO, possibly because of the inhibition of their

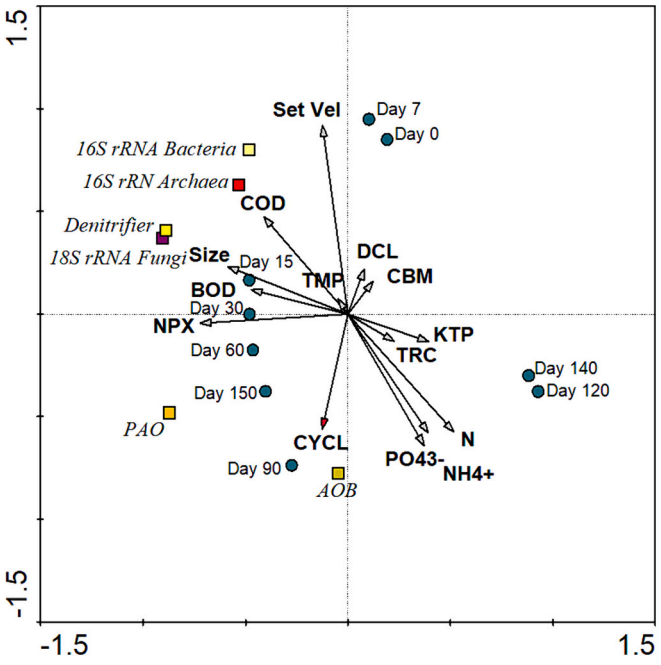

Fig. 7. Linkage of the number of gene copies with physico-chemical performance and drug degradation using multivariate redundancy analysis.

metabolism [40]. In contrast, the results highlighted the positive correlation of ammonia oxidation with *amoA* genes. Higher COD and BOD removal rates were strongly and positively correlated with the abundances of *Bacteria*, *Archaea*, *Fungi* and, more specifically, denitrifiers. As denitrifying microorganisms usually use the organic matter as a carbon source, this can be explained by the ubiquitous heterotrophic denitrifying activity [26].

The RDA performed for archaeal, bacterial and fungal populations (Figs. S8A, S8B, S8C, respectively) related to the physico-chemical parameters demonstrated that all studied communities changed progressively over time, despite the toxicity and oscillations of the raw water; interestingly, the community was stable from Days 90 to 150. The communities showed a positive intercorrelation on Days 90, 120, 140 and 150, highlighting the robustness of the granular biofilm regardless of the influent composition.

In the RDA performed for archaeal dynamics, two compounds (TRC, KTP) were positively correlated with the steady-state period, whereas four (TMP, DCL, CBM, NAP) were positively correlated with the first month of operation. In this way, the dominant OTUs from the inoculum showed a reduced evenness, with a massive proliferation of OTUs 01 and 03, both belonging to *Bathyarchaeota*. Despite the overgrowth of OTU 01 and OTU 03 during the first month of operation, only OTU 01 was correlated with TMP and DCL removal. On the other hand, the high abundance achieved by OTU 02 from Day 30 could be related to the resistance to pharmaceutical compounds, with a strong and positive correlation with KTP and TRC as well as with nitrogen and phosphate removal. The OTU 02, taxonomically affiliated to *Methanocaldococcus* is known by its implication in the nitrogen biogeochemical cycle. Recently, this genus has attracted increased interest because of its role in diazotrophic metabolism and its implications for the origin of nitrogenase [83,84].

The huge abundance of dominant bacterial OTUs prevents a clear recognition of the role of each of them in the performance of the reactor. Overall, 69 OTUs were correlated with a good performance in terms of TMP, NPX, CYCL, TRC, KTP, BOD, NH_4^+, PO_4^{3-} and N removal, of which only 20 OTUs were correlated with the steady-state on Days 90, 120, 140 and 150; these 20 OTUs were exclusively linked with N, KTP, TRC, NH_4^+, PO_4^{3-} and BOD removal. The granular properties were negatively

intercorrelated, indicating that larger granules settle more slowly. It has therefore been assumed that a better settling velocity is related to the compactness and density of the granules [44].

The RDA of the fungal community corroborates the ability of fungal phylotypes to growth on biofilm, and results demonstrate how granules act as an ecological niche with a large fungal diversity. This implies that fungi play an essential role in the structural core and granulation processes of this biotechnological approach [16,18,74]. In this study, 23 OTUs were positively correlated with a high drug removal efficiency throughout the experimental period.

4. Conclusions

To date, no study has demonstrated the ability of granular aerobic systems to reduce PhCs concentrations in real HWW. This study demonstrates the robustness and stability of granular biomass for the treatment of real hospital wastewater, regardless of the characteristics of the influent. BOD_5 and nitrogen removal were high (100 % and 70 %–90 %, respectively), whereas phosphate removal reached 50 % of removal. The results highlight the ability of the system to degrade TMP, CMP, CLP and TRC at high influent loads, whereas release peaks were suspected for NSAIDs. The PhCs modified the bacterial and fungal populations, increasing diversity and evenness due to competition between them. The dynamics of the archaeal population showed a progressive adaptation phase. The absolute abundance of genes was not significantly affected, except for PAO and fungal organisms. Despite the larger granule size and the decrease in settling ability over time, the stability of the granular structure was not compromised. The aerobic granular sludge technology is a feasible technology for treating hospital wastewater in order to reduce the contaminant load prior to disposal to the UWW.

CRediT authorship contribution statement

Lizandra Pérez-Bou: Data curation, Investigation, Writing – review & editing. **Aurora Rosa-Masegosa:** Conceptualization, Data curation, Formal analysis, Resources, Supervision, Writing – original draft. **Ramiro Vilchez-Vargas:** Formal analysis, Funding acquisition, Investigation, Software, Writing – review & editing. **Alexander Link:** Funding acquisition, Resources, Writing – review & editing. **Alejandro Gonzalez-Martinez:** Supervision, Writing – review & editing. **Jesus Gonzalez-Lopez:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. **Barbara Muñoz-Palazon:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Supervision, Writing – original draft.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jwpe.2024.105206.

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