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Description of new single-chamber continuous-flow reactors of aerobic granular sludge: Technical and biological study

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ABSTRACT

Aerobic granular sludge reactors usually operate in sequential batch mode, although this configuration limits the treatment of large volumes of wastewater, and they require a storage system. To implement this technology at full-scale, it would be necessary to design a simple and compact continuous-flow bioreactor able to treat larger volumes of wastewater. In this study, four aerobic granular sludge single-chamber continuous-flow reactors (R1, R2, R3 and R4) were designed and operated at the lab scale to evaluate and select a bioreactor configuration that achieves high organic matter removal performance while maintaining a stable granulation for long-term operation, promoting the washout of filamentous microorganisms. Results confirmed that the bioreactor including a lateral decanter (R1), was able to work in a steady state without loss of granular biomass and reached 95% of organic matter removal performance. Its granules had excellent compaction, with settling velocity values above 100 $m \cdot h^{-1}$. The R1 bioreactor also allowed rapid biomass adaptation and therefore a fast start-up (11 days). Results of this preliminary study at the lab scale suggested that the new and simple bioreactor design could be promising for its implementation at full scale. For that, future research is required to optimise the current model and to determine the most suitable operational parameters to treat domestic wastewater at full scale.

1. Introduction

Aerobic granular sludge (AGS) is a promising biological system for the treatment of wastewater due to its advantages in comparison with other technologies, such as conventional activated sludge (CAS) [1–3]. The hydrodynamic shear force and the continuous circular motion generate compact granules formed by microorganisms fixed and stabilised in a polymeric matrix [4,5]. This dense structure gives rise to the advantages of this technology because the high density of AGS promotes a better settleability [6,7], which can be translated into the implementation of more compact wastewater treatment plants (WWTPs) in comparison with CAS, due to lower time and space required to separate liquid-solid phases [2,5,8]. Moreover, the dense granular structure encourages a high accumulation of biomass. According to Nancharaiah et al. [9], AGS technology can reach a biomass concentration greater than 10 $g \cdot L^{-1}$. Besides this, the round shape maximises the granular surface, and the high compactness promotes mass transfer, which creates differences in terms of oxygen and nutrients from the external to internal layers [10–12]. This fact promotes the stratification of microorganisms along the layers, and consequently, it is possible to find different metabolisms in the same granule [2–4,13]. Therefore, AGS can remove organic matter, phosphorous, nitrogen and other substances, including pharmaceuticals, endocrine disrupters, phenolic compounds, dyes, heavy metals, particulate matter, nuclear waste and sulphur amino acids in the same chamber [1,3,8,13–17]. Changes in abiotic factors could modify influent characteristics [18]. However, AGS is a robust technology able to adapt to oscillating influent composition [19]. This capability is promoted by the large amount of extracellular polymeric substances (EPS) excreted by microorganisms in these aggregates, encouraging resistance to toxic compounds and high organic loading rates [13,20]. For all these reasons, AGS systems can be used to treat urban and industrial wastewater [13,21] as well as drinking water [22].

This technology has usually been operated in sequential batch reactors (SBRs), with the following cycles: 1) reactor filling with the raw water, 2) aeration, 3) settling of granules to separate the treated water from the biomass and 4) effluent discharge. In the last stage, light flocs

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and filamentous microorganisms are washed out using short settling times to select the fastest biomass [2]. Filamentous microorganisms are essential for granule formation because they establish the structural core [23]. Nevertheless, an excess of these microorganisms would give rise to a destabilisation of the system, causing biomass washout due to the loss of granular density [24]. Thus, the wash-out of the excess filamentous microorganisms is a key step for maintaining granular structural stability.

Recently, research has been carried out on the development of AGS continuous-flow reactors (CFRs), as they offer more advantages than SBRs. A CFR is easier to build, operate and maintain. Moreover, the constant flow permits the treatment of larger volumes of wastewater and the compaction of technology, as it does not require the previous storage system [25–28]. Nowadays, the challenge is to find a good design for AGS-CFR that concurrently allows granular biomass retention and the promotion of the wash-out of filamentous bacteria and fungi.

Diverse designs of CFRs for AGS have been proposed. They are based on bubble columns with baffles, serial multiple chambers, the use of clarifiers, CFRs with submerged membranes or hybrid SBR-CFR systems [3]. However, the mentioned configurations present restrictions, such as granulation limitation, granular destruction by the sludge return system, proliferation of filamentous microorganisms or complexity of construction and maintenance [29,30]. So, further studies are necessary to optimise the design and configuration for the selection pressure of biomass, enhance the operational control and permit full-scale implementation [29,30].

In this study, four novel and simple AGS-CFR configurations, each one equipped with different baffles, were designed and operated. The goal of this research was to achieve a successful performance using the most effective design of AGS-CFR, whose configuration allows satisfactory granular growth and stability, as well as the removal of excess filamentous microorganisms. The new bioreactors had a simpler design in comparison with those built previously to facilitate their construction and maintenance while reducing costs. It was also expected that there would be fewer granular instability problems because a sludge return system was not required, differentiating itself from most of the preceding models.

2. Material and methods

2.1. Bioreactors configuration and baffles design

The four bioreactors consisted of a methacrylate cylindrical tube of 10 cm in internal diameter and 72 cm in height. The bottom part was a polyvinyl chloride (PVC) cone where an air sparger was placed. Bioreactors 1, 2 and 3 (R1, R2 and R3, respectively) had eccentric cones, and bioreactor 4 (R4) had a concentric cone (Fig. 1). The total operational volume of the reactors was 6 L. The influent inlet was located at half the height of the reactor, and the treated effluent output was located at the top of the bioreactor (Fig. 1). Four different baffles were configurated to evaluate the most efficient one to avoid the loss of granules simultaneously to filamentous microorganisms wash-out. First, R1′s baffle was a truncated, conical PVC decanter 6.3 cm in diameter placed in the water outlet zone and coupled to an elbow tube [31] (Fig. 1). The

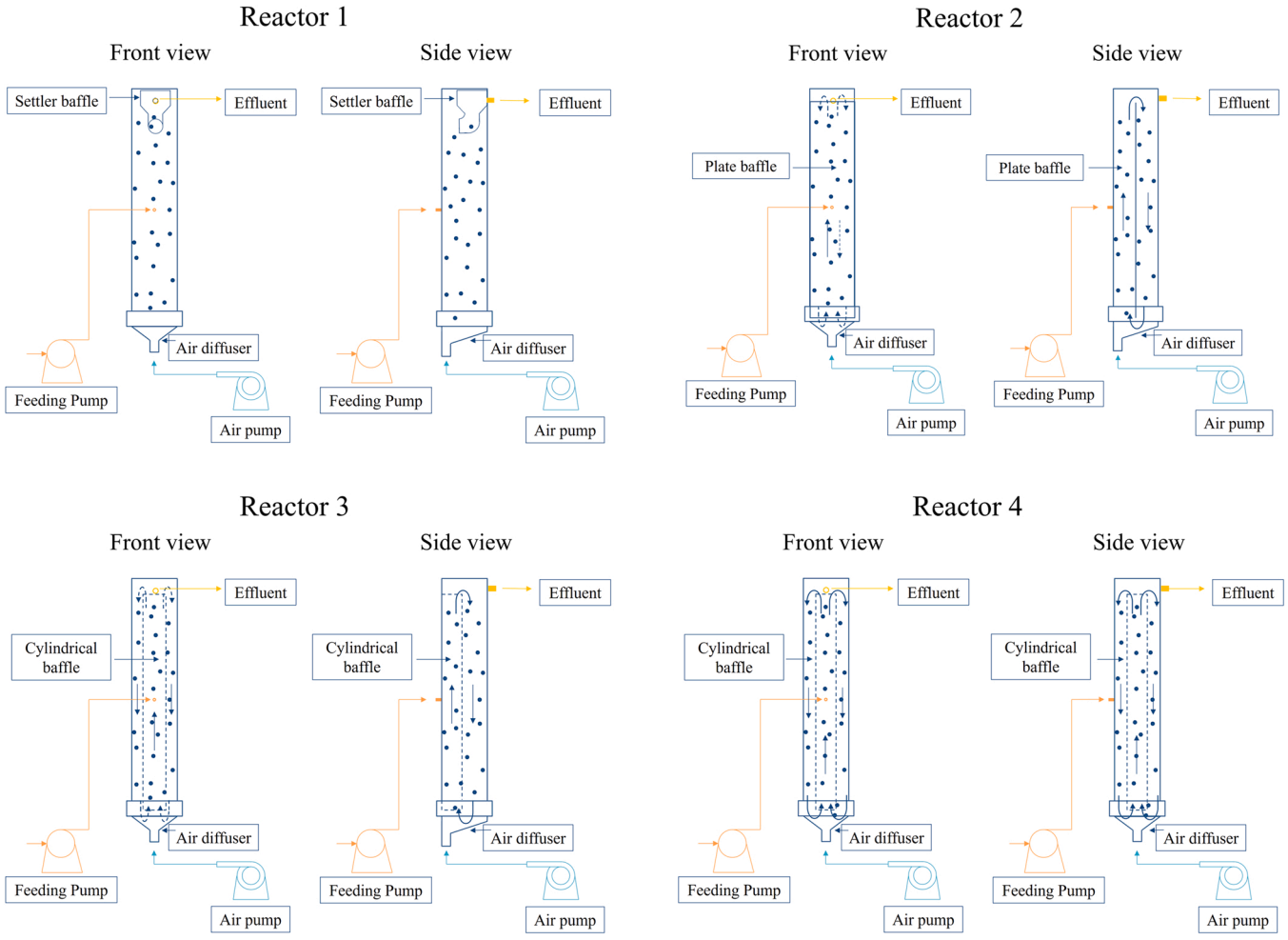

Fig. 1. CFR diagrams from front and side views; reactor 1 with a settler in the superior part; reactor 2 with a vertical plate dividing the cylinder; reactor 3 with an eccentric position tube; reactor 4 with a concentrical position tube.

baffle configuration allowed the remotion of floc biomass because flocs, in contrast to the granular biomass, could reach the overflow zone. This configuration of baffles allows the free movement of the granules throughout the reactor. Secondly, R2′s baffle consisted of a vertical plate of methacrylate placed 2.5 cm above the air diffusor to permit the flow of granular biomass. The diffusor created a circular movement that forced granules to go up along one column side and fall along the opposite column side due to the eccentricity of the bottom. The R2 configuration was based on a suction force created on top, which constrains the dense particles to go down, while filamentous microorganisms could outflow. This plate restricted the free movement of granules. Thirdly, R3′s baffle was an internal tube 4.6 cm in internal diameter circumscribed by the bioreactor's internal structure and was placed at 2.5 cm along the air diffuser. The goal was similar to the R2 baffle, to create a streamflow that enforces granule settling but allows the output of undesirable flocs. Finally, the baffle placed in R4 consisted of an internal concentric tube 5.4 cm in internal diameter placed at 2.5 cm along the air diffuser. The difference with the R3 design was the position of the internal tube because R4′s baffle was positioned at the middle of the reactor, due to its bottom cone being concentric. The premise was that the streamflow generated would force the settling of compact biomass, while floccules would be discarded.

2.2. Start-up

The four AGS-CFRs were operated for 55 days with 6 h of hydraulic retention time. Influent wastewater was introduced by Watson Marlow peristaltic pumps (United Kingdom). The synthetic medium simulating urban wastewater was composed of 1.16 $g{\cdot}L^{-1}$ $CH_3COONa{\cdot}3\ H_2O$, 0.25 $g{\cdot}L^{-1}$ NH_4Cl, 0.04 $g{\cdot}L^{-1}$ KCl, 0.1 $g{\cdot}L^{-1}$ $MgSO_4{\cdot}7\ H_2O$, 0.085 $g{\cdot}L^{-1}$ K_2HPO_4 and 0.030 $g{\cdot}L^{-1}$ KH_2PO_4 according to Muñoz-Palazon et al. [32], with slight modification related to carbon source and the trace solution. Air flowed through a diffuser placed at the bottom of each bioreactor, giving rise to the hydrodynamic motion.

Each CFR was inoculated with 600 mL of granular biomass from a lab-scale AGS-SBR located in the Water Research Institute (Granada, Spain).

2.3. Determination of physicochemical parameters

During the experiment, physicochemical characterisation was periodically analysed. Mix liquor suspended solids (MLSS) were determined in triplicate for all reactors using standard methods for the examination of water and wastewater [33]. Granular size and settling velocity were evaluated to characterise the biomass properties, following Moy et al. [34]. To determine the organic matter removal efficiency, chemical oxygen demand (COD) and biological oxygen demand at day 5 (BOD_5) were quantified during the experiment by standard methods [33].

2.4. Carbon mass balance

To perform a carbon mass balance in the system, two main reactions should be considered. There is one corresponding to the removal of organic matter to CO_2 by biological processes, according to the following chemical oxidation reaction:

$$C_nH_aO_b + 1/4\ (4\ n + a - 2b)\ O_2 \rightarrow nCO_2 + (a/2)\ H_2O.$$

where, for the second term of the equation, the amount of oxygen required for organic matter oxidation represents the COD.

Since the synthetic wastewater used in the experiments is composed of a known carbon source (acetate), it is possible to define the stoichiometry of the oxidation reaction of the organic matter contained in the wastewater:

$$CH_3COO^- + (7/4)\ O_2 \rightarrow 2CO_2 + (3/2)\ H_2O.$$

The reaction of substrate transformation into new biomass was also determined. On this occasion, although the stoichiometry involved in this reaction is unknown, it can be experimentally measured as the increment of mixed liquor volatile suspended solids (MLVSS). Assuming that new cells can be represented as $C_5H_7NO_2$, it is possible to calculate the oxygen equivalent (COD) for the generation of new biomass, which can be considered approximately 1.42 gCOD/gMLVSS [35].

$$C_5H_7NO_2 + 5\ O_2 \rightarrow 5CO_2 + NH_3 + 2\ H_2O.$$

$$(\Delta O_2/\Delta C_5H_7NO_2) = (5 \times 32\ g/mol\ O_2)\ /\ (113\ g/mol\ C_5H_7NO_2) = 1.42\ gO_2/gMLVSS.$$

Therefore, by applying a global mass balance to the system, it is possible to calculate the generation of carbon dioxide (CO_2) in each of the four reactors from the results of the COD analyses of influent and effluent samples and the MLVSS analyses.

2.5. Biological sample collection and DNA extraction

The granular biomass samples were collected at days 0, 15, 30 and 55 for each bioreactor. Representative granules were taken in a 15-mL Falcon tube and immediately centrifuged at 5000 rpm for 20 min at 4 °C. Afterwards, the supernatants were discarded, and pellets were stored at − 20 °C. The extraction of DNA from the samples stored was performed using the FastDNA SPIN Kit for Soil (MP Biomedicals, USA) according to the manufacturer's protocol. For the final step, the DNA pools were suspended in 150 μL of deionised water.

2.6. Absolute quantification of target genes

The quantification of the number of copies of the bacterial and archaeal 16 S rRNA gene, fungal 18 S rRNA gene and functional genes *amoA* and *nosZ I* was carried out by quantitative polymerase chain reaction (qPCR) using Quant Studio 3 equipment. Plasmid standards were used to create the calibration curves. For the bacterial 16 S rRNA gene, 10^7–10^3 copies·μL^{-1} serial dilutions were used. For the archaeal 16 S rRNA gene and fungal 18 S rRNA gene, 10^7–10^2 dilutions were used; for *amoA*, 10^8–10^4 dilutions were used, and for *nosZ I*, 10^8–10^3 dilutions were used. The reaction mixture contained 2.5 μL of buffer with $MgCl_2$, 0.5 μL of dNTPs (10 mM), 0.15 μL of forward and reverse primers (10 μM), 0.125 μL of SYBR Green, 0.125 μL of Taq polymerase, 0.0625 μL of BSA and 2 μL of DNA samples diluted 1:25. The primers and qPCR conditions used were described by Muñoz-Palazon et al. [36].

2.7. Next-generation sequencing and post-processing

Illumina next-generation sequencing was performed using a pair of primers: 16 S Pro341 forward 5′-CCTACGGGNBGCASCAG-3′ and Pro805 reverse 5′-GACTACNVGGGTATCTAATC -′3 for 16 S rRNA of Prokarya and Euk1391F 5′-GTACACACCGCCCGTC-3′ and reverse EukB 5′-TGATCCTTCTGCAGGTTCACCTAC-3′ for 18 S rRNA of Eukarya [37, 38]. The raw data were analysed using Mothur v 1.39.4 [39]. First, forward and reverse reads were merged into contigs. Then they were screened to remove sequences with >8 homopolymers and >0 ambiguous bases. After that, reads were aligned against the SiLVA seed v132 database using the Needleman conditions. Following, sequences that were not correctly aligned in the forward and reverse positions were removed. The VSEARCH algorithm was used for chimera detection, using the sample reads as the reference [40]. The chimeras were removed, and the remaining sequences were classified against SiLVA nr v132 database through the k-nearest-neighbour method, and then they were clustered into operational taxonomic units (OTUs) with a similarity cut-off of 97% for Prokarya and 95% for Eukarya [41].

2.8. Ecological and statistical analysis

The α-diversity and β-diversity were calculated for prokaryotic and eukaryotic sample groups using PAST3.14 software. Bioreactor differences were statistically significant for physicochemical data, and qPCR results were calculated using one-way PERMANOVA using PAST3.14 software. The similarity percentages analysis (SIMPER) was done to

quantify the contribution of each OTU to the dissimilarity between pairs of samples. To calculate SIMPER, PAST3.14 software was employed using the Bray-Curtis algorithm. Principal component analysis (PCA) was carried out using R project v4.2.1 software, complemented with the transformation to centred logarithm using CoDaPack software to cluster the compositional data of the microbial community distribution.

Multivariate redundancy analysis was determined to evaluate the relationship between the microbial community with more than 1% of relative abundance, operational parameters and bioreactor performance. Due to the variability of bioreactor performance during the start-up phase, the physicochemical data were expressed as average values for periods corresponding to the following operational days: Inoculum: from day 0 to day14, Day 15: from day 15 to day 29, Day 30: from day 30 to day 44 and Day 45: from day 45 to day 55. Before the analysis, physicochemical and diversity data were transformed according to the following equation: log (data + 1). Then, 999 Monte Carlo simulations under a full permutation model were computed using CANOCO 4.5 software [42].

3. Results and discussion

3.1. Characterisation of granular sludge

The mean size and the settling time are parameters providing valuable information about the compactness and stability of granules. Regarding the mean size (Fig. 2), no differences were detected among the granules of R2, R3 and R4 reactors during the first 15 days, with a value of around 5 mm. In the same period, the R1 bioreactor had slightly larger granules, reaching 8 mm. After day 15, the mean R1 and R2 biomass size increased until a value of around 12 mm, while the granular size of R3 and R4 enlarged only to 7 mm.

Concerning the settling velocity of granules, there were no great differences between the different CFR designs during the first 15 days, a period in which the settleability decreased, from a velocity of 50 $m \cdot h^{-1}$ to 35 $m \cdot h^{-1}$ (Fig. 2). Afterwards, there was an increase in the settling velocity of granules from the R1 bioreactor exceeding 100 $m \cdot h^{-1}$, whereby it became the bioreactor with the fastest granules, which

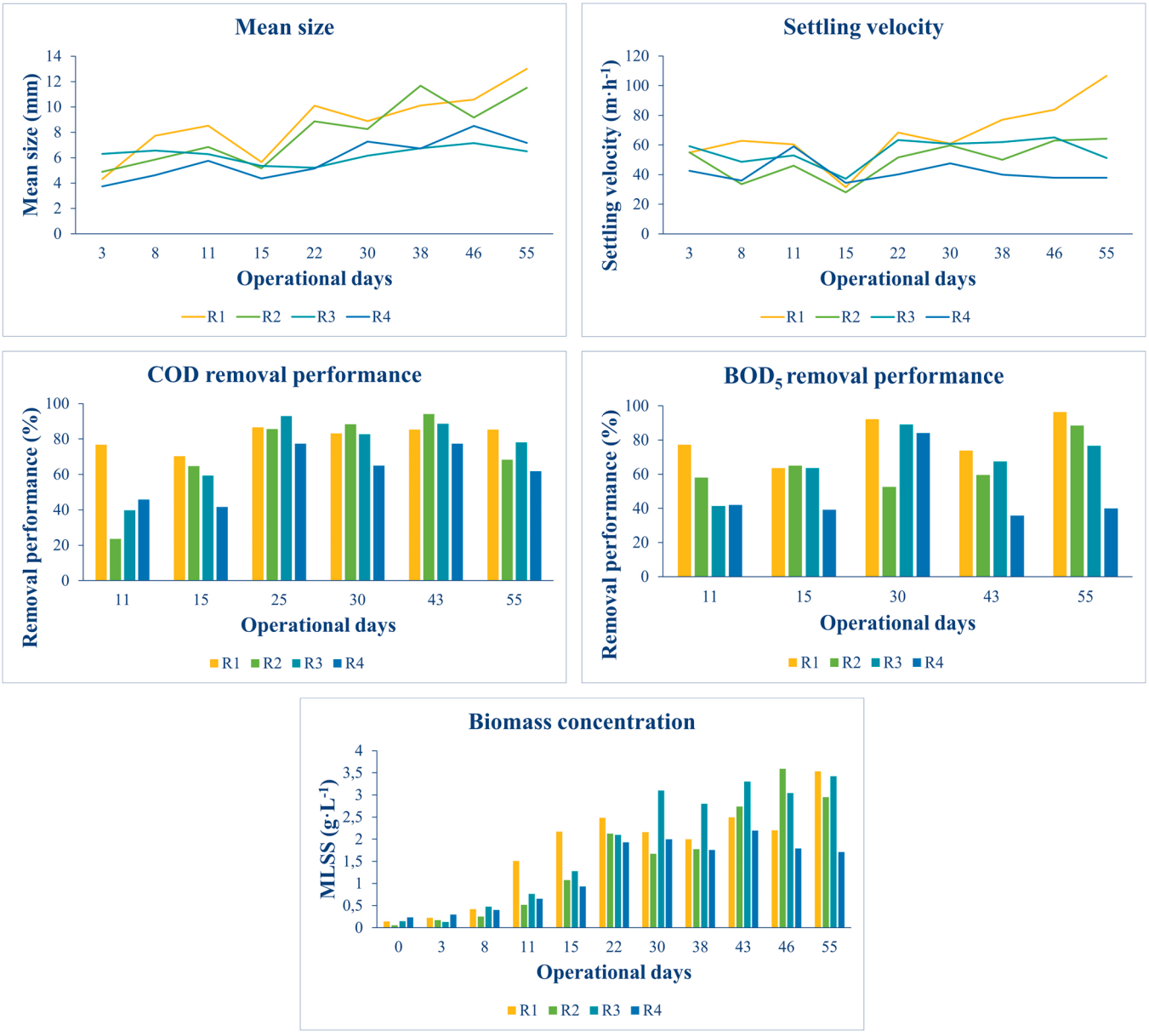

Fig. 2. Granular properties (mean size and settle velocity), organic matter removal (COD and BOD_5) and biomass concentration for each reactor during the whole experimentation.

means that those granules were the most compact [6,7]. On the other hand, R4 was the configuration with the highest presence of filamentous microorganisms, due to the settling velocity of its granules only reaching 40 $m \cdot h^{-1}$. The settleability of granules from the R2 and R3 bioreactors achieved intermediate values, ranging from 50 to 65 $m \cdot h^{-1}$. The granular properties pointed out that the configuration of R1 was the most optimal design to promote compact granules and the wash-out of filamentous microorganisms. The R1 design produced granules that achieved higher settling velocities than other aerobic granular systems, regardless of whether they were operated in continuous-flow or sequential batch mode. On the same operational day (day 55), R1′s granules had a greater average settling velocity (106.5 $m \cdot h^{-1}$) than those operated in the SBR reported by Muñoz-Palazon et al. [36], with values ranging from 65 to 90 $m \cdot h^{-1}$, emphasising the fast start-up of the R1 bioreactor. Also, the values for R1 were higher than the velocities registered (68–74 $m \cdot h^{-1}$) in the SBR operated by Kocaturk and Erguder [43]. In addition, it was clear that the R1 bioreactor had the fastest granular biomass than other CFRs. For example, Liu et al. [44] stated a maximum of 39.6 $m \cdot h^{-1}$, while Cofré et al. [45] reported a maximum of 113 $m \cdot h^{-1}$ but needed two chambers. Thus, despite the optimum value achieved under the best operating conditions, their system was disadvantageous because of the added difficulties of construction and operation of the reactor in two chambers. For a high settling velocity that was translated to an excellent granular conformation and no proliferation of filamentous microorganisms, the R1 configuration is possibly a more profitable design relative to the previously described by other authors.

The in vivo observations during the whole experiment corroborated the settling velocity results. It was possible to see how R1′s granules were the most compact (Fig. S1), followed by the granular biomass of R2 and R3 bioreactors, while the R4 configuration had flocculent biomass over operational time.

The monitoring of granule stability was also carried out considering the biomass concentration. During the first week, no statistically significant differences were detected between reactors (with a *p*-value of 0.5749 based on Bray-Curtis PERMANOVA), in which the biomass concentration in terms of MLSS was less than 0.5 $g \cdot L^{-1}$ (Fig. 2). From day 11, the R1 bioreactor reached values of 1.5 $g \cdot L^{-1}$, while the R2, R3 and R4 reactors had a concentration below 1 $g \cdot L^{-1}$. After day 22, the difference in biomass concentration between the R1 and the other configurations was declining, and all the reactors had oscillating values ranging from 1.5 to 3 $g \cdot L^{-1}$. At the end of the experimental period, the R2 and R3 bioreactors had an increase in MLSS concentration, but this rise was principally due to an increment of floc biomass instead of granular biomass in the bioreactors. Briefly, all assayed CFR configurations reached a significant biomass concentration (higher than 1.5 $g \cdot L^{-1}$) but comparatively, the R4 bioreactor showed the lowest values of biomass concentration, while the R1 bioreactor showed the highest values. In addition, all biomass in the R1 bioreactor was integrated by granules of high quality in terms of size and compactness, with a rapid start-up period of 11 days.

These results could be positively compared with AGS reactors operated on sequential batch cycles, because MLSS concentration, as well as the settling velocity and mean size, were similar to previously described research [36,46–49]. Additionally, this research reported how the settling ability of granules and the wash-out of flocs are essential parameters, demonstrating the success of the R1 configuration for both factors, even in absence of feast-famine periods, which promote the selection pressures in AGS [50].

3.2. Organic matter removal performance and carbon mass balance

The organic matter removal performance was evaluated to decide on a better continuous bioreactor design and setup. This parameter was determined by COD and BOD_5. During the first 15 days, the reactor with the highest COD and BOD_5 removal capacity was the R1 bioreactor (Fig. 2), which reached mean removals of 75% and 70% respectively. Throughout the experiment, the CFRs achieved stability and increased their organic matter removal rate. The COD removal capacity of the R1, R2 and R3 bioreactors increased to around 90%, while for the R4 bioreactor, fluctuations around 60–78% were recorded. Similar results were observed for the BOD_5 values, with the R1 bioreactor presenting, in general terms, a greater capacity (95%) of BOD_5 removal than the other configurations, particularly relative to bioreactor R4.

All these facts suggested that again, the R1 configuration, whose baffle was a small settler located in the water outlet zone, was the more suitable design to maintain a rapid stable granulation and high organic matter removal rates. This conclusion was corroborated by the effluent characteristics since the treated water from the R1 bioreactor was clear. On the contrary, the water treated in the bioreactors R2, R3 and R4, presented a murky effluent with evident turbidity and suspended solids. Also, bioreactors R2, R3 and R4 showed an unstable granulation, particularly in R3 and R4, where the growth of filamentous microorganisms was remarkably greater than in the R1 bioreactor.

Table 1 shows the carbon mass balance for each reactor. The results showed that the R1 bioreactor achieved higher organic load remotion, with values of 9.84 and 7.32 g $O_2 \cdot d^{-1}$ of COD and BOD_5, respectively. On the contrary, the lowest average values in terms of removal efficiency were obtained in the R4 bioreactor, with values of 6.72 and 4.62 g $O_2 \cdot d^{-1}$ of COD and BOD_5, respectively. These results promoted the selection of R1′s design as the most profitable configuration. The average values of CO_2 generation were the highest for the R1 reactor and lowest for the R4 reactor. Part of the consumed carbon was incorporated into new biomass, reflected in MLVSS values, and the rest was emitted as CO_2 gas from the oxidation of organic matter by microorganisms. No remarkable differences were detected in terms of the production of biomass (MLVSS) among reactor configurations. However, biomass concentration was not the only important parameter since it would need to be granular compact biomass to be functional in terms of performance, as was reported in Section 3.1.

3.3. Dynamics of microbial community

The changes in prokaryotic and eukaryotic communities were analysed to identify the microorganisms present in the different CFRs and their population dynamics.

3.3.1. Prokaryotic community dynamics

The prokaryotic community in the reactors was represented by 62 OTUs with more than 1.0% of the total relative abundance, all of them from the superkingdom *Bacteria* (Fig. 3). The populations were different, following a clear pattern marked by the baffle design and reactor configuration. Among the dominant OTUs in the inoculum sample, the most dominant phylotype was OTU02, affiliated with the *Chitinophagaceae* family, with 8.8% of total relative abundance, followed by OTU04 (8.3%), which belonged to the *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* genus, OTU01 (7.1%), affiliated with the *Brevundimonas* genus, and OTU06 (5.9%), which belonged to the *Xanthobacteraceae* family. All these OTUs represented more than 30% of the total bacteria population in the aerobic granular sludge used as inoculum, which was obtained from a lab-scale AGS-SBR bioreactor. *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* has been reported by He et al. [51] as contributing to nitrate removal in an up-flow fixed-bed bioreactor. Denitrifying activity has been described for the *Chitinophagaceae* family [52] and for the *Brevundimonas* genus [53], which was also reported for its excellent ability to secrete extracellular polymeric substances (EPS), which are essential for granule formation [54]. According to Hurtado-Martinez et al. [22], the *Xanthobacteraceae* family was able to remove nitrogen by partial denitrification and anammox processes.

The CFRs operation produced changes in the bacterial communities of granules employed as inoculum. Firstly, a reduction of OTU01 affiliated with *Brevundimonas* was detected on the R1 bioreactor, which

Table 1
Carbon mass balance of each bioreactor.

		Q ($L \cdot d^{-1}$)	INFLUENT		EFFLUENT		REMOTION		COD ΔMLVSS (g $O_2 \cdot d^{-1}$)	CO_2 GENERATION (g $CO_2 \cdot d^{-1}$)
			COD (g $O_2 \cdot d^{-1}$)	BOD_5 (g $O_2 \cdot d^{-1}$)	COD (g $O_2 \cdot d^{-1}$)	BOD_5 (g $O_2 \cdot d^{-1}$)	COD (g $O_2 \cdot d^{-1}$)	BOD_5 (g $O_2 \cdot d^{-1}$)		
R1	**Average**	24.00	12.44	9.36	2.93	2.04	9.84	7.32	0.05	15.38
	SD	0.00	1.18	1.82	1.20	1.50	0.21	0.84	0.07	0.22
R2	**Average**	24.00	13.67	9.42	6.80	3.36	7.44	6.06	0.06	11.59
	SD	0.00	1.46	2.38	3.54	1.89	3.67	0.69	0.06	5.67
R3	**Average**	24.00	12.92	9.78	5.49	3.54	7.73	6.24	0.05	12.07
	SD	0.00	0.85	1.75	2.76	2.17	2.14	1.56	0.05	3.29
R4	**Average**	24.00	13.42	10.14	6.96	5.52	6.72	4.62	0.02	10.52
	SD	0.00	1.19	2.08	2.03	1.53	0.76	1.40	0.03	1.15

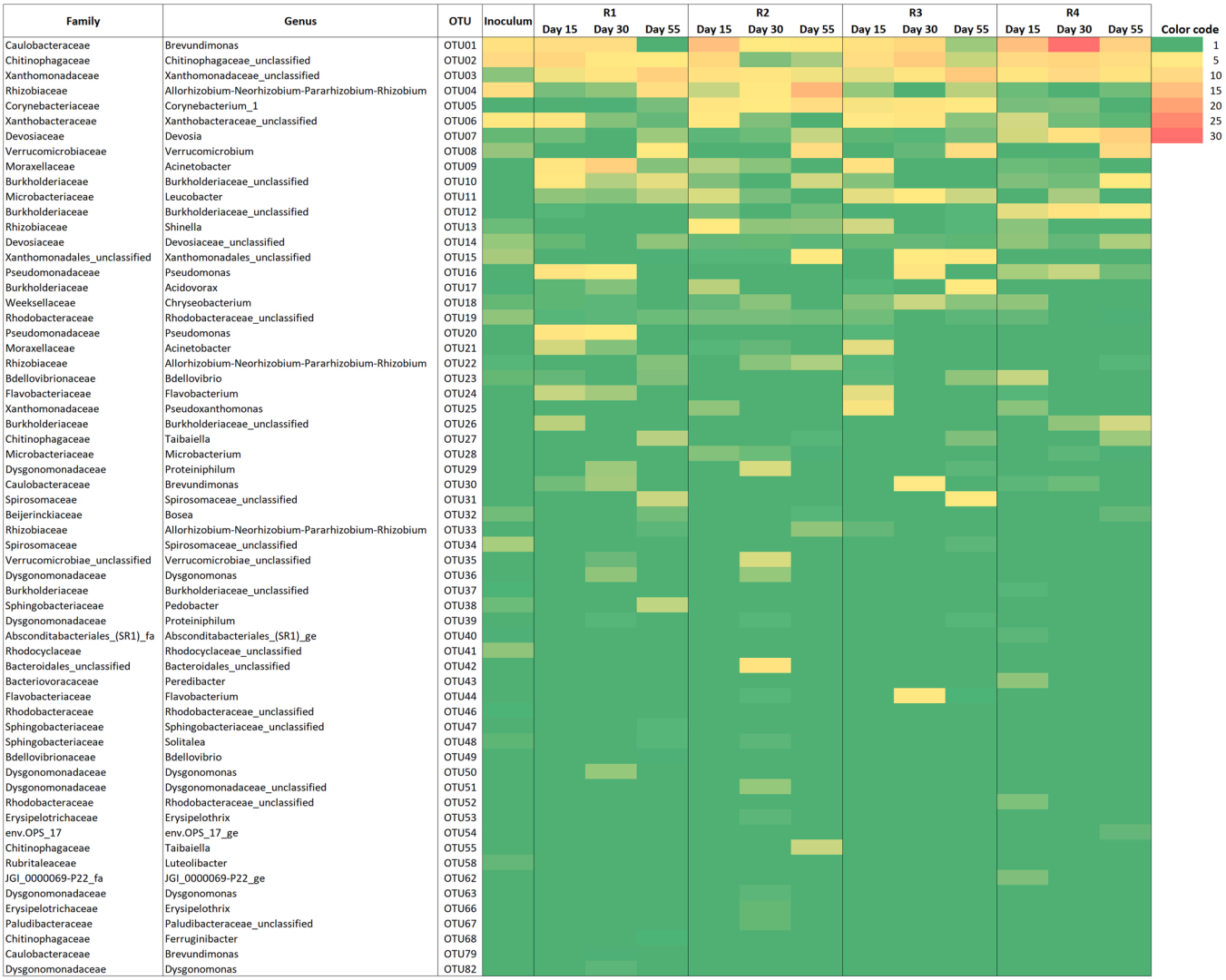

Fig. 3. Heat map of the dominant prokaryotic OTUs affiliated with the *Bacteria* superkingdom, with more than 1% of total relative abundance.

decreased from 8.2% on day 15–0.6% of total relative abundance on day 55. The *Xanthobacteraceae* family, which was dominant in the inoculum, was a detriment in the R1 bioreactor, from 5.9% to 1.4% of total relative abundance at the end of experimentation. A reduction in the *Chitinophagaceae* family (OTU02) was also observed, but it was less pronounced than for *Brevundimonas*, from 8.8% to 5.5% of relative abundance. *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* decreased their relative abundance, but at the end of the experiment, it recovered to 8.1%. OTU03 appeared and represented 11.1% of the total relative abundance at the end of the experiment and was taxonomically affiliated with the *Xanthomonadaceae* family. This family has been reported to have a capacity for EPS excretion, and some genera have even been described with nitrifying and denitrifying activity [55,56]. Other dominant genera in the R1 bioreactor during the experiment were *Acinetobacter* (OTU09 and OTU21) and *Pseudomonas* (OTU16 and OTU20). *Acinetobacter* had been reported as filamentous bacteria with denitrifying activity [52]. The initial proliferation and the following detriment of the total relative abundance of this microorganism could be justified by the granule formation role that *Acinetobacter* played [57]. So, its presence probably indicated a granular maturation process during the first

month of operation in the R1 bioreactor design. *Acinetobacter* and *Pseudomonas* have been reported as having some strains with strong self-aggregation ability and syntrophy capacity [58,59]. These results pointed out that the granular compactness in the R1 configuration could be related to the high relative abundance of these two genera. The results suggested that the prokaryotic community of granular biomass in the R1 bioreactor was stable, and it was quickly selected in response to the changes in operational conditions, which suggests a short start-up period.

In bioreactor R2, OTU01 and OTU02, affiliated with the *Brevundimonas* genus and the *Chitinophagaceae* family, increased their total relative abundances during the first 15 days of the experiment (14.2% and 9.3%, respectively). Later, their relative abundances had an evident decrease to 4.8% and 2.8%, respectively. There was also a strong depletion of the relative abundance of OTU06, from 5.9% in the inoculum to 0.2% at the end of the experiment. On the contrary, OTU04, taxonomically affiliated with the *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* genus, increased their relative abundance during the experiment until reaching 16.2%. A similar pattern was observed for OTU05, OTU08 and OTU15, which belonged to the *Corynebacterium* and *Verrucomicrobium* genera and the *Xanthomonadales* order, respectively, and were non-dominant OTUs in the inoculum. It is essential to point out that OTU13, belonging to *Shinella*, achieved 4.9% of total relative abundance during the first 15 days, suggesting its potential role in the granular aggregation process. Cydzik-Kwiatkowska et al. [60] reported the role played by the *Shinella* genus in granular formation processes due to its capability to produce and segregate a significant amount of EPS. Similarly, the increment at day 55 of *Devosia* (3.4%), represented by OTU07, suggested that granules were not yet well structured nor stabilised at the end of the experiment, because this genus is well-known for its capacity to excrete EPS, which is essential in the initial stages of granular formation [61]. Therefore, results could indicate that the R2 configuration needs more time than the R1 configuration to reach a stable microbial population able to maintain granular biomass in terms of physical parameters such as mean size and settling velocity.

The bacterial community in the R3 bioreactor showed, as in the case of the R1 and R2 bioreactors, temporal dynamics relative to the inoculum initially used for its start-up. The OTUs OTU01, OTU02 and OTU06 increased their relative abundance during the first month of the experiment. Many dominant OTUs were displaced at day 30, leaving the final dominance, with more than 5% of total relative abundance, to the *Xanthomonadaceae* family (13.5%), the *Verrucomicrobium* genus (8.3%) and the *Acidovorax* genus (5.6%), corresponding to OTU03, OTU08 and OTU17, respectively. At day 55, a strong depletion of the inoculum-dominant phylotypes was noticed, with 2.7%, 3.3%, 3.2% and 2.2% of total relative abundance for OTU01, OTU02, OTU04 and OTU06, respectively. Again, the results suggested that the configuration of the CFR was strongly linked to the selection of a granule-forming prokaryotic community and consequently to the functionality of the technology.

In the same way, as in the previous cases, the bacterial communities of the R4 bioreactor responded to their design characteristics. Thus, a proliferation of the *Brevundimonas* genus (OTU01) was recorded for microbial populations, achieving more than 30% of the relative abundance at day 30. On the last day of operation, *Brevundimonas* decreased to 11.0%, and the population was codominant with OTU07 from the *Devosia* genus, with 11.4% of the relative abundance. Therefore, these results suggested the encouragement of *Devosia* in the presence of *Brevundimonas*, modifying the dominant bacterial structure. The assistance of *Devosia* in granular formation reported by Alves et al. [61] was not corroborated in this reactor because results obtained in terms of settling velocity, as well as the in situ observation of the reactor, pointed out the loss of granular biomass compactness. These differences could suggest that the promotion of granular compactness in Alves et al. [61] was not only due to *Devosia*, but a syntrophic consortium with other genera. Other microbial dominant phylotypes at day 55 were OTU02 (8.3%), OTU08 (9.9%) and three OTUs belonging to the *Burkholderiaceae* family (OTU10, OTU12 and OTU26), achieving more than 14% of relative abundance. The dominance of *Brevundimonas*, which are potential denitrifying bacteria, demonstrated that the R4 design did not exert the same selection pressure on the microbial community.

Results suggested that the different configurations of AGS-CFR bioreactors tested in our study not only had different hydraulic conditions and performance but also had differences in the bioreactor prokaryotic communities that influenced the granulation process and the stability of the granules formed. According to these results, R1 was the bioreactor that allowed the fastest start-up period due to its successful microbial selection.

3.3.2. Eukaryotic community dynamics

In terms of eukaryotic communities, 15 OTUs dominated the bioreactors with more than 1% of the total relative abundance (Fig. 4). Firstly, the inoculum presented a low diversity, due to the dominance of OTU01, a microorganism from the *Ascomycota* phylum with 63.3% of relative abundance, OTU02 and OTU04, which belonged to the *Hypocreales* order (13.1%) and the *Trichosporonaceae* family (12.5%) respectively, followed by OTU10, affiliated with the *Tremellomycetes* class with 3.7% relative abundance. *Ascomycota* has been reported to lead to organic compound biodegradation, detoxification and aggregation of sludge flocs [62] and can act as a core for granule formation given their filamentous nature [2]. The *Trichosporonaceae* family has been reported by having an essential role to form the granular structural core and acting as a union bridge for microbial colonisation [15]. The *Tremellomycetes* class has also been found in aerobic granular sludge, possibly related to permitting a well-granulated structure and hence related to good settling ability [32].

The eukaryotic community in the R1 bioreactor was dominated by OTU01 (affiliated with *Ascomycota*), with 62.1% of the total relative abundance at the end of the experiment. However, some changes were produced regarding the rest of the OTUs. A reduction of total relative abundance was detected for the *Hypocreales* order and the *Trichosporonaceae* family that corresponded with OTU02 and OTU04, respectively. At day 55, they only represented 5.5% and 2.3% of the total relative abundance of the eukaryotic community, respectively, while OTU10 and OTU09 almost disappeared. On the contrary, OTU03, which was affiliated with the *Peronosporomycetes* class (formerly the *Oomycota* class), was selected for, especially at the end of the experiment, when it reached 15.8% of the total relative abundance. The *Oomycota* class is a group of decomposer and pathogen microorganisms of other eukaryotes [63]. In the same way, OTU07 was encouraged, reaching 8.1% of relative abundance.

In the R2 bioreactor, after 15 days of inoculation, OTU03, from *Peronosporomycetes* and with 44.6% of the total relative abundance, together with OTU05 (13.5%), taxonomically affiliated with *Ascomycota,* and OTU07 (10.3%) produced a displacement of OTU01 that decreased sharply their relative abundance until they reached 16.5%. However, this situation did not endure since a strong increment in the relative abundance of OTU01 was recorded, achieving the 92.5% of the system representation on days 30 and 55. At that moment, the rest of the OTUs almost disappeared, except OTU02, with 4.1% and 3.1% of total relative abundance at days 30 and 55, respectively.

Regarding the R3 bioreactor, OTU01 continued its dominant role on days 15 and 55, with total relative abundances of 71.6% and 74.5%, respectively. However, on day 30, there were changes due to the proliferation of OTU02 and OTU03.

The eukaryotic community in the R4 reactor did not respond in the same way as in the rest of the CFRs tested. The diversity of the community was higher, and contrary to the other designs, OTU01 was not the dominant phylotype. At day 15, OTU03 dominated, with 74.3% of total relative abundance, while a strong reduction was observed in inoculum-dominant OTUs, whose relative abundance decreased below 5%. After that, diverse OTUs shared the dominance of the eukaryotic community, such as OTU02, OTU05, OTU01 and OTU03, which

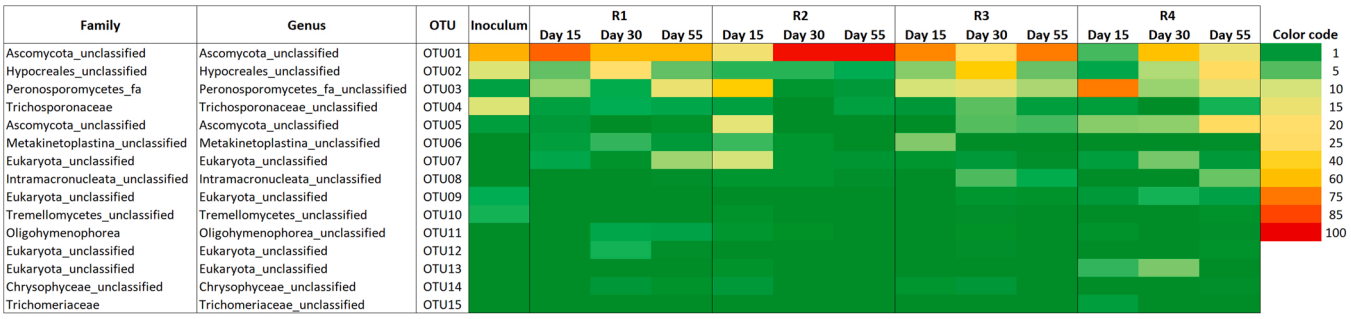

Fig. 4. Heat map that showed the dominant eukaryotic OTUs with more than 1% of total relative abundance.

codominated with 26.5%, 25.7%, 15.5% and 14.5% of the relative abundance at day 55, respectively. This change in the population could be linked to the system destabilisation observed in the R4 bioreactor. Some representative phylotypes of the *Ascomycota* phylum, such as OTU01, may be related to the stability of the granular microbial community.

The study of the eukaryotic community in the AGS-CFRs demonstrated a diversity linked with the design of the bioreactor. It was evident that the different configurations of the system affected not only the bacterial community but also the eukaryotic microbial structure. As a result, the high representation of the *Ascomycota* fungi in the granules was linked with better granular configuration and stability. This could be observed in the R4 bioreactor, where granules disintegrated, possibly caused by the detriment of these organisms over operational time.

3.4. α and β-diversity

The α-diversity indices for the prokaryotic community are summarised in Table S1. A loss of diversity was recorded in all reactors; however, regarding the Simpson and Shannon-Wienner indices, results showed that the R1 bioreactor had the most diverse population at the end of the experiment. Concerning evenness, all reactors at day 15 experienced a strong depletion, according to the obtained Pielou's Evenness values. After that, reactors recovered their evenness except for the R4 bioreactor, in which a pronounced depletion was perceived, which was corroborated in the community study by the massive proliferation of the *Brevundimonas* genus at day 30. In the final period, the reactor with more evenness was also R1. There was a general decrease in terms of species richness (Chao-1 index) between the inoculum and the final period of experimentation. Results suggested that the R4 configuration had an important detriment in terms of diversity, evenness and species richness generated by the proliferation of OTU01 (*Brevundimonas*), while the most diverse and evenness samples belonged to the R1 bioreactor.

These indices were also calculated for the eukaryotic community (Table S2). In general terms, the prokaryotic community was more diverse and had more species richness than the eukaryotic community, following a common trend described by Muñoz-Palazon et al. [14]. According to the Simpson and Shannon-Wienner indices, R4 was the reactor that had more eukaryotic diversity, followed by R1. A sharp depletion in diversity was detected in the R2 bioreactor, an aspect that could be corroborated by the community study (Fig. 4), where it was shown how, at days 30 and 55, an unclassified *Ascomycota* represented 92.5% of the total relative abundance. Pielou's evenness followed the same pattern as previously cited indices, with a rise in the R4 and R1 bioreactors, while R2 and R3 experienced a reduction of evenness. Regarding the Chao-1 index, there was a very low species richness. However, this indicator increased for the R1 bioreactor at days 30 and 55 and for the R3 bioreactor at day 55.

The β-diversity indices were calculated for pairs of samples to compare the microbial community and over the entire experiment on the different CFR configurations. For the prokaryotic community (Fig. S2), differences between the inoculum and the R1 bioreactor were produced at the beginning of the experiment, while for the rest of the reactors, the differences occurred more progressively and slowly. With these results, R1′s baffle was able to more quickly select the microbial species of the biomass, as was previously described in the dynamics of the microbial community section. Also, it was remarkable that the R3 bioreactor at day 30 had strong dissimilarities when it was compared with the rest of the reactors and operational times. This could be due to the proliferation of OTU06, which had 44.4% of the total relative abundance. For the eukaryotic community (Fig. S2), differences between samples were less pronounced than for the prokaryotic community. High dissimilarities were found between the inoculum and the R1 bioreactor, with a Whittaker index of 0.48 from day 15 to the end of the experiment. On the other hand, R2 and R3 were the reactors that kept their community more similar to the granular sludge used as inoculum. Moreover, the R4 bioreactor had strong differences from the R1 and R2 bioreactors. These results emphasised the efficient selection of biomass produced by the R1 configuration.

3.5. Similarity Percentages analysis

The SIMPER analysis showed which OTUs were responsible for dissimilarities between pairs of samples (Fig. S3A). OTU01 belonged to *Brevundimonas* and was responsible for the dissimilarities between R4 and the inoculum as well as the rest of CFR configurations due to strong proliferation, as was corroborated in the prokaryotic community study (Fig. 3). OTU02, OTU03 and OTU04 also caused differences between reactors, especially OTU04 when it was compared to the R3 and R4 bioreactors versus both the inoculum and R2 bioreactor. In addition, exclusively for the inoculum and the R1 configuration, the OTUs that most contributed to dissimilarities were OTU09, OTU20 and OTU16, affiliated with *Acinetobacter* and two OTUs affiliated with *Pseudomonas*. This fact reinforces the cooperation and syntrophic relations of the bacterial population of the R1, suggesting the role played by these OTUs in the high performance of R1′s granules. On the other hand, OTU07 and OTU12 only contributed to the difference between R4 and the rest of the reactors, whose relative abundances were notably higher on all operational days in the R4, but it was irrelevant in the rest of the reactors.

Regarding the SIMPER analysis for the eukaryotic community (Fig. S3B), it was possible to see how OTU01, belonging to the *Ascomycota* phylum, was the main microorganism that produced dissimilarities in all reactors (46.9–78.6%), with a remarkable record in R2. The *Hypocreales* order (OTU02) was responsible for dissimilarities for all samples, especially to differentiate the inoculum for the reactors and R3 against the rest of the CFR configurations. Moreover, the *Peronosporomycetes* class (OTU03) produced dissimilarities when comparing all samples, especially for R4. The *Trichosporonaceae* family (OTU04) was responsible for causing divergences between the inoculum and the different CFR designs, due to its high representation in the inoculum sample and its posterior depletion in the rest of the samples, as was

corroborated by the community population study (Fig. 4). On the other hand, OTU09 and OTU10 were specifically associated with dissimilarities in the inoculum when it was compared with the reactors. To a lesser degree, OTU05, OTU06, OTU07 and OTU08 contributed to the dissimilarities between the different bioreactor configurations. The OTUs that caused preservation of similarity between samples were two unclassified Eukaryotes (OTU12 and OTU13) and OTU11, corresponding with a ciliate of the *Oligohymenophorea* family, which did not have the importance and dominance as was reported by some authors [14,64].

3.6. Multivariate redundancy analyses

Multivariate redundancy analyses (RDA) were carried out to determine the relationship between the microbial OTUs, biological samples and physicochemical performance of each configuration of CFR. The RDAs for dominant prokaryotic OTUs (logarithm), operational days and operational parameters (logarithm) of each reactor are shown in Fig. 5. For the R1 bioreactor, the RDA (Fig. 5A) indicated that all parameters measured had a positive linkage between them and OTU03, OTU05, OTU07, OTU10, OTU11, OTU12, OTU22, OTU27, OTU28, OTU31, OTU33, OTU49, OTU54 and OTU68. There was a strong correlation between the BOD_5 removal ratio and OTU07 belonging to the *Devosia* genus, OTU22 related to the *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* genus, OTU27 and OTU55, taxonomically affiliated to *Taibaiella*, OTU31 from the *Spirosomaceae* family, OTU49 belonging to the *Bdellovibrio* genus, OTU54, a microorganism from the *env.OPS_17*

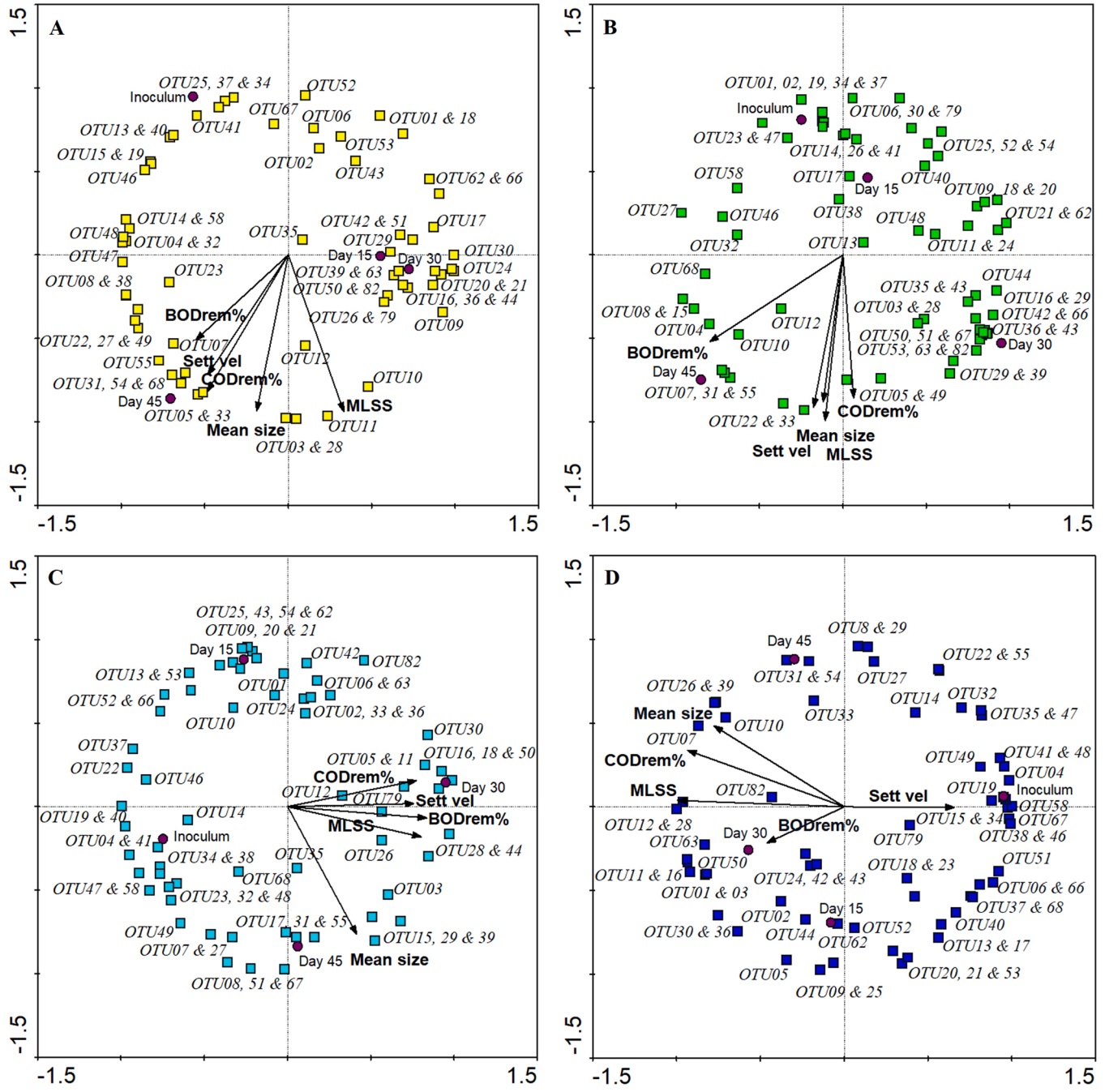

Fig. 5. Multivariate redundancy analysis of the most abundant prokaryotes with physicochemical parameters (logarithm) for CFR R1(A), R2(B), R3 (C) and R4 (D) bioreactors; operational days correspond with periods: inoculum: from day 0 to day 14, Day 15: from day 15 to day 29, Day 30: from day 30 to day 44 and Day 45: from day 45 to day 55.

genus and OTU68 affiliated with *Ferruginibacter*. Some of these bacteria have been reported as hydrolyser microorganisms for acetate and other substrates, for example, OTU27, OTU54, OTU55 and OTU68 [48,65,66]. This agrees with our results due to simple hydrolysis of acetate corresponds, with an almost complete BOD_5 removal performance. Contrarily, OTU01 and OTU02, which were dominant phylotypes, had a negative and opposite correlation with high physicochemical performance and operational parameters. Besides, higher organic matter removal performance was reached at the end of the operation, on days 45–55, as well as the achievement of the largest mean diameter size and fastest settling velocity. On the contrary, the beginning of the experiment had a negative correlation with the physicochemical parameters.

The RDA of the R2 reactor is shown in Fig. 5B. The BOD_5 removal ratio in the R2 bioreactor was positively correlated with OTU04, OTU07, OTU08, OTU10, OTU12, OTU15, OTU31 and OTU55. Moreover, COD removal, MLSS, settling velocity and mean size had a strong linkage with OTU05, OTU22, OTU33 and OTU49. OTU05 was a bacterium affiliated with the genus *Corynebacterium*, which has been reported as a heterotrophic nitrifier with COD removal capacity [67], a fact that agreed with our results.

For the R3 bioreactor (Fig. 5C), OTU05, OTU11, OTU12, OTU16, OTU18, OTU26, OTU28, OTU30, OTU44, OTU50 and OTU79 had a positive linkage with the BOD_5 and COD removal ratio, MLSS and settling velocity. These parameters obtained a stronger correlation with the period between 30 and 44 days, which suggested that a higher organic matter removal ratio was achieved in this period, while later,

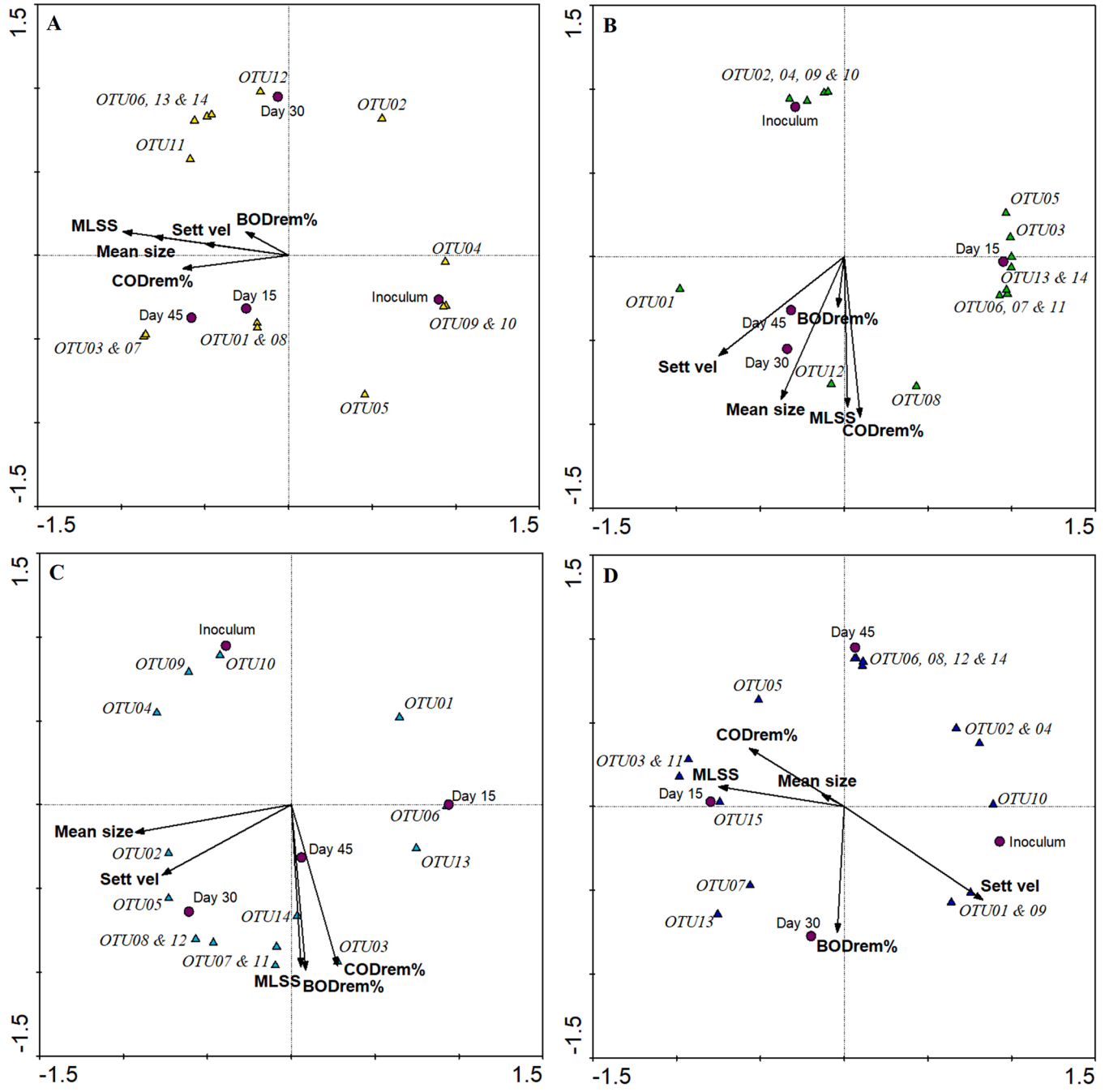

Fig. 6. Multivariate redundancy analysis of the most abundant eukaryotes with physicochemical parameters (logarithm) for CFR R1(A), R2(B), R3(C) and R4(D) bioreactors; operational days correspond with periods: Inoculum: from day 0 to day 14, Day 15: from day 15 to day 29, Day 30: from day 30 to day 44 and Day 45: from day 45 to day 55.

the R3 bioreactor decreased in its functional stability in terms of performance. Mean size was positively correlated with OTU03, OTU15, OTU17, OTU29, OTU31, OTU35, OTU39 and OTU55.

Finally, RDA calculated for the R4 bioreactor showed that a high number of its dominant OTUs had a negative correlation with settling velocity (Fig. 5D). These OTUs were OTU01, OTU03, OTU07, OTU12 and, to a lesser degree, OTU10 and OTU02. This fact, joined with the positive correlation between the inoculum and the settling velocity, indicated that granules lost density and stability during the experimentation time. Moreover, this result corroborated the idea to reject this CFR configuration, due to it did not allow the conformation of compact granules for long-term operation. Concerning the rest of the parameters, MLSS had a positive linkage with OTU12, OTU28 and OTU82, BOD_5 removal performance with OTU01, OTU03, OTU11, OTU16, OTU50 and OTU63, and finally, mean size and COD removal rate were correlated positively with OTU07, OTU10, OTU26 and OTU39.

The RDA calculated for eukaryotic communities concerning the R1 (Fig. 6A), showed that all parameters studied had a positive linkage with OTU01, which was the most dominant phylotype in the R1, as well as with OTU03 and OTU07 (also dominant OTUs), followed by OTU08 and OTU11, while a negative correlation with OTU04 from the *Trichosporonaceae* family, an unclassified Eukaryote (OTU09) and OTU10 belonging to the *Tremellomycetes* class. The positive correlation of all physicochemical factors and the most dominant phylotypes demonstrated the high performance and granular structure that the R1 was able to achieve.

The RDA analysis for the R2 bioreactor (Fig. 6B), showed that OTU01, OTU08 and OTU12, as well as the period from day 30 to day 55 (corresponding with days 30 and 45 in RDA), had a positive correlation with all the physicochemical parameters. In the same way as the R1 bioreactor, all parameters had a negative linkage with OTU04, OTU09 and OTU10, with the addition of OTU02, a fungus that belongs to the *Hypocreales* order. Moreover, OTU03 and OTU05, which were dominant in this reactor on day 15, were negatively correlated with the settling ability, proving the slow conformation of stable granular biomass.

Regarding the R3 configuration, the RDA showed that granular conformation, represented by the mean size and settling ability, was positively correlated with OTU02 and OTU05, which were well represented in the system, especially on day 30 (Fig. 6C). On the other hand, organic matter removal and MLSS had a positive linkage with OTU03, OTU07, OTU11 and OTU14. However, OTU01, which represented more than 70% of total relative abundance at days 15 and 55, had a strong negative correlation with the settling velocity, also explaining why this reactor did not have well-conformed and dense granules.

Finally, for the RDA related to the R4 bioreactor (Fig. 6D), the COD removal ratio, MLSS and mean size were positively correlated with OTU03, OTU05, OTU11 and OTU15, while settling velocity had a positive linkage with OTU01 and OTU09. On the contrary, other OTUs that dominated during the whole experimentation (OTU03 and OTU05) were negatively correlated with the settling velocity. This fact could suggest the destabilisation of granular biomass in the R4 bioreactor.

4. Conclusions

Four aerobic granular sludge continuous-flow reactors were designed in a single-chamber configuration to evaluate their ability to maintain a stable granulation and high removal performance at a steady state. The bioreactor that included a small settler located in the upper lateral part coupled to an elbow tube (R1 configuration) achieved the fastest start-up (11 days) and the most efficient results for stable granule conformation, granular biomass with high microbial diversity (functional diversity) and values above 95% of organic matter removal. Moreover, the R1 bioreactor was able to remove floccular biomass and formed highly compact granules, reaching more than 100 $m \cdot h^{-1}$ of settling velocity. Analysis of the bioreactors' microbial community showed how the R1 bioreactor produced a quick selection of microorganisms adapted to the CFR operational conditions. However, other CFR designs, especially the R4 configuration, were not capable of maintaining stable granules, giving rise, after 55 days of operation, to effluents with evident turbidity because of the presence of biomass not retained in the bioreactors. The R1 configuration at the lab scale could be used as a model for future research to achieve the implementation of AGS-CFR at full-scale, due to the simple design and the excellent results obtained, such as high removal performance, ability to establish dense and well-structured granular biomass for steady-state and short times of start-up. For this reason, further research should be tested the design and determine optimal operational parameters to treat wastewater at full scale.

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CRediT authorship contribution statement

Aurora Rosa-Masegosa: Methodology, Investigation, Data curation, Formal analysis, Writing – original draft preparation. **Barbara Muñoz-Palazon**: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Writing – original draft. **Susanna Gorrasi:** Methodology, Investigation, Writing – review & editing. **Massimiliano Fenice:** Data curation, Investigation, Writing – review & editing. **Alejandro Gonzalez-Martinez:** Conceptualization, Supervision, Writing – review & editing. **Jesus Gonzalez-Lopez:** Funding acquisition, Supervision, Project administration, Writing – review & editing.

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. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jece.2023.109938.

References

[1] C.L. Amorim, A.S. Maia, R.B.R. Mesquita, A.O.S.S. Rangel, M.C.M. van Loosdrecht, M.E. Tiritan, P.M.L. Castro, Performance of aerobic granular sludge in a sequencing batch bioreactor exposed to ofloxacin, norfloxacin and ciprofloxacin, Water Res. 50 (2014) 101–113, https://doi.org/10.1016/j.watres.2013.10.043.

[2] Y.V. Nancharaiah, G.K.K. Reddy, Aerobic granular sludge technology: mechanisms of granulation and biotechnological applications, Bioresour. Technol. 247 (2018) 1128–1143, https://doi.org/10.1016/j.biortech.2017.09.131.

[3] A. Rosa-Masegosa, B. Muñoz-Palazon, A. Gonzalez-Martinez, M. Fenice, S. Gorrasi, J. Gonzalez-Lopez, New advances in aerobic granular sludge technology using continuous flow reactors: engineering and microbiological aspects, Water 13 (2021) 1–20, https://doi.org/10.3390/w13131792.

[4] S.L. de Sousa Rollemberg, A.R. Mendes Barros, P.I. Milen Firmino, A. Bezerra dos Santos, Aerobic granular sludge: cultivation parameters and removal mechanisms,

Bioresour. Technol. 270 (2018) 678–688, https://doi.org/10.1016/j.biortech.2018.08.130.
[5] B.M. Wilén, R. Liébana, F. Persson, O. Modin, M. Hermansson, The mechanisms of granulation of activated sludge in wastewater treatment, its optimization, and impact on effluent quality, Appl. Microbiol. Biotechnol. 102 (2018) 5005–5020, https://doi.org/10.1007/s00253-018-8990-9.
[6] E. Suja, Y.V. Nancharaiah, T.V. Krishna Mohan, V.P. Venugopalan, Denitrification accelerates granular sludge formation in sequencing batch reactors, Bioresour. Technol. 196 (2015) 28–34, https://doi.org/10.1016/j.biortech.2015.07.045.
[7] M.K.H. Winkler, R. Kleerebezem, M. Strous, K. Chandran, M.C.M.Van Loosdrecht, Factors influencing the density of aerobic granular sludge, Appl. Microbiol. Biotechnol. 97 (2013) 7459–7468, https://doi.org/10.1007/s00253-012-4459-4.
[8] J.P. Bassin, D.C. Tavares, R.C. Borges, M. Dezotti, Development of aerobic granular sludge under tropical climate conditions: the key role of inoculum adaptation under reduced sludge washout for stable granulation, J. Environ. Manag. 230 (2019) 168–182, https://doi.org/10.1016/j.jenvman.2018.09.072.
[9] Y.V. Nancharaiah, M. Sarvajith, T.V. Krishna Mohan, Aerobic granular sludge: the future of wastewater treatment, Curr. Sci. 117 (2019) 395–404, https://doi.org/10.18520/cs/v117/i3/395-404.
[10] R.D.G. Franca, H.M. Pinheiro, M.C.M. van Loosdrecht, N.D. Lourenço, Stability of aerobic granules during long-term bioreactor operation, Biotechnol. Adv. 36 (2018) 228–246, https://doi.org/10.1016/j.biotechadv.2017.11.005.
[11] L. van den Berg, C.M. Kirkland, J.D. Seymour, S.L. Codd, M.C.M. van Loosdrecht, M.K. de Kreuk, Heterogeneous diffusion in aerobic granular sludge, Biotechnol. Bioeng. 117 (2020) 3809–3819, https://doi.org/10.1002/bit.27522.
[12] M.K.H. Winkler, C. Meunier, O. Henriet, J. Mahillon, M.E. Suárez-Ojeda, G. Del Moro, M. De Sanctis, C. Di Iaconi, D.G. Weissbrodt, An integrative review of granular sludge for the biological removal of nutrients and recalcitrant organic matter from wastewater, Chem. Eng. J. 336 (2018) 489–502, https://doi.org/10.1016/j.cej.2017.12.026.
[13] F. Cai, L. Lei, Y. Li, Y. Chen, A review of aerobic granular sludge (AGS) treating recalcitrant wastewater: refractory organics removal mechanism, application and prospect, Sci. Total Environ. 782 (2021), 146852, https://doi.org/10.1016/j.scitotenv.2021.146852.
[14] B. Muñoz-Palazon, A. Rodriguez-Sanchez, M. Hurtado-Martinez, I.M. de Castro, B. Juarez-Jimenez, A. Gonzalez-Martinez, J. Gonzalez-Lopez, Performance and microbial community structure of an aerobic granular sludge system at different phenolic acid concentrations, J. Hazard. Mater. 376 (2019) 58–67, https://doi.org/10.1016/j.jhazmat.2019.05.015.
[15] B. Muñoz-Palazon, A. Mikola, A. Rosa-Masegosa, R. Vilchez-Vargas, A. Link, J. Gonzalez-Lopez, A. Gonzalez-Martinez, Novel application of aerobic granular biofilm systems for treating nitrate-polluted groundwater at low temperature: microbial community and performance, J. Environ. Chem. Eng. 10 (2022), https://doi.org/10.1016/j.jece.2022.107818.
[16] A. Rosa-Masegosa, L. Perez-Bou, B. Muñoz-Palazon, A. Monteoliva-García, A. Gonzalez-Martinez, J. Gonzalez-Lopez, D. Correa-Galeote, Effects of sulphur amino acids on the size and structure of microbial communities of aerobic granular sludge bioreactors, Amino Acids (2022), https://doi.org/10.1007/s00726-022-03168-y.
[17] S.J. Sarma, J.H. Tay, A. Chu, Finding knowledge gaps in aerobic granulation technology, Trends Biotechnol. 35 (2017) 66–78, https://doi.org/10.1016/j.tibtech.2016.07.003.
[18] S. Borzooei, R. Teegavarapu, S. Abolfathi, Y. Amerlinck, I. Nopens, M.C. Zanetti, 2019. Impact Evaluation of Wet-Weather Events on Influent Flow and Loadings of a Water Resource Recovery Facility, in: New Trends Urban Drain. Model., 2019: pp. 706–711. https://doi.org/10.1007/978–3-319–99867-1_122.
[19] M. Geng, S. You, H. Guo, F. Ma, X. Xiao, J. Zhang, X. Ma, Response of aerobic granular sludge to loading shock: performance and proteomic study, Chem. Eng. J. 444 (2022), 136458, https://doi.org/10.1016/j.cej.2022.136458.
[20] H. Chen, A. Li, D. Cui, C. Cui, F. Ma, Evolution of microbial community and key genera in the formation and stability of aerobic granular sludge under a high organic loading rate, Bioresour. Technol. Rep. 7 (2019), 100280, https://doi.org/10.1016/j.biteb.2019.100280.
[21] L.D.A. Purba, H.T. Ibiyeye, A. Yuzir, S.E. Mohamad, K. Iwamoto, A. Zamyadi, N. Abdullah, Various applications of aerobic granular sludge: a review, Environ. Technol. Innov. 20 (2020), 101045, https://doi.org/10.1016/j.eti.2020.101045.
[22] M. Hurtado-Martinez, B. Muñoz-Palazon, V.M. Robles-Arenas, A. Gonzalez-Martinez, J. Gonzalez-Lopez, Biological nitrate removal from groundwater by an aerobic granular technology to supply drinking water at pilot-scale, J. Water Process Eng. 40 (2021), https://doi.org/10.1016/j.jwpe.2020.101786.
[23] H. Aqeel, M. Basuvaraj, M. Hall, J.D. Neufeld, S.N. Liss, Microbial dynamics and properties of aerobic granules developed in a laboratory-scale sequencing batch reactor with an intermediate filamentous bulking stage, Appl. Microbiol. Biotechnol. 100 (2016) 447–460, https://doi.org/10.1007/s00253-015-6981-7.
[24] L.L. Moura, K.L.S. Duarte, E.P. Santiago, C.F. Mahler, J.P. Bassin, Strategies to re-establish stable granulation after filamentous outgrowth: Insights from lab-scale experiments, Process Saf. Environ. Prot. 117 (2018) 606–615, https://doi.org/10.1016/j.psep.2018.06.005.
[25] K. Qi, Z. Li, C. Zhang, X. Tan, C. Wan, X. Liu, L. Wang, D.J. Lee, Biodegradation of real industrial wastewater containing ethylene glycol by using aerobic granular sludge in a continuous-flow reactor: Performance and resistance mechanism, Biochem. Eng. J. 161 (2020), 107711, https://doi.org/10.1016/j.bej.2020.107711.
[26] Y. Sun, B. Angelotti, Z.W. Wang, Continuous-flow aerobic granulation in plug-flow bioreactors fed with real domestic wastewater, Sci. Total Environ. 688 (2019) 762–770, https://doi.org/10.1016/j.scitotenv.2019.06.291.
[27] D. Zhang, Y. Sun, B. Angelotti, Z.W. Wang, Understanding the dewaterability of aerobic granular sludge formed in continuous flow bioreactors treating real domestic wastewater: Is it really better than that of activated sludge? J. Water Process Eng. 36 (2020), 101253 https://doi.org/10.1016/j.jwpe.2020.101253.
[28] J. Zou, Y. Tao, J. Li, S. Wu, Y. Ni, Cultivating aerobic granular sludge in a developed continuous-flow reactor with two-zone sedimentation tank treating real and low-strength wastewater, Bioresour. Technol. 247 (2018) 776–783, https://doi.org/10.1016/j.biortech.2017.09.088.
[29] J.L. Yan, Y.W. Cui, J.L. Huang, Continuous flow reactors for cultivating aerobic granular sludge: configuration innovation, principle and research prospect, J. Chem. Technol. Biotechnol. 96 (2021) 2721–2734, https://doi.org/10.1002/jctb.6791.
[30] D. Xu, J. Li, J. Liu, X. Qu, H. Ma, Advances in continuous flow aerobic granular sludge: a review, Process Saf. Environ. Prot. 163 (2022) 27–35, https://doi.org/10.1016/j.psep.2022.05.018.
[31] A. Gonzalez-Martinez, J. Gonzalez-Lopez, A. Rosa-Masegosa, 2022. Biorreactor granular aeróbico, 2022.
[32] B. Muñoz-Palazon, C. Pesciaroli, A. Rodriguez-Sanchez, J. Gonzalez-Lopez, A. Gonzalez-Martinez, Pollutants degradation performance and microbial community structure of aerobic granular sludge systems using inoculums adapted at mild and low temperature, Chemosphere 204 (2018) 431–441, https://doi.org/10.1016/j.chemosphere.2018.04.062.
[33] APHA, 2017. Standard Methods for the Examination of Water and Wastewater , 2017.
[34] B.Y.P. Moy, J.H. Tay, S.K. Toh, Y. Liu, S.T.L. Tay, High organic loading influences the physical characteristics of aerobic sludge granules, Lett. Appl. Microbiol. 34 (2002) 407–412, https://doi.org/10.1046/j.1472-765X.2002.01108.x.
[35] S.R. Hoover, N. Porges, Assimilation of dairy wastes by activated sludge: II. The equation of synthesis and rate of oxygen utilization, Sew. Ind. Waste 24 (1952) 306–312.
[36] B. Muñoz-Palazon, A. Rodriguez-Sanchez, M. Hurtado-Martinez, F. Santana, J. Gonzalez-Lopez, L. Mack, A. Gonzalez-Martinez, Polar Arctic Circle biomass enhances performance and stability of aerobic granular sludge systems operated under different temperatures, Bioresour. Technol. 300 (2020), 122650, https://doi.org/10.1016/j.biortech.2019.122650.
[37] T. Stoeck, D. Bass, M. Nebel, R. Christen, M.D.M. Jones, H.W. Breiner, T. A. Richards, Multiple marker parallel tag environmental DNA sequencing reveals a highly complex eukaryotic community in marine anoxic water, Mol. Ecol. 19 (2010) 21–31, https://doi.org/10.1111/j.1365-294X.2009.04480.x.
[38] S. Takahashi, J. Tomita, K. Nishioka, T. Hisada, M. Nishijima, Development of a prokaryotic universal primer for simultaneous analysis of Bacteria and Archaea using next-generation sequencing, PLoS One 9 (2014), https://doi.org/10.1371/journal.pone.0105592.
[39] P.D. Schloss, S.L. Westcott, T. Ryabin, J.R. Hall, M. Hartmann, E.B. Hollister, R. A. Lesniewski, B.B. Oakley, D.H. Parks, C.J. Robinson, J.W. Sahl, B. Stres, G. G. Thallinger, D.J. Van Horn, C.F. Weber, Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities, Appl. Environ. Microbiol. 75 (2009) 7537–7541, https://doi.org/10.1128/AEM.01541-09.
[40] A. Rodriguez-Sanchez, B. Muñoz-Palazon, M. Hurtado-Martinez, P. Maza-Marquez, J. Gonzalez-Lopez, R. Vahala, A. Gonzalez-Martinez, Microbial ecology dynamics of a partial nitritation bioreactor with Polar Arctic Circle activated sludge operating at low temperature, Chemosphere 225 (2019) 73–82, https://doi.org/10.1016/j.chemosphere.2019.03.012.
[41] B. Muñoz-Palazon, A. Rodriguez-Sanchez, M. Hurtado-Martinez, J. Gonzalez-Lopez, P. Pfetzing, A. Gonzalez-Martinez, Performance and microbial community structure of aerobic granular bioreactors at different operational temperature, J. Water Process Eng. 33 (2020), 101110, https://doi.org/10.1016/j.jwpe.2019.101110.
[42] A. Rodriguez-Sanchez, B. Muñoz-Palazon, M. Hurtado-Martinez, A. Mikola, J. Gonzalez-Lopez, R. Vahala, A. Gonzalez-Martinez, Analysis of microbial communities involved in organic matter and nitrogen removal in a full-scale moving bed biofilm reactor located near the Polar Arctic Circle, Int. Biodeterior. Biodegrad. 146 (2020), 104830, https://doi.org/10.1016/j.ibiod.2019.104830.
[43] I. Kocaturk, T.H. Erguder, Investigation of the use of aerobic granules for the treatment of sugar beet processing wastewater, Environ. Technol. (U. Kingd.) 36 (2015) 2577–2587, https://doi.org/10.1080/09593330.2015.1039070.
[44] H. Liu, Y. Li, C. Yang, W. Pu, L. He, F. Bo, Stable aerobic granules in continuous-flow bioreactor with self-forming dynamic membrane, Bioresour. Technol. 121 (2012) 111–118, https://doi.org/10.1016/j.biortech.2012.07.016.
[45] C. Cofré, J.L. Campos, D. Valenzuela-Heredia, J.P. Pavissich, N. Camus, M. Belmonte, A. Pedrouso, P. Carrera, A. Mosquera-Corral, A. Val del Río, Novel system configuration with activated sludge like-geometry to develop aerobic granular biomass under continuous flow, Bioresour. Technol. 267 (2018) 778–781, https://doi.org/10.1016/j.biortech.2018.07.146.
[46] M.F. Khan, L. Yu, J.H. Tay, G. Achari, Coaggregation of bacterial communities in aerobic granulation and its application on the biodegradation of sulfolane, J. Hazard. Mater. 377 (2019) 206–214, https://doi.org/10.1016/j.jhazmat.2019.05.076.
[47] B. Muñoz-Palazon, A. Rosa-Masegosa, R. Vilchez-Vargas, A. Link, S. Gorrasi, J. Gonzalez-Lopez, A. Gonzalez-Martinez, Biological removal processes in aerobic granular sludge for treating synthetic hospital wastewater: effect of temperature, J. Water Process Eng. 47 (2022), https://doi.org/10.1016/j.jwpe.2022.102691.
[48] M. Sarvajith, G.K.K. Reddy, Y.V. Nancharaiah, Textile dye biodecolourization and ammonium removal over nitrite in aerobic granular sludge sequencing batch

reactors, J. Hazard. Mater. 342 (2018) 536–543, https://doi.org/10.1016/j.jhazmat.2017.08.064.
[49] M.K.H. Winkler, J.P. Bassin, R. Kleerebezem, R.G.J.M. van der Lans, M.C.M. van Loosdrecht, Temperature and salt effects on settling velocity in granular sludge technology, Water Res. 46 (2012) 5445–5451, https://doi.org/10.1016/j.watres.2012.07.022.
[50] T.R. Kent, C.B. Bott, Z.W. Wang, State of the art of aerobic granulation in continuous flow bioreactors, Biotechnol. Adv. 36 (2018) 1139–1166, https://doi.org/10.1016/j.biotechadv.2018.03.015.
[51] X. He, S. Zhang, Y. Jiang, M. Li, J. Yuan, G. Wang, Influence mechanism of filling ratio on solid-phase denitrification with polycaprolactone as biofilm carrier, Bioresour. Technol. 337 (2021), 125401, https://doi.org/10.1016/j.biortech.2021.125401.
[52] Q. He, J. Zhang, S. Gao, L. Chen, W. Lyu, W. Zhang, J. Song, X. Hu, R. Chen, H. Wang, J. Yu, A comprehensive comparison between non-bulking and bulking aerobic granular sludge in microbial communities, Bioresour. Technol. 294 (2019), https://doi.org/10.1016/j.biortech.2019.122151.
[53] S.T. O'Donnell, B.E. Rittmann, E. Kavazanjian, Factors controlling Microbially Induced Desaturation and Precipitation (MIDP) via denitrification during continuous flow, Geomicrobiol. J. 36 (2019) 543–558, https://doi.org/10.1080/01490451.2019.1581858.
[54] T. Song, X. Zhang, J. Li, The formation and distinct characteristics of aerobic granular sludge with filamentous bacteria in low strength wastewater, Bioresour. Technol. 360 (2022), https://doi.org/10.1016/j.biortech.2022.127409.
[55] J. Xia, L. Ye, H. Ren, X.X. Zhang, Microbial community structure and function in aerobic granular sludge, Appl. Microbiol. Biotechnol. 102 (2018) 3967–3979, https://doi.org/10.1007/s00253-018-8905-9.
[56] C.E.D. dos Santos, R.B. Costa, C.A.B.S. Rabelo, A.D.N. Ferraz Júnior, G.F. Persinoti, E. Pozzi, E. Foresti, M.H.R.Z. Damianovic, Hacking biofilm developed in a structured-bed reactor (SBRRIA) with integrated processes of nitrogen and organic matter removal, Bioprocess Biosyst. Eng. 44 (2021) 1841–1851, https://doi.org/10.1007/s00449-021-02564-0.
[57] R. Liébana, O. Modin, F. Persson, E. Szabó, M. Hermansson, B.M. Wilén, Combined deterministic and stochastic processes control microbial succession in replicate granular biofilm reactors, Environ. Sci. Technol. 53 (2019) 4912–4921, https://doi.org/10.1021/acs.est.8b06669.
[58] C. Wan, X. Yang, D.J. Lee, X.Y. Wang, Q. Yang, X. Pan, Aerobic granulation of aggregating consortium X9 isolated from aerobic granules and role of cyclic di-GMP, Bioresour. Technol. 152 (2014) 557–561, https://doi.org/10.1016/j.biortech.2013.11.052.
[59] X. Han, Y. Jin, J. Yu, Rapid formation of aerobic granular sludge by bioaugmentation technology: A review, Chem. Eng. J. 437 (2022), 134971, https://doi.org/10.1016/j.cej.2022.134971.
[60] A. Cydzik-Kwiatkowska, P. Rusanowska, M. Zielińska, K. Bernat, I. Wojnowska-Baryła, Microbial structure and nitrogen compound conversions in aerobic granular sludge reactors with non-aeration phases and acetate pulse feeding, Environ. Sci. Pollut. Res. 23 (2016) 24857–24870, https://doi.org/10.1007/s11356-016-7709-7.
[61] O.I.M. Alves, J.M. Araújo, P.M.J. Silva, B.S. Magnus, S. Gavazza, L. Florencio, M. T. Kato, Formation and stability of aerobic granular sludge in a sequential batch reactor for the simultaneous removal of organic matter and nutrients from low-strength domestic wastewater, Sci. Total Environ. 843 (2022), https://doi.org/10.1016/j.scitotenv.2022.156988.
[62] D. Correa-Galeote, A. Roibás, A. Mosquera-Corral, B. Juárez-Jiménez, J. González-López, B. Rodelas, Salinity is the major driver of the global eukaryotic community structure in fish-canning wastewater treatment plants, J. Environ. Manag. 290 (2021), https://doi.org/10.1016/j.jenvman.2021.112623.
[63] M. Thines, Oomycetes, Curr. Biol. 28 (2018) R812–R813, https://doi.org/10.1016/j.cub.2018.05.062.
[64] S.H. Chan, M.H. Ismail, C.H. Tan, S.A. Rice, D. McDougald, Microbial predation accelerates granulation and modulates microbial community composition, BMC Microbiol. 21 (2021) 1–18, https://doi.org/10.1186/s12866-021-02156-8.
[65] E. Szabó, R. Liébana, M. Hermansson, O. Modin, F. Persson, B.M. Wilén, Microbial population dynamics and ecosystem functions of anoxic/aerobic granular sludge in sequencing batch reactors operated at different organic loading rates, Front. Microbiol. 8 (2017) 1–14, https://doi.org/10.3389/fmicb.2017.00770.
[66] L. Zhang, B. Long, J. Wu, Y. Cheng, B. Zhang, Y. Zeng, S. Huang, M. Zeng, Evolution of microbial community during dry storage and recovery of aerobic granular sludge, Heliyon 5 (2019), e03023, https://doi.org/10.1016/j.heliyon.2019.e03023.
[67] Y. Zhao, J. Huang, H. Zhao, H. Yang, Microbial community and N removal of aerobic granular sludge at high COD and N loading rates, Bioresour. Technol. 143 (2013) 439–446, https://doi.org/10.1016/j.biortech.2013.06.020.