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Development of a new generation of continuous-flow aerobic granular sludge bioreactors for urban wastewater treatment: technical and biological study

Department of Microbiology

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Departamento de Microbiología

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“La historia de los hombres se refleja en la historia de las cloacas” (Víctor Hugo en Los Miserables).

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List of acronyms

AGS	Aerobic granular sludge
AOB	Ammonia-oxidising bacteria
APHA	American Public Health Association
BGS	Biorreactores granulares secuenciales
BOD_5	Biological oxygen demand at day 5
BRFC	Biorreactor granular de flujo continuo
BSA	Bovine serum albumin
CAS	Conventional activated sludge
CBG	Concentración de biomasa granular
CFR	Continuous-flow reactor
CLR	Centered logarithm ratio
COD	Chemical oxygen demand
COT	Carbono orgánico total
DBO_5	Demanda biológica de oxígeno
DNA	Desoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DQO	Demanda química de oxígeno
EES	Expected effect size
EPS	Extracellular polymeric substances
FGA	Fango granular aeróbico
GAO	Glycogen-accumulating organism
HRT	Hydraulic retention time
MLSS	Mixed liquor suspended solids

MLVSS	Mixed liquor volatile suspended solids
OLR	Organic loading rate
OTU	Operational taxonomic unit
PAO	Polyphosphate-accumulating organism
PCA	Principal component analysis
PVC	Polyvinyl chloride
qPCR	Quantitative polymerase chain reaction
RDA	Redundancy analysis
RFC	Reactor de flujo continuo
RMO	Rendimiento de eliminación de materia orgánica
RNT	Rendimiento de eliminación de nitrógeno total
SBR	Sequential batch reactor
SVI	Sludge volume index
TRH	Tiempo de retención hidráulico
VD	Velocidad de decantación
WWTP	Wastewater treatment plant

Summary

In accordance with Sustainable Development Goal 6: clean water and sanitation, wastewater treatment technologies must be at the forefront in minimizing the impact of used water on both human and environmental health. Under this perspective, aerobic granular sludge (AGS) technology represents an excellent option for wastewater treatment due to its low area requirements, pollutants removal capacity and resilience under diverse conditions.

Traditionally, AGS systems have been operated in sequential batch reactors, with the aim of exerting a selection pressure able to retain granular biomass and wash out light flocs. However, the scientific community has focused on developing continuous-flow reactors (CFRs) to facilitate operation and maintenance while treating larger wastewater volume in a practical mode. The challenge of CFRs resides in retaining dense granular biomass within the bioreactor and washing out filamentous flocs in concordance with continuous-flow mode.

Trying to address this challenge, several authors have designed diverse AGS-CFR configurations, but they have complex designs and operation, hindering their implementation at full scale. Thus, a simpler and functional CFR design was still required.

Within this Ph.D. thesis, four single-chamber CFRs were developed and assessed at lab scale. After their evaluation, the most suitable configuration was selected for further research. Posteriorly, this novel design was operated under different organic loading rates (OLRs) and hydraulic retention times (HRTs), with the aim to elucidate its treatment capacity and observe its physicochemical and microbial response under different operational conditions.

In detail, **chapter 1** describes the four novel CFRs configurations. All designs were based on a single column with an element to prevent granule loss and enhance withdrawal of flocs. Bioreactor 1 had a settler-baffle before the water output which prevented dense biomass to reach the output due to the height and the curved configuration. Bioreactor 2 had a longitudinal plate which divided the reactor in two parts and caused difference of pressure, creating a circular movement which enhanced dense biomass to descend to the bottom and avoid the water output. Bioreactors 3 and 4 had a

longitudinal tube in eccentric and concentric position, respectively. The difference of pressure in and out the tube should force granules to go down and prevent granule loss while promoting flocs washout. Results showed that bioreactor 1 promoted a rapid biomass adaptation and a fast start-up (11 days). Additionally, the novel configuration was able to select and retain stable granules with high compaction, achieving settling velocities above 100 $m \cdot h^{-1}$. Furthermore, this bioreactor had excellent organic matter removal (reaching 95 %). For these reasons, this CFR design was selected for further research, in order to determine its operational capacity.

Chapter 2 assessed the selected design for a wide range of OLR. This configuration was operated under different OLRs (0.45, 0.90, 1.40 and 1.85 kg $COD \cdot m^{-3} \cdot d^{-1}$). The results revealed that this single-chamber CFR was able to operate across a broad range of OLRs (0.45 to 1.85 kg $COD \cdot m^{-3} \cdot d^{-1}$) in urban wastewater. Bioreactors were able to adjust the biomass concentration in function of the OLR, achieving organic matter removal ratios above 80 % for all OLR tested. Results suggested that changes in influent did not compromise the stability of granules. Furthermore, the selection of microbial community depended on the OLR, with lower diverse granules at higher OLRs. In this sense, OLRs in the range of 0.45 to 0.90 kg $COD \cdot m^{-3} \cdot d^{-1}$ favoured the *OPB56* order and the *Rhodobacteraceae* and *Rhizobiaceae* families. On the other hand, the *Spirosomaceae* family and the *Pseudomonas* genus were enhanced at OLRs from 1.40 to 1.85 kg $COD \cdot m^{-3} \cdot d^{-1}$. *Hypocreales* order acquired an important role in mature granules of all bioreactors.

In **chapter 3**, four bioreactors with the selected configuration were operated at HRTs from 2 to 8 hours. Results showed that 2 hours of HRT promoted an excessive granular sludge production, damaging the hydrodynamic movement in the system, and the continuous withdrawal of granules hindered its physicochemical removal performance. However, HRTs from 4 to 8 hours achieved a high organic pollutants removal. It suggests that this bioreactor is able to treat large wastewater volume in short times, being 4 hours the most cost-effective HRT in terms of organic matter removal. Nutrient removal was enhanced by the longest HRT (6 and 8 hours), although the optimal conditions for nutrient removal have not yet been achieved. HRT modulated the microbial community, although some taxa such as *Hypocreales*, *Spirosomaceae* and *Brevundimonas* were ubiquitous in all conditions. The presence of the *Chitinophagaceae* family was

enhanced at HRT from 4 to 8 hours. The shortest HRTs promoted deeper changes in microbial dynamics.

Briefly, this Ph.D. thesis developed a novel single-chamber AGS-CFR configuration. This bioreactor has an effective selection pressure for keeping dense granules and high biomass retention within it, even at very short HRTs and without the need of feast-famine regime. It achieves a fast start-up and is able to treat a wide range of OLRs, maintaining long-term granular stability and achieving more than 80% of organic matter removal. Additionally, it can be operated at different HRT, being 4 h the most cost-effective in terms of removal performance and granular stability. The developed design during this Ph. D. thesis, characterised by a simple single-chamber configuration, eliminated the need for biomass recirculation and complex control. For these reasons, this advantageable design is suitable for full-scale implementation.

Resumen

De acuerdo con el Objetivo de Desarrollo Sostenible 6: agua limpia y saneamiento, las tecnologías de tratamiento de aguas residuales deben estar a la vanguardia para minimizar el impacto del agua usada en la salud humana y medioambiental. Bajo esta perspectiva, la tecnología de fango granular aeróbico (FGA) representa una excelente opción para el tratamiento de aguas residuales debido a sus bajos requerimientos de área, capacidad de eliminación de contaminantes y resiliencia bajo diversas condiciones.

Tradicionalmente, los sistemas FGA han sido operados en reactores secuenciales discontinuos, con el objetivo de ejercer una presión de selección capaz de retener la biomasa granular y lavar los flóculos ligeros. Sin embargo, la comunidad científica se ha centrado en el desarrollo de reactores de flujo continuo (RFC) para facilitar el funcionamiento y mantenimiento al tiempo que se trata de forma práctica un volumen mayor de agua residual. El reto de los RFC reside en retener la biomasa granular densa dentro del biorreactor y lavar los flóculos filamentosos en consonancia con el modo de flujo continuo.

Tratando de abordar este reto, varios autores han diseñado diversas configuraciones de FGA-RFC, pero su diseño y funcionamiento son complejos, lo que dificulta su aplicación a escala real. Así pues, seguía siendo necesario un diseño de RFC más sencillo y funcional.

En esta tesis doctoral se desarrollaron y evaluaron cuatro RFC de una sola cámara a escala de laboratorio. Tras su evaluación, se seleccionó la configuración más adecuada con el objetivo de seguir investigando sobre ella. Posteriormente, este novedoso diseño fue operado bajo diferentes cargas orgánicas y tiempos de retención hidráulico (TRH), con el objetivo de esclarecer su capacidad de tratamiento y observar su respuesta fisicoquímica y microbiológica bajo diferentes condiciones operativas.

En detalle, el **capítulo 1** describe las cuatro novedosas configuraciones de RFC. Todos los diseños estaban basados en una única columna con un elemento para evitar la pérdida de gránulos y mejorar la eliminación de flóculos. El biorreactor 1 tenía un deflector-decantador antes de la salida del agua que impedía que la biomasa densa llegara

a la salida debido a la altura y al diseño curvo. El biorreactor 2 tenía una placa longitudinal que dividía el reactor en dos partes y provocaba una diferencia de presión, creando un movimiento circular que favorecía que la biomasa densa descendiera hacia el fondo y así evitara la salida del agua. Los biorreactores 3 y 4 tenían un tubo longitudinal en posición excéntrica y concéntrica, respectivamente. La diferencia de presión dentro y fuera del tubo debería obligar a los gránulos a descender y evitar la pérdida de biomasa densa, al tiempo que favorecía el lavado de los flóculos. Los resultados mostraron que el biorreactor 1 promovió una rápida adaptación de la biomasa y una rápida puesta en marcha (11 días). Además, la novedosa configuración fue capaz de seleccionar y retener gránulos estables con alto grado de compactación, alcanzando velocidades de sedimentación superiores a 100 $m \cdot h^{-1}$. Asimismo, este biorreactor tuvo una excelente eliminación de materia orgánica (alcanzando el 95 %). Por estas razones, este diseño de RFC fue seleccionado para futuras investigaciones, con el fin de determinar su capacidad operativa.

En el **capítulo 2** se evaluó el diseño seleccionado en un amplio rango de carga orgánica. Esta configuración fue operada bajo diferentes cargas orgánicas (0,45, 0,90, 1,40 y 1,85 kg $DQO \cdot m^{-3} \cdot d^{-1}$). Los resultados revelaron que este RFC de cámara única fue capaz de operar en un amplio rango de carga orgánica (de 0,45 a 1,85 kg $DQO \cdot m^{-3} \cdot d^{-1}$) en aguas residuales urbanas. Los biorreactores fueron capaces de ajustar la concentración de biomasa en función de la carga orgánica, alcanzando ratios de eliminación de materia orgánica superiores al 80 % para todas las cargas orgánicas probadas. Los resultados sugirieron que los cambios en el influente no comprometieron la estabilidad de los gránulos. Además, la selección de la comunidad microbiana dependió de la carga orgánica, con gránulos menos diversos a cargas orgánicas más altas. En este sentido, las cargas orgánicas en el rango de 0,45 a 0,90 kg $DQO \cdot m^{-3} \cdot d^{-1}$ favorecieron al orden *OPB56* y las familias *Rhodobacteraceae* y *Rhizobiaceae*. Por otro lado, la familia Spirosomaceae y el género Pseudomonas se vieron favorecidos por cargas orgánicas desde 1.40 a 1.85 kg $DQO \cdot m^{-3} \cdot d^{-1}$. El orden *Hypocreales* adquirió un papel importante en los gránulos maduros de todos los biorreactores.

En el **capítulo 3**, cuatro biorreactores con la configuración seleccionada fueron operados con TRH de 2 a 8 horas. Los resultados mostraron que 2 horas de TRH promovieron una producción excesiva de fango granular, dañando el movimiento hidrodinámico del sistema, además, la continua retirada de gránulos dificultó su

rendimiento de eliminación fisicoquímico. Sin embargo, los TRH de 4 a 8 horas consiguieron una elevada eliminación de contaminantes orgánicos. Esto sugiere que este biorreactor es capaz de tratar grandes volúmenes de agua residual en tiempos cortos, siendo 4 horas el TRH más rentable en términos de eliminación de materia orgánica. La eliminación de nutrientes mejoró a TRH más largos (6 y 8 horas), aunque no se alcanzaron las condiciones óptimas para la eliminación de nutrientes. El TRH moduló la comunidad microbiana, aunque algunos taxones como *Hypocreales*, *Spirosomaceae* y *Brevundimonas* fueron ubicuos en todas las condiciones. La presencia de la familia *Chitinophagaceae* aumentó con TRH de 4 a 8 horas. Los TRH más cortos promovieron cambios más profundos en la dinámica microbiana.

Resumiendo, esta tesis doctoral desarrolló una novedosa configuración FGA-RFC de una sola cámara. Este biorreactor tiene una presión de selección efectiva para mantener gránulos densos y una alta retención de biomasa en su interior, incluso a TRH muy cortos y sin necesidad de un régimen de "festín y hambruna". El diseño consigue una rápida puesta en marcha y es capaz de tratar un amplio rango de carga orgánica, manteniendo la estabilidad granular a largo plazo y consiguiendo más de un 80 % de eliminación de materia orgánica. Además, puede ser operado a diferentes TRH, siendo 4 h el más rentable en términos de rendimiento de eliminación y estabilidad granular. El diseño desarrollado durante esta tesis doctoral, caracterizado por una configuración simple de una sola cámara, eliminó la necesidad de recirculación de biomasa y de un control complejo. Por estas razones, este ventajoso diseño es adecuado para su aplicación a escala real.

I - GENERAL INTRODUCTION

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Urbanization, industrialization, and lifestyle changes have led to an increase in waste generation and a shift in its composition. In this context, urban and industrial wastewater can contain high concentrations of organic and inorganic nutrients, as well as harmful substances such as endocrine disruptors, heavy metals, dyes, pharmaceuticals, and microplastics, among others (Bashir et al., 2020). The discharge of untreated wastewater into the environment poses a serious threat, as it can disrupt ecosystem balance and negatively impact global health (Pratap et al., 2023). To address this issue, it is essential to raise awareness about the importance of reducing water consumption and wastewater generation, reusing it whenever possible, and ensuring proper treatment, in alignment with Sustainable Development Goal 6: clean water and sanitation.

1. Wastewater treatment

Wastewater treatment consist of copying natural physical, chemical or biological processes and intensifying them to handle larger volumes of water and higher concentrations of pollutants within the shortest possible time. The primary goal is to minimize the impact of used water on both human health and environment (Salgot and Folch, 2018). Sanitation systems have been in use since the Neolithic period, evolving from rudimentary methods to more advanced infrastructure, such as the roman “Cloaca Maxima”, and continuing its development up to the present day (Cooper, 2001; Lofrano and Brown, 2010). Wastewater treatment technologies are based on physical, chemical and biological processes. They may be applied in combination among them to take advantage of complementary benefits, especially when wastewater composition is very recalcitrant. Biological technologies are based on the activity of organisms to degrade pollutants. The application of biological technologies does not produce secondary toxic waste as a byproduct of treatment; moreover, these systems are more environmentally friendly and cost-effective (Ahmed et al., 2021). These engineering systems are inhabited by a diverse range of microorganisms, including bacteria, archaea, fungi, protozoa, algae, and other microbial groups (Reineke and Schlömann, 2023; Sharma et al., 2023). The most used treatment is conventional activated sludge (CAS), which was developed in 1914 by Ardern and Lockett (1914). This technology is based on suspended biomass grouped in light flocs and it consists of an aerobic bioreactor followed by a secondary

settler (Pariente et al., 2022). Subsequently, the single aerobic bioreactor was replaced by a series of anaerobic, anoxic, and aerobic bioreactors, with the aim of operating under a sequence of different redox conditions to achieve the complete removal of pollutants that require distinct environmental conditions for their metabolic degradation, such as nitrogen and phosphorus. In each chamber, microorganisms break down pollutants according to the specific operational conditions. Following this process, the biomass and treated water are directed to a secondary clarifier, where the two phases are separated, and the effluent is either discharged into the environment or sent for tertiary treatment. In the secondary settler, the settled sludge is recirculated to the head of the secondary treatment to retain biomass within the bioreactor, preventing excess sludge from being washed out.

Conventional activated sludge is an efficient technology; however, it requires a large surface, high energetic and economical costs, and the removal of macro and micro-pollutants encompassing nutrients and priority and emerging pollutants is limited (Cicekalan et al., 2023; Khan et al., 2022).

To protect water bodies and ensure their safety and long-term sustainable use, increasingly stringent restrictions on effluent discharges are being established (Directive 2000/60/EC). In response, significant efforts are being made to develop technologies capable of meeting these new requirements (Hasan et al., 2021). Among these are biofilm-based technologies, which rely on microbial growth on surfaces or on self-aggregating microbial communities embedded in a matrix of extracellular polymeric substances (EPS). These technologies enhance pollutants removal, offer shorter hydraulic retention times, generate minimal excess sludge, tolerate highly contaminated wastewater, and support the growth of slow-growing microorganisms, among other benefits (Saini et al., 2023). Examples of biofilm-based technologies include moving bed biofilm reactors, fluidized bed biofilm reactors, trickling filters, biodiscs, integrated fixed-film activated sludge, and granular sludge systems (Hasan et al., 2021; Saini et al., 2023).

In the recent years, research has focused on the development of aerobic granular sludge (AGS), due to its strengths in comparison with CAS or even over other biofilm-based technologies, in terms of area requirements, nutrient removal capacity and no carrier needs.

2. Aerobic granular sludge technology

Aerobic granular sludge system is a wastewater treatment technology in which microorganisms are grouped into dense and spherical biofilm structures without the need of carriers. Granular sludge was reported in 1980 by Lettinga et al. (1980) when they were working with an up-flow anaerobic sludge blanket. They showed that granule formation was enhanced by short hydraulic retention times (HRT) and high organic loads. Moreover, they stated that hydrodynamic shear force and EPS excretion were determinant for granulation. During the following decade, Mishima and Nakamura (1991) reported for first time the cultivation of AGS in an aerobic up-flow sludge blanket, and Morgenroth et al. (1997) achieved stable aerobic granulation using a sequential batch reactor (SBR). The discovery that this technology could be operated under aerobic conditions had high impact because aerobic metabolism has a faster kinetic; it is able to remove not only organic matter, but nutrients; it is less sensitive to parameters such as temperature or pH. Therefore, this is translated into offering time and space savings, easier operational control and higher quality effluents (Zieliński et al., 2023).

2.1. Granule formation

According to Lettinga et al. (1980), hydrodynamic force and EPS excretion have an important role in granule formation. This process is equivalent to biofilm formation, and it could be described in four stages (Fig. 1) (Liu and Tay, 2002; Rather et al., 2021; Sarma et al., 2017):

- Cell-cell contact. The movement generated in the bioreactor promotes the contact between cells. This first attachment is loosely and reversible. Some of the forces responsible are hydrodynamic, diffusion, gravity, cell mobility or Brownian movement.
- Microaggregate formation. Physical, chemical and biochemical attractive forces act to maintaining stable the multicellular contact. In this stage, filamentous microorganisms assist in the formation of aggregates, as they function as a surface on which other microorganisms are attached. The intracellular signalling molecule bis-(3'-5')-cyclic dimeric guanosine monophosphate plays an essential role restricting cell motility by flagella of microorganisms attached.

- EPS production. After microbial growth, microorganisms excrete EPS, a polymer that acts as glue and promotes the firmness of aggregates. This stage is mediated by quorum sensing and environmental signalling.
- Granule maturation. Hydrodynamic shear forces influence the shape and conformation of granular biomass. The compact structure of the biomass limits the diffusion of substances into the inner parts of the granules, creating a redox gradient that establishes different microbial niches and enables the stratification and maturation of the granules. After that, biofilms have a cyclic behaviour of dispersion (due to factors as EPS degradation or physical forces) and biofilm formation.

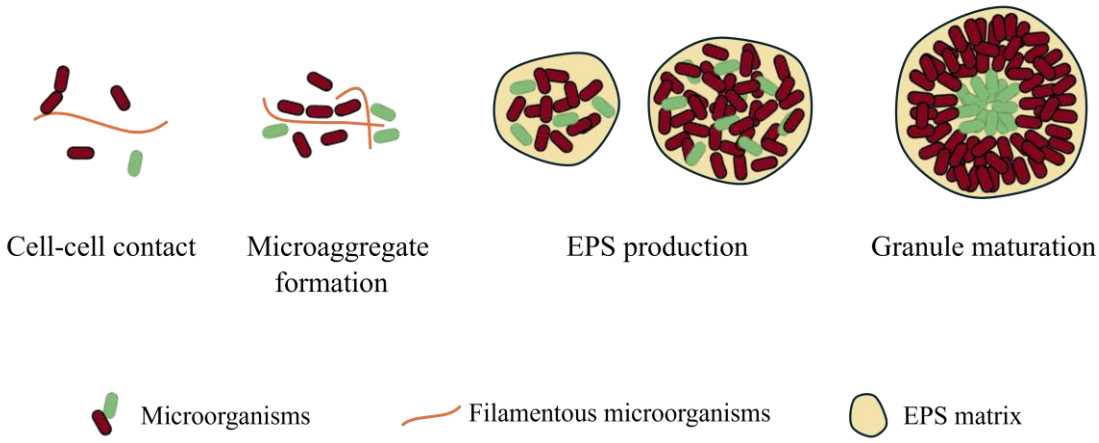

Figure 1. Scheme of granulation process during the successive stages.

2.2. Extracellular polymeric substances production and its role in aerobic granular sludge

Granular biofilm that conforms AGS is composed of a microbial consortium (10-25 %) immobilised in an EPS matrix (75-90 %) synthesised by themself (Rather et al., 2021).

EPS are composed of water, polysaccharides, extracellular proteins, extracellular DNA, lipids, humic compounds, and surfactants (Rather et al., 2021; Saini et al., 2023). Water allows microorganisms to live and keeps a nutrient flow between external medium and biofilm. Regarding polysaccharides, there are numerous types forming EPS, such as alginate, colanic acid, cellulose, or pel, among others. Their functions may be aggregative, protective or architectural, and it depends on the physicochemical properties

of each polysaccharide (Limoli et al., 2015). Extracellular proteins, including enzymes, proteases, and DNases, play a key role in reorganizing the biofilm structure (Fong and Yildiz, 2015). The functions of extracellular DNA within EPS include providing structural integrity, promoting stability, enhancing EPS production, and facilitating gene transfer between cells (Ibáñez de Aldecoa et al., 2017). Viscosin, emulsan and surfactin are some lipids with surface-active properties present in biofilms (Rather et al., 2021). They act retaining some substances and increasing hydrophobicity which facilitates biofilm definition, formation and dispersion. The EPS production and hence, the biofilm formation process is controlled by quorum sensing. It consists of the excretion of signal molecules that, when its concentration reaches a threshold, enter to cells and cause a signalling cascade which regulate gene expression implied in EPS production (Hou et al., 2021).

Several functions of EPS have been defined in biofilms (Flemming and Wingender, 2010):

- Adhesion and attachment to surfaces.
- Aggregation of cells.
- Determination of biofilm architecture.
- Cell-cell communication.
- Retention of water even in water-deficient environments.
- Protection against harmful substances such as pharmaceuticals.
- Sorption of organic compounds (nutrients or pollutants). This capacity makes this biomass useful for environmental detoxification.
- Enzymatic activity. Used for digestion and acquisition of nutrients and for biofilm restructuration.
- Storage of organic and inorganic nutrient that act as nutrient source in case of starvation conditions.
- Gene transfer.

2.3. Characteristics of aerobic granular sludge

Characteristics of AGS are mainly derived from the high compaction degree of microbial aggregates. The predominant features are described below:

- Regular shape

Aerobic granular sludge usually has a spherical shape and a uniform surface (Fig. 2). Biofilm conformation is modelled by the pressure of hydrodynamic shear force (Rosa-Masegosa et al., 2021). EPS and cells hydrophobicity helps in shaping and defining granules (Rather et al., 2021). Some protozoa also contribute to regulate microbial populations of granule surface (Chen et al., 2017).

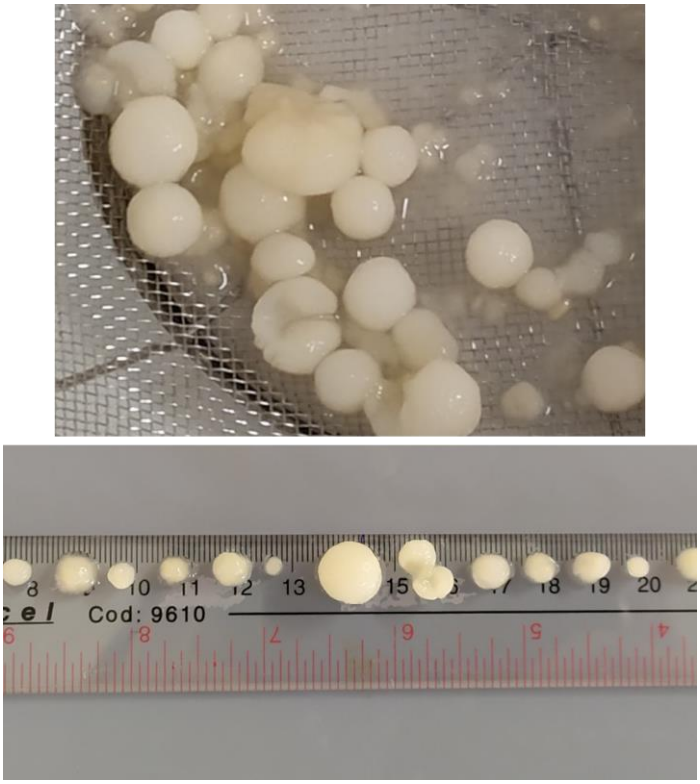

Figure 2. Images of AGS where it can be seen the spherical shape and the uniform surface.

- Dense structure

Hydrodynamic shear force contributes to compacting biomass (Hou et al., 2021). After that, selection pressure derived from operational conditions selects the densest granules, which remain within the bioreactor, and promotes the withdrawal of fluffy flocs, that could cause granular instability (Wei et al., 2020). This peculiarity gives rises to most of granular biomass characteristics.

- Good settleability

Biomass settling velocity is a crucial parameter in wastewater treatment plants, due to the great interest on separate biomass from treated water in an effective way. CAS systems have high sludge volume index (SVI) and need large surfaces and long time to separate water from flocs. However, granular sludge has a lower SVI and, moreover, SVI_5 and SVI_{30} are very similar, corresponding with an incomparable decanting ability, achieving up to 138 $m \cdot h^{-1}$, whereas CAS flocs show velocities below 10 $m \cdot h^{-1}$ (Muñoz-Palazon et al., 2020a; Winkler et al., 2018). This property is further enhanced by the high density and hydrophobicity of the granules, which is due to the negative charges present on their surface, preventing the granules from agglutinating. The liquid-solid separation between biomass and treated water is so effective that it is conducted in the same bioreactor, without the need to use a secondary settler and avoiding biomass recirculation (de Sousa Rollemberg et al., 2022). Excellent settleability also allows treatments operated at shorter HRTs. In addition, differences on settling velocity of granules and flocs are used as selection pressure to wash out fluffy structures and retain compact biomass (Zhang et al., 2019b).

- High biomass retention

The fact that granules are not removed from bioreactor promotes a high biomass concentration within it, reaching 10 $g \cdot L^{-1}$ (Othman et al., 2013). It leads a higher removal performance, enabling the treatment of high-strength influents and large amounts of wastewater in a smaller bioreactor in comparison to other technologies such as CAS (Ekholm et al., 2023). In addition, this property allows the proliferation of slow-growing microorganisms, encouraging the microbial diversity and several metabolic activities (Liébana et al., 2023).

- Tolerance to changing influents and toxics compounds

The high density of granules and biomass retention also confers capacity to withstand changing influents (Zhang et al., 2018). During famine periods, granules have the capacity to obtain nutrients from EPS and stored products, whereas in periods with shock organic loading rates, biofilm confers protection to single cells. (Li et al., 2022; Oliveira et al., 2021).

Moreover, granular biomass is able to withstand and degrade toxics compounds such as pharmaceuticals, pesticides, phenols, heavy metals, nuclear waste, among others (Franca et al., 2018; Nancharaiah and Kiran Kumar Reddy, 2018). Thus make AGS an advantageous technology for the treatment of industrial and recalcitrant wastewaters.

- Ability to remove different pollutants simultaneously

The high compaction of granules limits the mass transfer. Nutrients are consumed mainly in the external layers and their metabolites are transported to the inner layers. In case of dissolved oxygen, the limitation it its diffusion creates different redox conditions through layers (Nancharaiah and Sarvajith, 2019; Winkler et al., 2018). Different microorganisms proliferate in each niche, which generate high diversity of metabolic pathways (Fig. 3). This results in a strong consortium with syntrophic relationships that achieve a complete degradation of pollutants in a unique space, reducing required area and operational time (Purba et al., 2020; Winkler et al., 2018). In this sense, AGS is able to remove organic matter, nitrogen, phosphorus, and even recalcitrant pollutants concurrently (Franca et al., 2018; Purba et al., 2020). On the contrary, CAS would need serial chambers with different redox conditions for removing N and P, requiring larger surface and operational time (Franca et al., 2018).

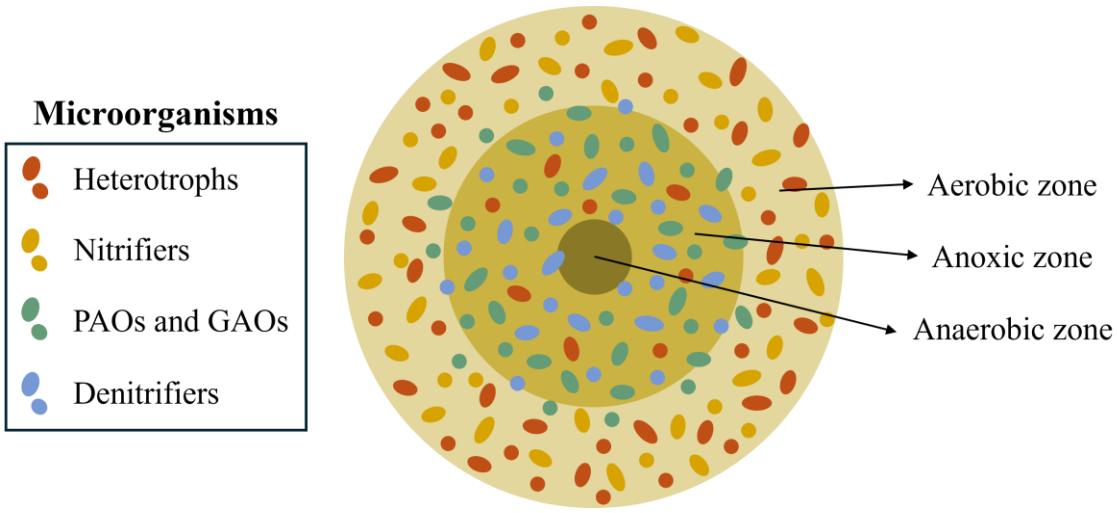

Figure 3. Schematic distribution of different microorganisms through the layers of a granule.

2.4. Advantages of aerobic granular sludge

The characteristics previously described motivate the advantages of AGS technology, although the main feature is the high compactness of biomass granular, which generates the rest of the properties.

Due to the rapid separation between biomass and treated water, AGS eliminates the need for a secondary settler and sludge return, reducing both energy consumption and space requirements in wastewater treatment plants (Franca et al., 2018; Gonzalez-Martinez et al., 2017). Moreover, AGS is able to treat a larger amount of wastewater in smaller bioreactors in comparison with CAS (Ekholm et al., 2023). The tolerance against recalcitrant influents, together with the development of slow-growing microorganisms and the high diversity achieved driven by the granular interlayers, allow the simultaneous removal of an elevate variety of pollutants and the treatment of wastewater from different origins, which is traduced in economic and investing savings and a higher quality effluent in comparison to other technologies such as CAS (Franca et al., 2018).

Furthermore, anaerobic digestion of purged granular sludge achieves a stabilised sludge with comparable yields of biomethane production to CAS (Zaghloul et al., 2023).

In summary, AGS could be applied for the treatment of different influents achieving great removal performance of pollutants and a high-quality effluent. Moreover, according to various authors, AGS systems minimizes the land footprint up to 75 %, energy up to 50 % and capital cost up to 50 % than CAS (Cicekalan et al., 2023; Kosar et al., 2023; Nancharaiah and Sarvajith, 2019).

2.5. Applications of aerobic granular sludge systems

As it was previously mentioned, advantageous characteristics of AGS allow the application of this technology to the removal of a wide range of pollutants by mean of biodegradation, bioaccumulation and biosorption processes (Purba et al., 2020). This capacity makes possible the treatment of urban wastewater, different industrial wastewater, even nitrate and pesticide-polluted groundwater for supplying drinking water (Guo et al., 2024; Hurtado-Martínez et al., 2024).

Some processes of pollutants removal and types of wastewaters to which AGS can be applied are exposed below:

- Nutrients removal from wastewater

The release of nitrogen and phosphorus compounds to environment can cause environmental problems such as eutrophication. To mitigate these problems, regulations governing nitrogen and phosphorus discharges are becoming increasingly stringent, requiring wastewater treatment technologies to effectively remove these pollutants.

In the case of nitrogen, the most common inorganic form found in wastewater is ammonium. Its removal involves the oxidation of ammonium to nitrite, followed by its conversion to nitrate. Subsequently, nitrate is reduced to nitrite, nitric oxide, nitrous oxide, and finally to nitrogen gas (Purba et al., 2020). These processes, known as nitrification and denitrification, require aerobic and anoxic conditions, respectively. In CAS systems, these conditions are typically achieved using multiple chambers. However, in AGS, the dense structure of the granules allows for the coexistence of both conditions, enabling simultaneous nitrification and denitrification, even in an aerated bioreactor (Franca et al., 2018). Under this nitrogen removal pathway, AGS operated in continuous flow mode has been shown to remove more than 90% of total nitrogen (Chen et al., 2024). An alternative is the ammonium removal over nitrite, in which ammonium is only oxidised to nitrite, by restricting the proliferation of nitrite oxidising bacteria, and then, nitrite is reduced to nitrogen gas. This pathway reduces the oxygen requirement and thus, it reduces the economic inputs (Nancharaiah and Kiran Kumar Reddy, 2018). Zou et al. (2024) reported that ammonium removal over nitrite achieved more than 95 % of total nitrogen removal treating an ammonium-rich wastewater by mean of AGS technology operated in SBR.

Regarding biological phosphorus removal, aerobic and anoxic conditions are also required. During absence of oxygen, PAOs hydrolyse poly-P to obtain energy and release phosphorus into the environment, whereas in aerobic conditions, PAOs remove phosphorus from wastewater, which is accumulated by cells (Liu et al., 2023b). This process is carried out simultaneously in granules, without needing different bioreactors. Chen et al. (2024) achieved more than 94 % of total phosphorus removal through AGS operated in continuous flow mode.

- Phenols compounds

The presence of phenolic compounds in waterbodies is due to natural and anthropogenic causes, such as the decomposition of vegetal material or activities derived

by olive, paper, pharmaceutical or plastic industries, among others. Several authors have reported the application of AGS systems for treatment of wastewater polluted with these substances (Muñoz-Palazon et al., 2023a). Muñoz-Palazon et al. (2019) reported the stability and removal capacity of AGS treating up to 300 $mg \cdot L^{-1}$ of a mixture of caffeic acid, hydroxybenzoic acid and protocatechuic acid. Ho et al. (2010) concluded that AGS is able to resist higher concentrations of phenolic compounds without inhibition than CAS.

- High-strength wastewater: livestock

Farms usually have a high-strength wastewater. Despite this, AGS is able to withstand this kind of influent and achieve a high removal performance of contaminants. Othman et al. (2013) reported a removal performance of 74 %, 73 % and 70 % for chemical oxygen demand (COD), total nitrogen and total phosphorus, respectively, treating a livestock wastewater with 9 kg $COD \cdot m^{-3} \cdot d^{-1}$ and operated in a SBR system at 8 h of hydraulic retention time.

In addition, these wastewaters are often rich in sulfur-containing amino acids. Their presence can negatively impact the efficiency of wastewater treatment processes by inhibiting the activity of certain microorganisms. Aerobic granular sludge systems have been reported for achieving an excellent sulphur amino acids removal rate without impacting the organic matter elimination (Rosa-Masegosa et al., 2022).

- Pharmaceuticals and personal care products

Pharmaceutical compounds are present in wastewater originated in domestic environments, hospitals, pharmaceutical industries, even in farms. These priority and emerging concern pollutants are not able to be removed by mean of conventional wastewater treatments, due to the toxicity produced by these compounds over microbial communities of biological processes, while technologies such as advanced oxidation or membrane bioreactors are expensive and not feasible for hospitals or farms (Nivedhita et al., 2022). However, AGS has tolerance to toxic compounds and AGS processes have been postulate as a great alternative to remove these substances. Zhao et al. (2015) reported the capacity of AGS for removing sulfamethoxazole, norfloxacin, prednisolone, naproxen and ibuprofen. Another study has revealed that AGS is able to biodegrade trimethoprim, carbamazepine, triclosan and cyclophosphamide, whereas nonsteroidal anti-inflammatory drugs are more affected by bioadsorption-desorption processes (Pérez-

Bou et al., 2024). These authors indicated that AGS could be applied as pretreatment before to be discharge in the sanitation sewer, to reduce the load of pharmaceutical compounds and diminish its negative effect in the urban WWTP.

- Nitrate and pesticide-polluted drinking water

Wastewater is not the unique water that could be treated by AGS technology. Aerobic granular biomass has been proven for removing nitrate and pesticide-polluted groundwater with the objective to supply drinking water in small towns (Hurtado-Martínez et al., 2024; Muñoz-Palazon et al., 2023b). Carbendazim had a removal rate near 100 %, while Diuron was very recalcitrant, proving that pesticide nature affects its removal (Muñoz-Palazon et al., 2023b). According to Hurtado-Martinez et al. (2021), AGS achieved 70 % of nitrate removal, reaching to remove more than 98 % of organic matter applied.

2.6. Microbial communities of aerobic granular sludge

Microorganisms present in AGS depend on different factors, such as origin of inoculum, influent characteristics, bioreactor design, operational parameters or age of biomass (Pishgar et al., 2021; Rosa-Masegosa et al., 2021; Wilén et al., 2018).

During the granulation process, some microorganisms play a special important role. In this sense, EPS producers such as *Flavobacterium*, *Thauera*, *Devosia* or *Stenotrophomonas* contribute to granular stability (Chen et al., 2019b; Xia et al., 2018). Stalked ciliates and other protozoa enhance granular compaction by controlling suspended and peripheral bacteria (Wilén et al., 2018). Additionally, filamentous bacteria and fungi act as backbone on which other microorganisms grow, being essential for first steps of granulation process (Han et al., 2022; Purba et al., 2020). However, an overgrowth of filamentous microorganisms such as *Thiothrix*, *Sphaerotilus* or *Geotrichum* could damage the compactness and settleability of granules (Ebrahimi et al., 2010; Li et al., 2016b; Sharaf et al., 2019).

In mature granules, microorganisms are distributed based on the redox conditions (Rosa-Masegosa et al., 2024b). Thus, the outer layers contain ordinary heterotrophic organisms, which are involved in the remotion of organic compounds, such as the family *Xanthomonadaceae* or the genus *Flavobacterium* (de Sousa Rollemberg et al., 2018).

Furthermore, these niches host ammonia-oxidising and nitrite-oxidising bacteria, including among them the genera *Nitrosomonas* and *Nitrobacter*, respectively, although some bacteria such as *Nitrospira* are able to complete the two steps of ammonia oxidation by themselves (Burzio et al., 2022). Denitrification has been reported in both aerobic and anaerobic conditions (Utom et al., 2020; Xi et al., 2022). It should be noted the role played by *Hydrogenophaga*, *Pseudomonas* or *Thauera* genera in denitrification process (Bucci et al., 2020; Sharaf et al., 2019).

In anoxic microenvironments polyphosphate-accumulating organisms (PAOs) and glycogen-accumulating organisms (GAOs) are encouraged. Both are considered slow-growing microorganisms. The main PAO representative is *Candidatus* Accumulibacter phosphatis, although *Flavobacterium*, *Corynebacterium*, or *Pseudomonas* are also typical PAOs specimens (Rosa-Masegosa et al., 2024b). With regard to GAOs, *Candidatus* Competibacter is the main genus (Wei et al., 2020). PAOs have the ability to accumulate phosphate in aerobic conditions, in this way they remove phosphorus from wastewater. However, GAOs are direct competitors of PAOs, therefore there is an increasing interest in the study of reduction of GAOs activity to promote the removal of phosphorus (Liu et al., 2023b). Some PAOs have also denitrifying activity and need less aeration requirements. For that, the search for conditions that promote denitrifying PAOs, such as *Thauera*, has great interest (Ren et al., 2024).

Microorganisms present in granules not only remove pollutants, but they also produce substances with biotechnological interest. Alginate-like exopolysaccharides are produced mainly by *Pseudomonas*, *Azotobacter* and algae. *Thauera* or *Paracoccus* are some of the microorganisms implied in polyhydroxyalkanoates and tryptophan production. These products can be isolated and utilised as raw material in pharmaceutical, textile, paper or chemical industries, among others (Tavares Ferreira et al., 2021).

2.7. Modes of operation of aerobic granular sludge technology

2.7.1. Sequential batch reactors

Aerobic granular sludge has traditionally been operated in sequential batch reactors (SBR) (Silva et al., 2023) in order to wash out light flocs using settling time as selection pressure. This mode of operation works in cycles with the next stages (Fig. 4):

- Feeding: the bioreactor is filling with raw water.
- Aeration: air is introduced through a diffuser. It creates aerobic conditions, puts in contact biomass with wastewater, and provides the shear force for creating and maintaining granules conformation.
- Settling: air flow stops, and in a short time, dense biomass is deposited at the bottom of the reactor. In this way, biomass is separated from effluent, and it is retained within the bioreactor for the next cycle.
- Effluent discharge: treated water over the water output is withdrawn from the bioreactor

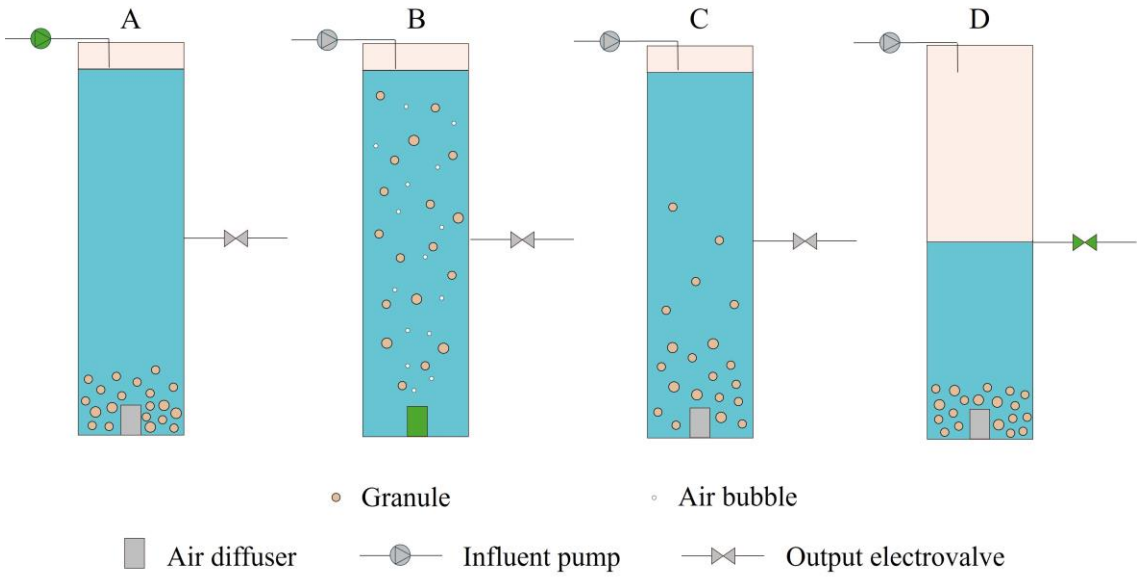

Figure 4. Stages diagram of a cycle in SBR operation. Filling (A); Aeration (B); Settling (C); and Effluent discharge (D). Element in green colour means “ON”, while element in grey colour means “OFF”.

This mode of operation promotes the stability of granular biomass by encouraging feast-famine conditions and selecting for denser biomass, which helps to eliminate floccular structures that could compete with and destabilize the system (Silva et al., 2023). A critical parameter for selecting dense biomass is the volume exchange ratio. The selection pressure varies based on the height of the water output; a higher volume exchange ratio increases the selection pressure, leading to improved settleability of the granules (Xavier et al., 2021). However, SBR is not the optimum mode of operation for urban wastewater treatment, because aging infrastructures of WWTP were structured in

continuous flow with the aim to treat higher flow rates (Winkler and van Loosdrecth, 2022). For that, during the last decade, scientific community has focused on developing continuous-flow reactors (CFRs) for AGS technology.

2.7.2. Continuous-flow reactors

CFRs operation promotes a simpler operation and maintenance than SBRs. In addition, continuous flow adapts to the existing infrastructures and allows treating greater quantities of wastewater in a practical mode (Kent et al., 2018).

CFR designs should find a selection pressure model that retains dense biomass and promotes the washout of flocs in concordance with the continuous-flow mode, with the objective to maintain the granular stability (Xu et al., 2022; Yan et al., 2021). With this aim, several authors have developed different configuration of potential CFRs. Most of these designs have been based on airlift reactors with baffles, serial multiple chambers, use of clarifiers, submerged membranes or hybrid SBR-CFR systems (Rosa-Masegosa et al., 2021; Yan et al., 2021).

2.7.2.1. Airlift reactors with baffles

Airlift reactors are predominantly cylindrical bioreactors characterised by a high height-to-diameter ratio. These reactors incorporate many structural components designed to prevent the loss of granular biomass. Chen et al. (2009) operated a bioreactor based on recirculation of biomass and water between an up-flow sludge bed and an aeration column (Fig. 5A). Zhou et al. (2013) operated a continuous-flow airlift fluidized bed reactor, consisting of two tubes where biomass ascended through the internal tube and descended via the external tube (Fig. 5B). A bubble burst was also positioned at the top of the bioreactor. However, this configuration led to issues with filamentous bulking, as reported by the authors. Li et al. (2020c) developed a bioreactor with a sedimentation tube placed at the centre of it (Fig. 5C). Aerators were placed surrounding the settler and granules that enter it were conducted again to the external part. Another tube located inside the settler prevented granules from reaching the water outlet, which consisted of a bypass that directed the effluent out of the bioreactor. A notable disadvantage was that while recirculation did not harm the granules, the complexity of the design hinders scaling. Furthermore, other designs were based on the use of a lamella responsible for

separating the main reactor from the effluent output zone (Fig. 5D) (Kishida et al., 2012; Li et al., 2020a; Xin et al., 2017) or the use of a sieve (Santorio et al., 2022).

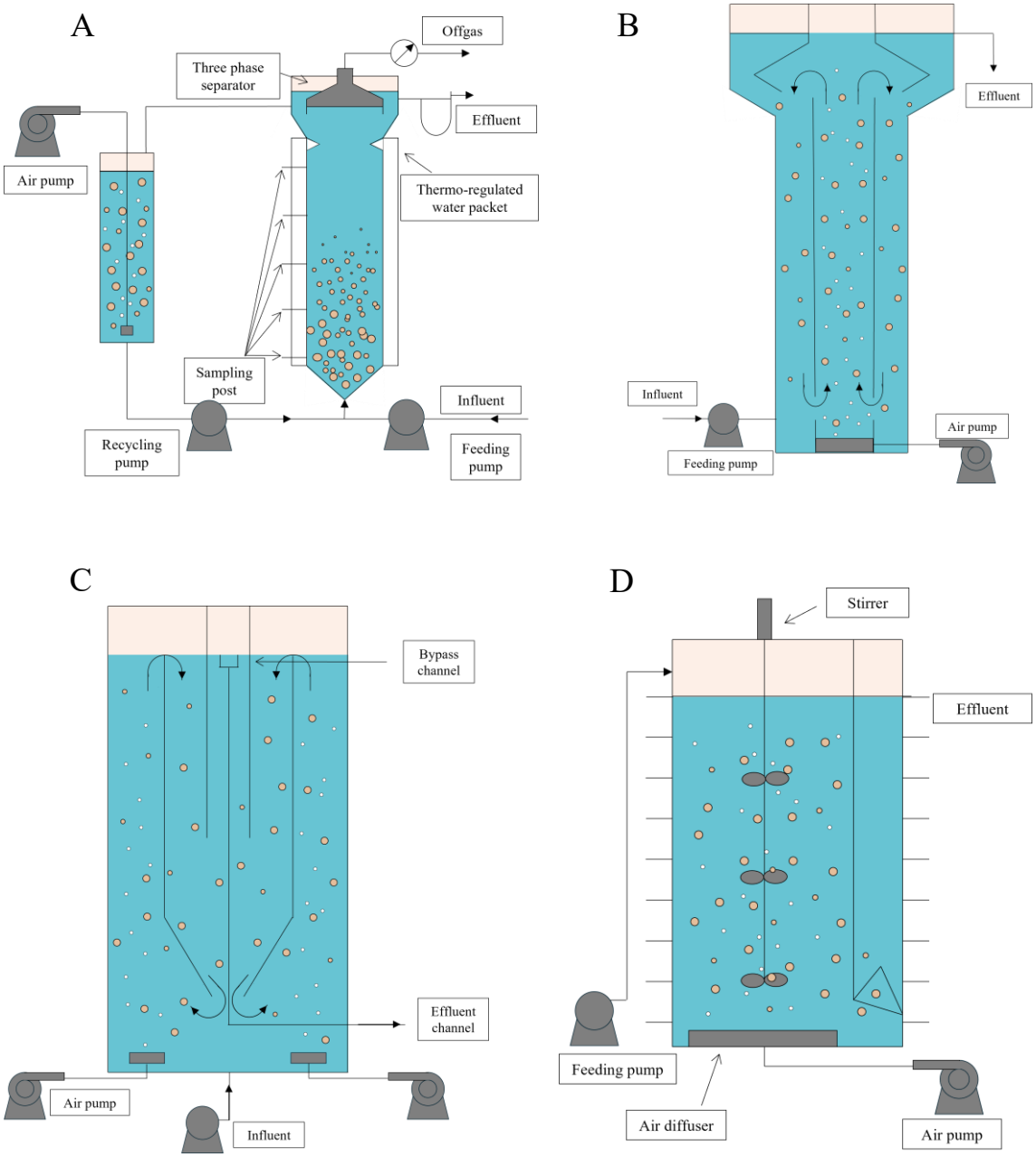

Figure 5. Diagram of aerobic granular sludge continuous-flow reactors based on airlift reactors with baffles, adapted from Chen et al. (2009) (A); Zhou et al. (2013) (B); Li et al. (2020c) (C); and Li et al. (2020a) (D)

2.7.2.2. Serial multiple chambers

Serial multiple chamber design consists of different chambers connected in series. Configuration proposed by Chen et al. (2024) had eight sequential chambers with different redox conditions. Water and granules were circulated through all tanks until reaching the last chamber, where effluent outlet was located. Prior to discharge, a baffle avoided the washout of granules, redirected them back to the first chamber. Another bioreactor configuration (Li et al., 2015) was based on the periodic change of direction of water flow when granules were accumulated in the last chamber (Fig. 6). Bioreactor was provided also by a stirrer in each extreme chamber, but only the stirrer placed on the influent chamber worked each time. In spite of the complex design and operation, this configuration enhances the feast-famine strategy.

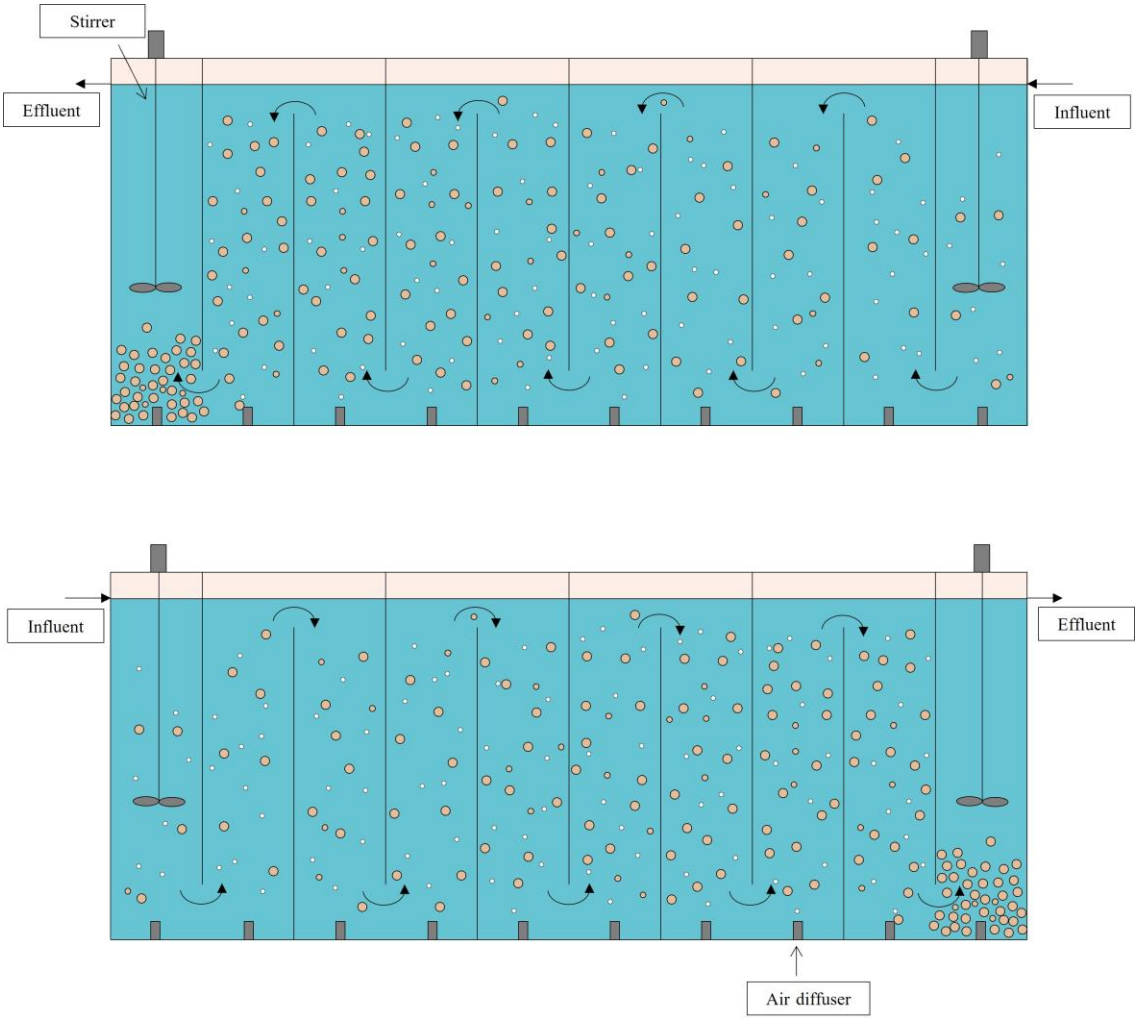

Figure 6. Diagram of aerobic granular sludge continuous-flow reactor based on serial multiple chambers, adapted from Li et al. (2015).

2.7.2.3. Use of clarifiers

Some AGS-CFR configurations are connected to a settling tank which retains biomass washed out with effluent and posteriorly, granules are returned to the main reactor. The design described by Long et al. (2015) consisted of a cylindrical bioreactor connected to a settling tank via an inclined tube. Within the settling tank, a removable baffle enabled the adjustment of the selection pressure. Granules retained within the clarifier were recirculated to the main bioreactor chamber. Other research had a similar configuration with some modifications, such as Chen et al. (2015), Li et al. (2016d) or Sun et al. (2021a, 2019), which applied the use of stirrers, different number of chambers previous to the clarifier, and a different selection pressure within it (Fig. 7A). Cofré et al. (2018) described the configuration of a CFR consisting of two tanks and a clarifier. Within tank 1, biomass was in contact with raw wastewater (feast conditions), while in tank 2, substrate concentration was reduced near zero (famine conditions). Granules from the second tank could be transported to the settler, facilitating the separation of granules from the treated water. Each 15 minutes, a peristaltic pump promoted the recirculation of settled granules to the tank 1, rising the water level and allowing the pass of biomass from tank 1 to tank 2. Bioreactors used by Xu et al. (2021) and Zou et al. (2018) were constituted by an aeration tank, two sedimentation tanks connected between them by a fixing and a movable baffle, which allowed to adjust the selection pressure (Fig. 7B). The first zone of sedimentation was connected with the aeration tank, where settled granules were returned. Bioreactor described by Li et al. (2014) was a circuit where wastewater was drove by stirrers along an aeration zone and a settling zone. The settling zone was equipped with inclined plates that enhanced the sedimentation of granules, which continued towards the aeration zone, while treated wastewater was discharged through the top of the settling area. A similar bioreactor configuration was operated by Xu et al. (2020). The settling area contained two clarifies instead of plates. The first clarifier released granules directly to the bioreactor, avoiding complex recirculation, whereas the sludge discharged from the second settler was post-treated and returned to aeration zone.

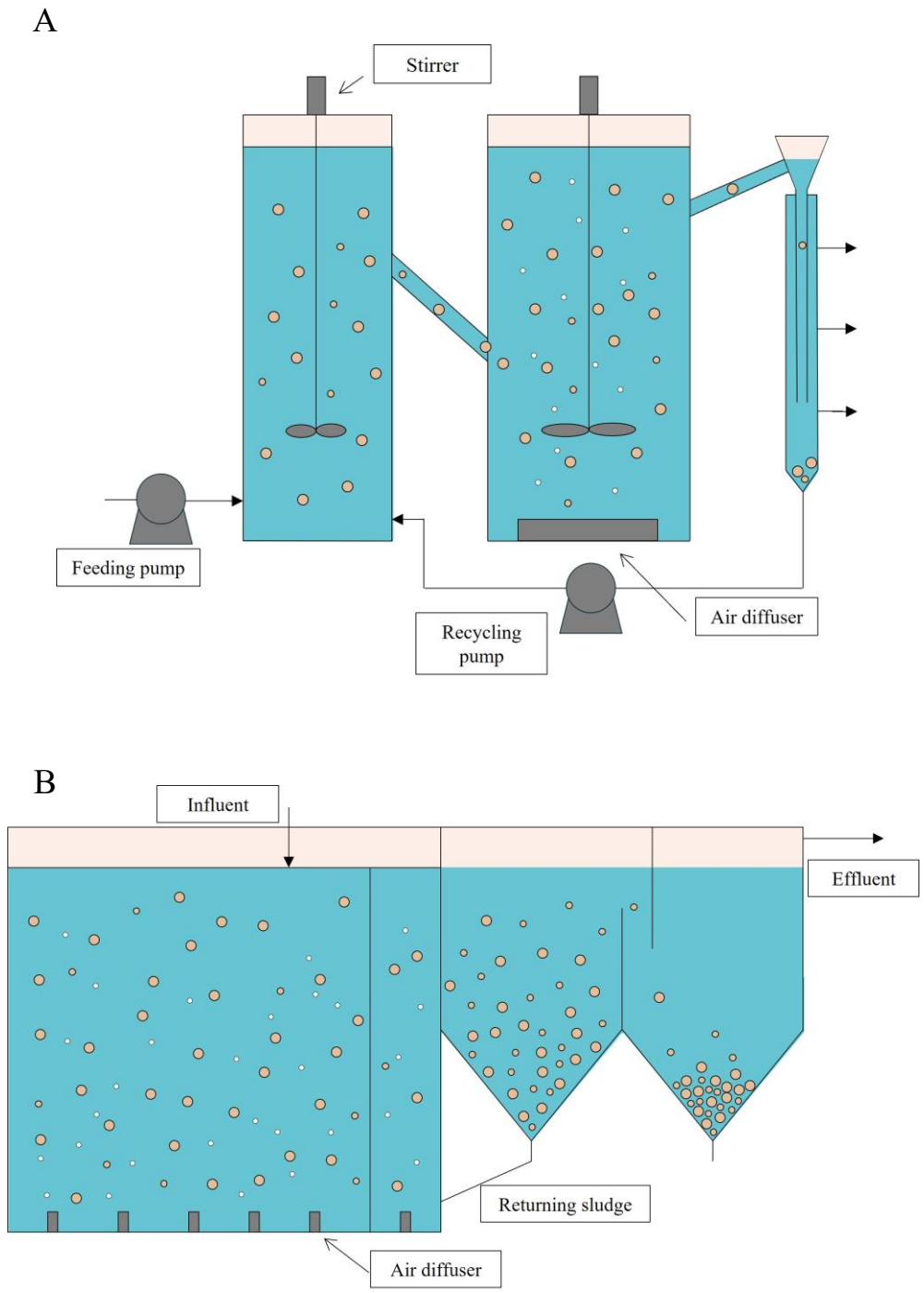

Figure 7. Diagram of aerobic granular sludge continuous-flow reactors based on the use of clarifiers, adapted from Li et al. (2016d) (A); and Xu et al. (2021) (B).

2.7.2.4. Submerged membranes

Advantages of wastewater treatment by mean of AGS-membrane systems are the generation of high-quality effluents reducing typical fouling issues in membranes due to

the use of granular biomass (Campo et al., 2021). In this sense, CFR designs based on membranes has been proposed. There are simpler designs that had a unique chamber with diffuser and a membrane through which the treated water was discharged (Iorhemen et al., 2019; Zhang et al., 2021). However, a primary challenge for these systems is maintaining stable granules over the long term. To enhance stability, some designs incorporate two or more chambers to promote feast-famine conditions. For example, the bioreactor described by Chen et al. (2017) featured an aeration zone where the hydrodynamic forces generated by bubbles facilitated the upward movement of granules. These granules then transitioned to a mixing zone, equipped with a stirrer that forced their descent, allowing them to circulate back to the aeration zone. Similarly, Corsino et al. (2016) utilized a design with multiple chambers in series, where water and granules were actively driven through the system (Fig. 8). This configuration included two mixing zones, functioning as high-load and low-load chambers, respectively.

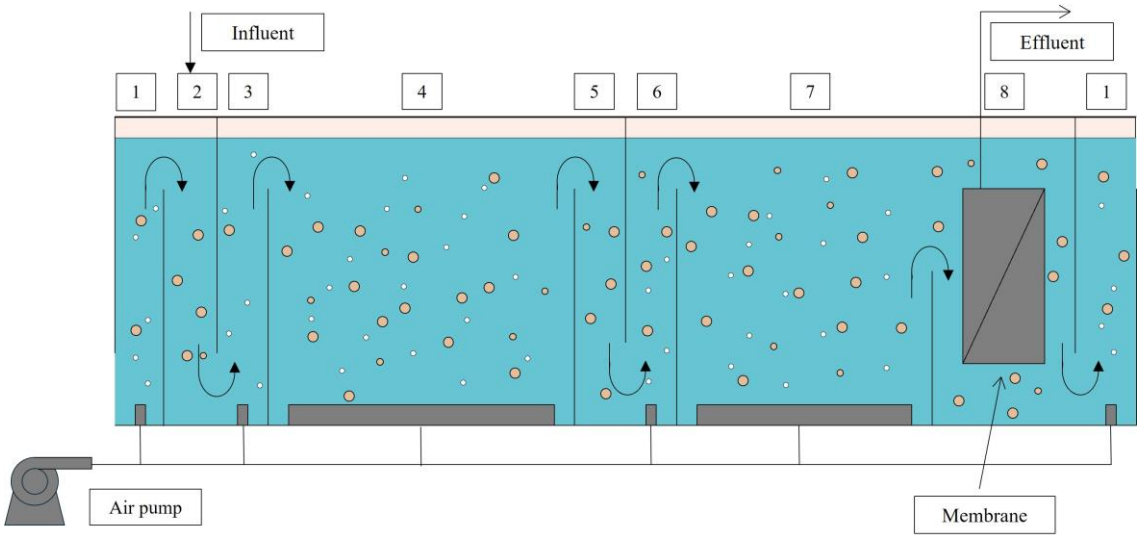

Figure 8. Diagram of aerobic granular sludge continuous-flow reactor based on the use of submerged membranes, adapted from Corsino et al. (2016).

2.7.2.5. Hybrid SBR-CFR systems

The configuration was proposed by Li et al. (2019) and consisted of four columns: one acting as an anaerobic chamber, two functioning as aerobic chambers and the fourth one acting as a settler (Fig. 9). Inflow direction rotated, as well as the activities of each chamber. This design aimed to keep the SBR advantages while maintaining a continuous flow.

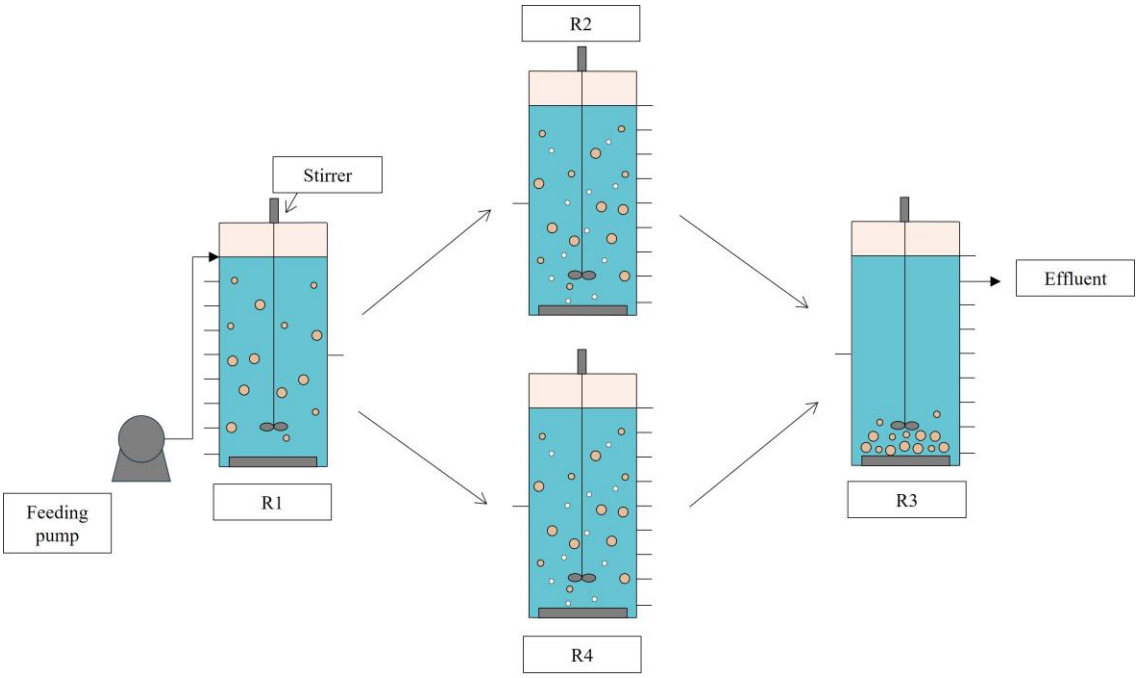

Figure 9. Diagram of aerobic granular sludge hybrid sequential batch reactor and continuous-flow reactor, adapted from Li et al. (2019).

Despite the effort to find an optimal CFR system to implement at full scale, most of these configurations have complex designs and operation. Moreover, some designs do not support stable granules for long-term or they are based on biomass recirculation, that causes granule damage and instability. These reasons make them suboptimal models for full-scale implementation; hence, a simple and optimal CFR configuration is required for AGS technology. Ideally, this novel configuration should maintain a shear-force and a selection pressure able to retain dense biomass and remove flocs from bioreactor.

As we have mentioned, there have been various proposals to generate continuous aerobic granular systems that allow the treatment of wastewater, although the complexity of the proposals has certainly not allowed them to be accepted from the point of view of their scaling up to the level of a real plant. In this doctoral thesis, we propose the development of a novel design for AGS-CFR, aimed at ensuring simplicity, effectiveness and efficiency for practical application. Thus, this thesis has developed and assessed a novel and simpler single-chamber CFR for full-scale implementation.

II - GENERAL OBJECTIVES

II - GENERAL OBJECTIVES

In response to the need of developing efficient and environmentally friendly biotechnological approaches for wastewater treatment, AGS technology is postulated as a viable alternative to CAS. However, granular instability required the operation of the AGS system in SBR mode, which is sub-optimal for treating urban wastewater. In contraposition, CFRs could offer advantages such as simpler construction, operation, and maintenance. Additionally, they are better adapted to existing sewage infrastructures, enabling the practical treatment of large volumes of wastewater. However, despite these advantages, the retention of granular biomass within the CFR, and the long-term granular stability pose a serious challenge problem in AGS-CFR systems designed to date. Efforts to identify a CFR configuration capable of addressing the challenges associated with AGS-CFR applications have resulted in the design of numerous AGS-CFR configurations. However, these designs are often complex to operate and challenging to implement at full scale. Furthermore, many of the abovementioned designs require granular recirculation processes, which can compromise granular stability. In the light of this situation, the development of a new simple CFR easy of scale, operate, without granule recirculating requirements represents the first step for the successful implementation of the technology at full scale.

The main objective of this doctoral thesis was to develop and evaluate a functional new single-chamber continuous-flow bioreactor for the treatment of urban wastewater from the technical and microbiological point of view.

To achieve it, secondary objectives were set:

1. To design and develop a novel and simple AGS-CFR at lab scale able for retaining granular biomass within the bioreactor and ensuring long-term granule stability.

2. To study the ability of macro-pollutants removal according to different operational conditions (organic loading rate and hydraulic retention time).

3. To know the microbial communities responsible for granulation processes and pollutants removal.

4. To link the physicochemical parameters, microbial communities and operational conditions for optimising the bioreactor operation and performance.

III - GENERAL MATERIALS AND METHODS

III - GENERAL MATERIALS AND METHODS

To achieve the objectives established in the present doctoral thesis, four continuous-flow bioreactors were designed and evaluated. The most advantageous configuration was assessed in terms of different operational parameters (OLR and HRT). For that, physicochemical and microbiological analysis were performed, and posteriorly, they were correlated in function of the operational parameters.

1. Continuous-flow bioreactors design

Four single-chamber CFRs were designed. They consisted of a methacrylate cylindrical tube of 72 cm in height and 10 cm in internal diameter. The bottom part consisted of a polyvinyl chloride (PVC) eccentric cone (for bioreactors R1, R2 and R3) and concentric cone (for R4 bioreactor). Bioreactors had a working volume of 6 L. Water discharge was produced by overflowing of treated water through a tube in the superior part of bioreactors. Each bioreactor had a different baffle to impede the washout of dense granules, but to promote the removal of fluffy flocs (Fig. 10).

Bioreactor 1 (R1) had a truncated conical PVC settle of 6.3 cm in diameter coupled to an elbow tube placed in the water outlet within the bioreactor. The function of this settle-baffle was to limit dense granules to reach the output zone, due to the height and the curved configuration.

Bioreactor 2 (R2) had a methacrylate vertical plate placed 2.5 cm above the air diffusor. The diffusor forced granules to exert a circular movement, going up along the column side where the diffuser was placed and falling by the opposite side. The suction force created due to differences in pression could force dense granules to go down, while fluffy flocs would be washed out.

Bioreactor 3 (R3)'s baffle was an internal tube of 4.6 cm in internal diameter placed eccentrically, 2.5 cm over the air diffuser. Its functioning was based on a streamflow created to enhance granules to decant, whereas flocs would not be so forced, and they would be withdrawn.

Baffle design for bioreactor 4 (R4) was an internal tube of 5.4 cm in internal diameter paced concentrically 2.5 cm over the air diffuser. Theoretically, its functioning would be as R3's baffle, differentiated by the position within the bioreactor.

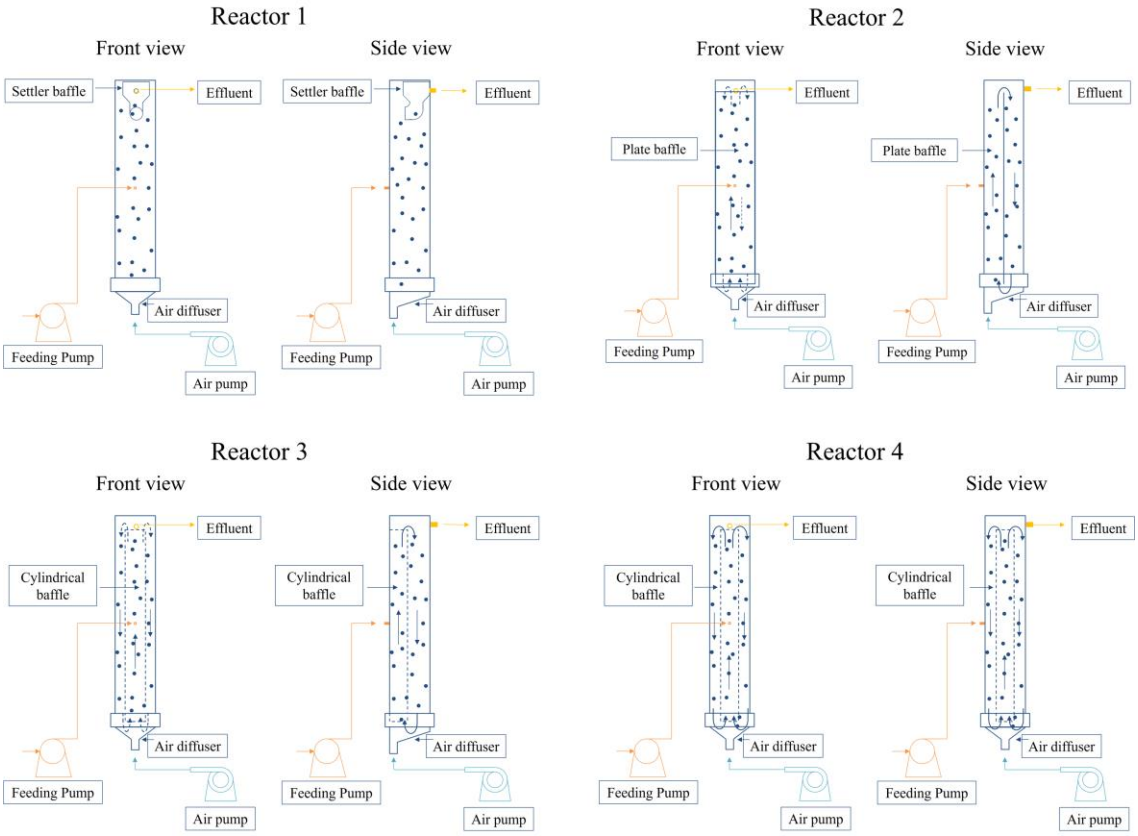

Figure 10. CFR diagrams from front and side views; reactor 1 with a settler in the superior part; reactor 2 with a vertical plate dividing the cylinder; reactor 3 with an eccentric position tube; reactor 4 with a concentrical position tube.

In Chapter 1, the four different bioreactor configurations were used. However, in that chapter, bioreactor R1 design was selected, and it was the bioreactor model used for Chapters 2 and 3.

2. Operational parameters

Synthetic medium simulating urban wastewater was used for all chapters. The composition of the medium was 0.250 $g \cdot L^{-1}$ of NH_4Cl, 0.100 $g \cdot L^{-1}$ of $MgSO_4 \cdot 7H_2O$, 0.085 $g \cdot L^{-1}$ of K_2HPO_4, 0.040 $g \cdot L^{-1}$ of KCl and 0.030 $g \cdot L^{-1}$ of KH_2PO_4. The organic source was $CH_3COONa \cdot 3H_2O$, and its concentration varied depending on the chapter. In this sense, for research described in Chapter 1, 1.160 $g \cdot L^{-1}$ were used; concentration used for the achievement of Chapter 2 differed among the bioreactors: 1.050 $g \cdot L^{-1}$ (R1), 0.787 $g \cdot L^{-1}$ (R2), 0.525 $g \cdot L^{-1}$ (R3), and 0.262 $g \cdot L^{-1}$ (R4); whereas for Chapter 3, 0.525 $g \cdot L^{-1}$ were

employed. The influent was introduced by the mid-height of reactors by mean of Watson Marlow peristaltic pumps (United Kingdom). Air flowed through a diffuser placed at the bottom conical base of bioreactors. Seed sludge used is described in each chapter.

3. Physicochemical determinations

During experiments, both granular biomass and wastewater (influent and effluent) were monitored. Mixed liquor suspended solids (MLSS) were periodically determined in triplicate according to the standard methods for the examination of water and wastewater of the American Public Health Association (APHA) (2017). Size and settling velocity of granules were measured following Moy et al. (2002) and Muñoz-Palazon et al. (2023c) with slight modifications. Chemical oxygen demand (COD), biological oxygen demand at day 5 (BOD_5) were quantified according to APHA methods (American Public Health Association, 2017). Ammonium, nitrate, nitrite and phosphate ions were determined using a MagICNet 33 Metrohm ion chromatograph. The anion column was Metrosep-A Supp 5-250/4.0, using sodic carbonate–bicarbonate solutions as eluent, whereas the cation column was Metrosep C6-150/4.0, using nitric acid-oxalic acid solution as eluent.

4. Carbon mass balance

The carbon mass balance was performed in base to two main reactions: the oxidation of organic matter to carbon dioxide and the transformation of substrate to new biomass.

Organic matter oxidation reaction was the following:

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

The amount of oxygen required for organic matter oxidation represents the COD.

Due to acetate was the organic carbon used in synthetic mediums, the stoichiometry of the oxidation reaction would be the following:

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

On the other hand, the reaction of substrate transformation into new biomass was also determined. The stoichiometry was experimentally quantified in base to the increase

of mixed liquor volatile suspended solids (MLVSS). Presuming that new cells can be represented as $C_5H_7NO_2$, the oxygen equivalent (COD) need for the generation of new biomass was approximately 1.42 g COD · g $MLVSS^{-1}$ (Hoover and Porges, 1952).

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

The oxygen demand per unit of biomass was:

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

Performing a global mass balance to the system, the amount of CO_2 generated could be estimated based on the COD analyses and MLVSS measurements.

5. Biomass sampling and DNA extraction

In each experiment, granular biomass samples were collected periodically and centrifuged during 20 minutes at 5000 rpm in a 15-mL Falcon tube. Posteriorly, supernatant was discarded, and pellet was stored at -20 ºC until DNA extraction.

FastDNA SPIN Kit for Soil (MP Biomedicals, USA) was employed for DNA extraction according to the manufacturer's protocol. Extracted DNA of each sample was stored at -20 ºC until use.

6. Absolute quantification of target genes

The number of copies of fungal 18S rRNA, and bacterial and archaeal 16S rRNA genes were quantified for determinate the abundance of total *Fungi*, total *Bacteria* and total *Archaea*. The bacterial ammonia monooxygenase (*amoA*) gene was used to estimate ammonia-oxidising bacteria (AOB). The nitrous oxide reductase (*nosZ I*) gene determined the abundance of denitrifying microorganisms. Finally, the *C.* Accumulibacter 16S rRNA gene served as a proxy for polyphosphate-accumulating organisms (PAOs). Quantitative polymerase chain reactions (qPCRs) were carried out using a QuantStudio-3 Real-Time PCR equipment (Applied Biosystems). The reaction mixture solution contained 2.5 µL of buffer with $MgCl_2$, 0.5 µL of deoxynucleotides triphosphate (dNTPs) (10 mM), 0.15 µL of forward primer (10 µM), 0.15 µL of reverse

primer (10 µM), 0.125 µL of SYBR Green, 0.125 µL of Taq polymerase, 0.0625 µL of bovine serum albumin (BSA) and 2 µL of DNA diluted. Pair of primers (Table 1) and cycling conditions (Table 2) were set according to Muñoz-Palazon et al. (2020b) and Correa-Galeote et al. (2021a) with slight modifications.

Table 1. Primers used for quantification of total *Bacteria*, *Archaea* and *Fungi*, ammonia-oxidising bacteria, denitrifying microorganisms and polyphosphate-accumulating organisms populations.

Group	Gene	Primer name	Primer sequence (5´-3´)	Reference
Total *Bacteria*	16S rRNA	341F	CCTACGGGAGGCAGCAG	(Muyzer et al., 1993)
		534R	ATTACCGCGGCTGCTGG	
Total *Archaea*	16S rRNA	ARCH915	AGGAATTGGCGGGGGAGCAC	(Yu et al., 2008)
		UNI-b-rev	GACGGGCGGTGTGTRCAA	
Total *Fungi*	18S rRNA	FungiQuantF	GGRAAACTCACCAGGTCCAG	(Liu et al., 2012a)
		FungiQuantR	GSWCTATCCCCAKCACGA	
AOB	*amoA*	AmoA-1F	GGGGTTTCTACTGGTGGT	(Rotthauwe et al., 1997)
		AmoA-2R	CCCCTCKGSAAAGCCTTCTTC	
Denitrifier	*nosZ*	nosZ1840F	CGCRACGGCAASAAGGTSMSSGT	(Henry et al., 2006)
		nosZ2090R	CAKRTGCAKSGCRTGGCAGAA	
PAO	16S rRNA	518F	CCAGCAGCCGCGGTAAT	(He et al., 2007)
		PAO-846R	GTTAGCTACGGCACTAAAAGG	

Table 2. Thermocycler conditions for quantification of the different genes by qPCR.

Group	Initial denaturalization	Amplification (x35 cycles)			Final elongation
		Denaturalization	Annealing	Elongation	
Total *Bacteria*	95 ºC, 7’	95 ºC, 30”	60 ºC, 40”	72 ºC, 30”	72 ºC, 2’
Total *Archaea*	95 ºC, 7’	95 ºC, 30”	65 ºC, 30”	72 ºC, 30”	72 ºC, 7’
Total *Fungi*	95 ºC, 7’	95 ºC, 30”	62 ºC, 30”	72 ºC, 45”	72 ºC, 7’
AOB	95 ºC, 7’	95 ºC, 30”	52 ºC, 30”	72 ºC, 30”	72 ºC, 5’
Denitrifying	95 ºC, 7’	95 ºC, 30”	65 ºC, 30” (x5) 60 ºC, 30” (x35)	72 ºC, 30”	72 ºC, 5’
PAO	95 ºC, 7’	95 ºC, 15”	55 ºC, 30”	72 ºC, 30”	72 ºC, 5’

7. Next-generation sequencing and bioinformatic analysis

Next-generation sequencing was performed through Illumina MiSeq platform. 16S rRNA Pro341 forward 5′- CCTACGGGNBGCASCAG-3′ and 16S rRNA Pro805 reverse 5′-GACTACNVGGGTATCTAATCC-3′ pair of primers were used to identify the prokaryotic community, whereas primers for eukaryotic community were *18S rRNA* Euk1391F 5′- GTACACACCGCCCGTC-3′ and 18S rRNA EukB 5′- TGATCCTTCTGCAGGTTCACCTAC-3′ (Stoeck et al., 2010; Takahashi et al., 2014). Raw data obtained were analysed through Mothur v 1.39.4 software (Schloss et al., 2009). Firstly, forward and reverse reads were merged into contigs, to which sequenced with more than eight homopolymers and more than 0 ambiguous bases were removed. Then, reads were aligned against the SiLVA seed v132 database using the Needleman conditions. After that, sequences which were not correctly aligned in the forward and

reverse positions were removed. Chimeras were detected using VSEARCH algorithm (Rodriguez-Sanchez et al., 2019). After chimera removal, the remaining sequences were classified against SiLVA nr v132 database through the k-nearest-neighbour method. Sequences with a similarity cut-off of 97% and 95% were clustered into operational taxonomic units (OTUs) for *Prokaryota* and *Eukaryota*, respectively (Muñoz-Palazon et al., 2020a).

8. Statistical and ecological analysis

The α-diversity (Shannon-Wiener, Simpson, Pielou's Evenness and Chao-1 indices) and the β-diversity (Whittaker) were calculated using PAST 3.14 software (Garcia-Ruiz et al., 2021). Statistically significant differences were detected by one-way PERMANOVA analysis under 999 bootstraps through PAST 3.14 software (Muñoz-Palazon et al., 2020b).

The similarity percentages analysis (SIMPER) revealed information about the contribution to dissimilarities of each OTU for pairs of samples. To conduce the analysis, Bray-Curtis algorithm was employed in PAST 3.14 software (Muñoz-Palazon et al., 2021). The expected effect size (EES) was calculated to assess the significant differences in OTUs abundance between bioreactors. Corrected OTU table without zero-counts and centered log-ratio (CLR)-transformed was used for EES computation, employing the ALDEx2 package in R v4.2.1 software (Rodriguez-Sanchez et al., 2018).

Samples were clustered according to their microbial community by mean of principal component analysis (PCA) analysis carried out in R v4.2.1 software (González-Martínez et al., 2021). For that, OTU table was corrected to avoid zero-counts, then data were transformed using a CLR transformation. After that, singular value decomposition calculation was performed to the corrected OTU table, and results were visualised through the PCA plot using the ALDEx2 package.

Multivariate redundancy analysis (RDA) was performed to determine the relationship between dominant OTUs and physicochemical parameters. Log(x + 1)-transformed data were used to compute 499 unconstrained Monte-Carlo simulations under a full-permutation model using CANOCO 4.5 software (Rodriguez-Sanchez et al., 2019).

9. Prediction of potential functions in prokaryotic community

The potential functions of the prokaryotic populations were predicted employing Functional Annotation of Prokaryotic Taxa (FAPROTAX) v1.2.7 software according to developers' descriptions (Louca et al., 2016). Relative abundances of each OTU involved in specific functions were determined, and functions representing more than 0.5 % of the total abundance were represented.

IV - RESULTS AND DISCUSSION

CHAPTER 1

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

CHAPTER 1

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

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 settler-baffle (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 optimize the current model and to determine the most suitable operational parameters to treat domestic wastewater at full scale.

Keywords: Aerobic granular sludge; continuous-flow reactor; granular stability; microbial community; qPCR; single-chamber.

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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) (Amorim et al., 2014; Nancharaiah and Kiran Kumar Reddy, 2018; Rosa-Masegosa et al., 2021). The hydrodynamic shear force and the continuous circular motion generate compact granules formed by microorganisms fixed and stabilised in a polymeric matrix (de Sousa Rollemberg et al., 2018; Wilén et al., 2018). This dense structure gives rise to the advantages of this technology, because the high density of AGS promotes a better settleability (Suja et al., 2015; Winkler et al., 2013), 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 (Bassin et al., 2019; Nancharaiah and Kiran Kumar Reddy, 2018; Wilén et al., 2018). Moreover, the dense granular structure encourages a high accumulation of biomass. According to Nancharaiah et al. (2019), 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 (Franca et al., 2018; van den Berg et al., 2020; Winkler et al., 2018). This fact promotes the stratification of microorganisms along the layers, and consequently, it is possible to find different metabolisms in the same granule (Cai et al., 2021; de Sousa Rollemberg et al., 2018; Nancharaiah and Kiran Kumar Reddy, 2018; Rosa-Masegosa et al., 2021). 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 (Amorim et al., 2014; Bassin et al., 2019; Cai et al., 2021; Muñoz-Palazon et al., 2022a, 2019; Rosa-Masegosa et al., 2022, 2021; Sarma et al., 2017). Changes in abiotic factors could modify influent characteristics (Borzooei et al., 2019). However, AGS is a robust technology able to adapt to oscillating influent composition (Geng et al., 2022). 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 (Cai et al., 2021; Chen et al., 2019b). For all these reasons, AGS systems can be used to treat urban and industrial wastewaters (Cai et al., 2021; Purba et al., 2020), as well as drinking water (Hurtado-Martinez et al., 2021).

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 and filamentous microorganisms are washed out using short settling times to select the fastest biomass (Nancharaiah and Kiran Kumar Reddy, 2018). Filamentous microorganisms are essential for granule formation, because they establish the structural core (Aqeel et al., 2016). Nevertheless, an excess of these microorganisms would give rise to a destabilization of the system, causing biomass washout due to the loss of granular density (Moura et al., 2018). Thus, the washout 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 (Qi et al., 2020; Sun et al., 2019; Zhang et al., 2020; Zou et al., 2018). Nowadays, the challenge is to find a good design for AGS-CFR that concurrently allows granular biomass retention and the promotion of the washout 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, use of clarifiers, CFRs with submerged membranes or hybrid SBR-CFR systems (Rosa-Masegosa et al., 2021). 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 (Xu et al., 2022; Yan et al., 2021). 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 (Xu et al., 2022; Yan et al., 2021).

In this study, four novel and simple AGS-CFRs 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. Bioreactor configurations 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 an eccentric cone 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 height of the reactor, and the treated effluent output was located at the top of the bioreactor. Four different baffles were configured to evaluate the most efficient one to avoid the loss of granules simultaneously to filamentous microorganisms washout. First, R1's baffle was a truncated, conical PVC settler of 6.3 cm in diameter placed in the water outlet zone and coupled to an elbow tube (Gonzalez-Martinez et al., 2024). The baffle configuration allowed the remotion of floccular 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 constrain 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 granules 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 was concentric. The premise was that the streamflow generated would force the settling of compact biomass, while floccules would be discarded.

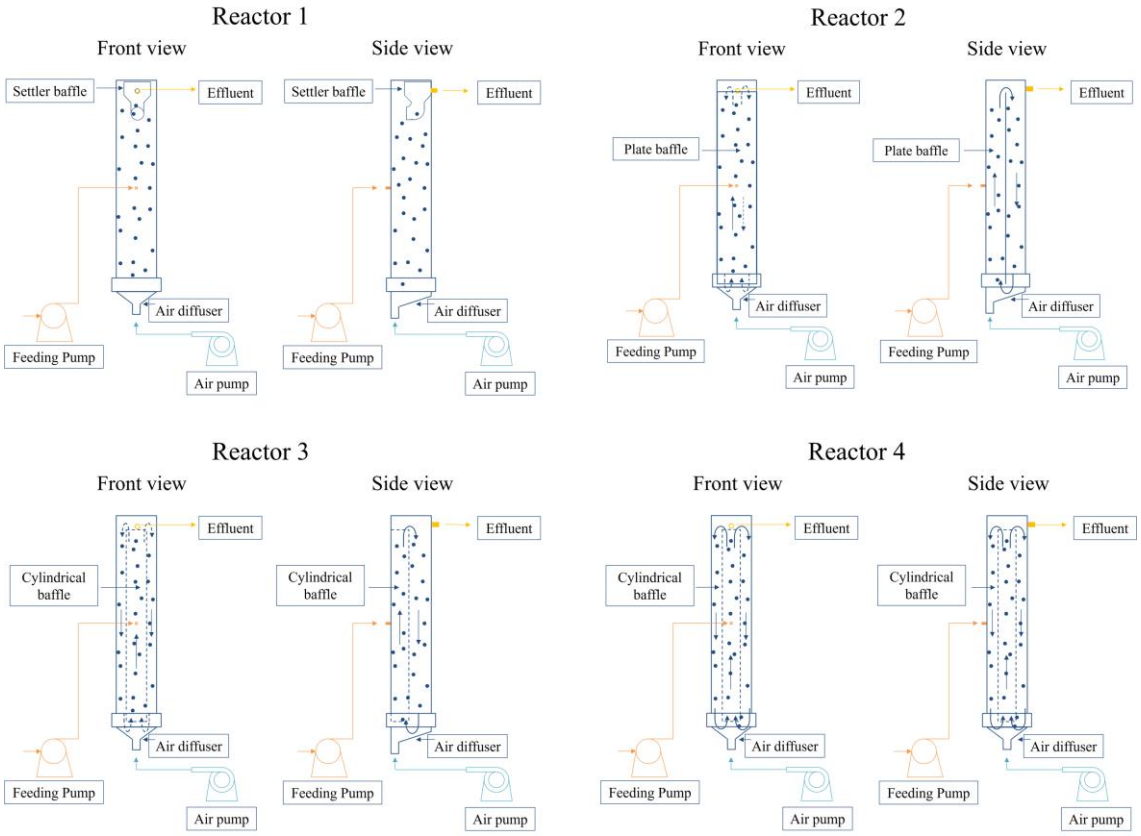

Figure 1. CFR diagrams from front and side views. Reactor 1 with a settler in the superior part; reactor 2 with a vertical plate dividing the cylinder; reactor 3 with an eccentric position tube; reactor 4 with a concentric position tube.

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 by 1.160 $g \cdot L^{-1}$ of $CH_3COONa \cdot 3H_2O$, 0.250 $g \cdot L^{-1}$ of NH_4Cl, 0.040 $g \cdot L^{-1}$ of KCl, 0.100 $g \cdot L^{-1}$ of $MgSO_4 \cdot 7H_2O$, 0.085 $g \cdot L^{-1}$ of K_2HPO_4, and 0.030 $g \cdot L^{-1}$ of KH_2PO_4 according to Muñoz-Palazon et al. (2018), 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 (American Public Health Association, 2017). Granular size and settling velocity were evaluated to characterise the biomass properties, following Moy et al. (2002). 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 (American Public Health Association, 2017).

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:

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

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 was 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

calculate the oxygen equivalent (COD) for the generation of new biomass, which can be considered approximately 1.42 g COD/g MLVSS (Hoover and Porges, 1952).

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

$(\Delta O_2 / \Delta C_5H_7NO_2) = (5 \cdot 32g/mol\ O_2) / (113\ g/mol\ C_5H_7NO_2) = 1.42\ g\ O_2 / g\ MLVSS$

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 samples collection and DNA extraction

The granular biomass samples were collected at day 0, 15, 30 and 55 for each bioreactor. Representative granules were taken in a 15-mL Falcon tube and immediately centrifuged at 5000 rpm during 20 min at 4ºC. Afterwards, the supernatants were discarded, and pellets were stored at -20ºC. 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 deionized water.

2.6. Absolute quantification of target genes

The quantification of number of copies of bacterial and archaeal 16S rRNA gene, fungal 18S 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 16S rRNA gene, 10^7-10^3 copies·µL^{-1} serial dilutions were used. For archaeal 16S rRNA gene and fungal 18S 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), 0.15µL of forwards 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 samples DNA diluted 1:25. The primers and qPCR conditions used were described by Muñoz-Palazon et al. (2020b).

2.7. Next generation sequencing and post-processing

Illumina next generation sequencing was performed using pair of primers: 16S Pro341 forward 5'-CCTACGGGNBGCASCAG-3' and Pro805 reverse 5′-GACTACNVGGGTATCTAATCC -′3 for 16S rRNA of Prokaryota and Euk1391F 5′-GTACACACCGCCCGTC-3′ and reverse EukB 5′-TGATCCTTCTGCAGGTTCACCTAC-3′ for 18S rRNA of Eukaryota (Stoeck et al., 2010; Takahashi et al., 2014). The raw data were analysed using Mothur v 1.39.4 (Schloss et al., 2009). 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 to chimera detection, using the samples reads as reference (Rodriguez-Sanchez et al., 2019). The chimeras were removed, and the remaining sequences were classified against SiLVA nr v132 database through k-nearest-neighbour method, and then they were clustered into operational taxonomic units (OTUs) with a similarity cut-off of 97% for Prokaryota and 95% for Eukaryota (Muñoz-Palazon et al., 2020a).

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 by one-way PERMANOVA using PAST3.14 software. The similarity percentages analysis (SIMPER) was done to quantify the contribution of each OTU to dissimilarity between pair 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 centered 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 (Rodriguez-Sanchez et al., 2020).

3. Results and discussion

3.1. Characterisation of granular sludge

The mean size and the settling velocity 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 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 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 means that those granules were the most compact (Suja et al., 2015; Winkler et al., 2013). On the other hand, R4 was the configuration with the highest presence of filamentous microorganisms, due to the settling velocity of its granules only reached 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 washout 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 SBR reported by Muñoz-Palazon et al. (2020b) 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 velocities registered (68–74 $m \cdot h^{-1}$) in the SBR operated by Kocaturk & Erguder (2015). In addition, it was clear that the R1 bioreactor had the fastest granular biomass than other CFRs. For example, Liu et al. (2012b) stated a maximum of 39.6 $m \cdot h^{-1}$, while Cofré et al. (2018) 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.

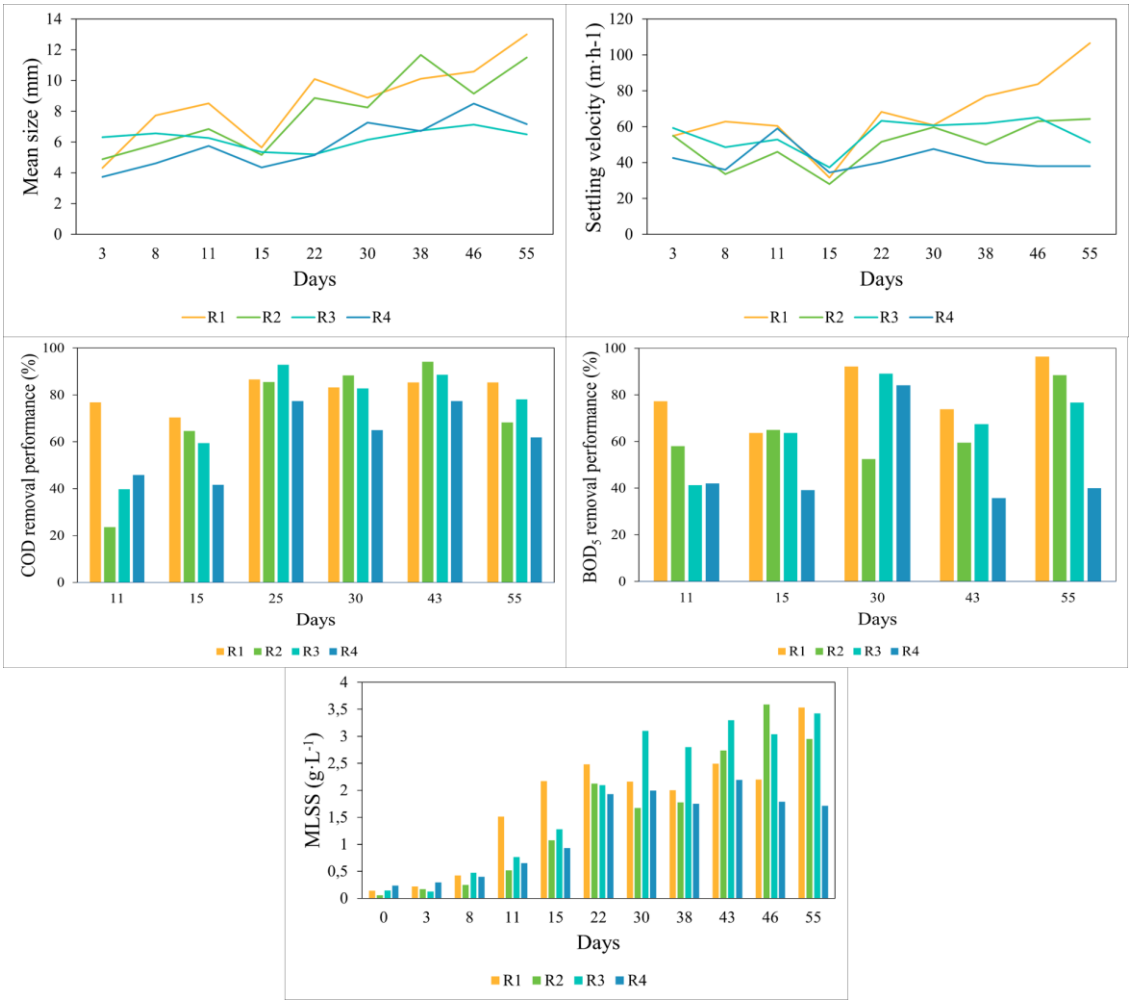

Figure 2. Granular properties (mean size and settling velocity), organic matter removal (COD and BOD_5) and biomass concentration for each reactor during the whole experimentation.

The *in vivo* observations during the whole experimentation corroborated the settling velocity results. It was possible to see how R1's granules were the most compact (Fig. S1), followed by granular biomass of R2 and R3 bioreactors, while 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 of 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, 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 (Khan et al., 2019; Muñoz-Palazon et al., 2022b, 2020b; Sarvajith et al., 2018; Winkler et al., 2012). Additionally, this research reported how the settling ability of granules and the washout 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 (Kent et al., 2018).

3.2. Organic matter removal performance and carbon mass balance

The organic matter removal performance was evaluated to decide on a better CFR 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 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, R1 configuration, whose baffle was a small settler located in the water outlet zone, was the most 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 the highest 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 term of removal efficiency were obtained in 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 the 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 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 reported in section 3.1.

Table 1. Carbon mass balance of each bioreactor.

		Q ($L \cdot d^{-1}$)	INFLUENT		EFFLUENT		REMOTION		COD ΔMLVSS	CO_2 GENERATION
			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 ($g\ O_2 \cdot d^{-1}$)	($g\ O_2 \cdot d^{-1}$)	($g\ CO_2 \cdot d^{-1}$)
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

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 AGS used as inoculum, which was obtained from a lab-scale AGS-SBR bioreactor. *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* has been reported by He et al. (2021) as contributing to nitrate removal in an up-flow fixed-bed bioreactor. Denitrifying activity has been described for the *Chitinophagaceae* family (He et al., 2019) and for the *Brevundimonas* genus (O'Donnell et al., 2019), which was also reported by its excellent ability to secrete extracellular polymeric substances (EPS), which are essential for granular formation (Song et al., 2022a). According to Hurtado-Martinez et al. (2021), 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 decreased from 8.2 % on day 15 to 0.6 % of total relative abundance on day 55. The *Xanthobacteraceae* family, which was dominant in the inoculum, was in 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 until 8.1 %. OTU03 appeared and represented the 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 (dos Santos et al., 2021; Xia et al., 2018). 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 (He et al., 2019). 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 (Liébana et al., 2019). So, its presence probably indicated a granular maturation process during the first month of operation into the R1 bioreactor design. *Acinetobacter* and *Pseudomonas* have been reported as having some strains with strong self-aggregation ability and syntrophy capacity (Han et al., 2022; Wan et al., 2014b). 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 conformed 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 abundance 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. (2016) 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 stabilized 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 (Alves et al., 2022). 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. (2022) 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. (2022) 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.

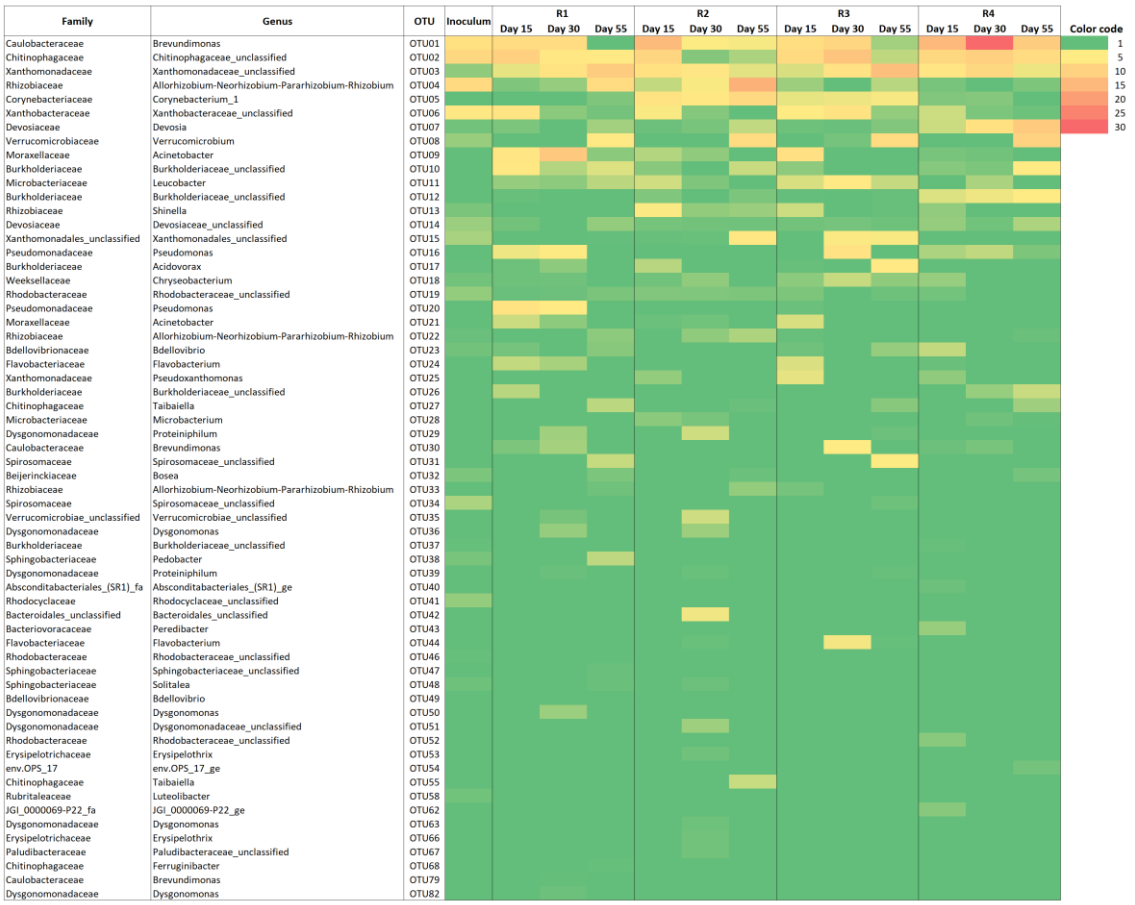

Figure 3. Heat map of the dominant prokaryotic OTUs affiliated with the *Bacteria* superkingdom, with more than 1 % of total relative abundance.

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 a 3.7 % of relative abundance. *Ascomycota* has been reported to lead to organic compound biodegradation, detoxification and aggregation of sludge flocs (Correa-Galeote et al., 2021b) and can act as a core for granule formation given their filamentous nature (Nancharaiah and Kiran Kumar Reddy, 2018). 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 colonization (Muñoz-Palazon et al., 2022a). The *Tremellomycetes* class has also been found in AGS, possibly related to permitting a well-granulated structure and hence related to good settling ability (Muñoz-Palazon et al., 2018).

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 OTUs. A reduction of total relative abundance was detected on the *Hypocreales* order and the *Trichosporonaceae* family that corresponded with OTU02 and OTU04, respectively. At day 55, they only represented the 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 (Thines, 2018). 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 abundance 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 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 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.

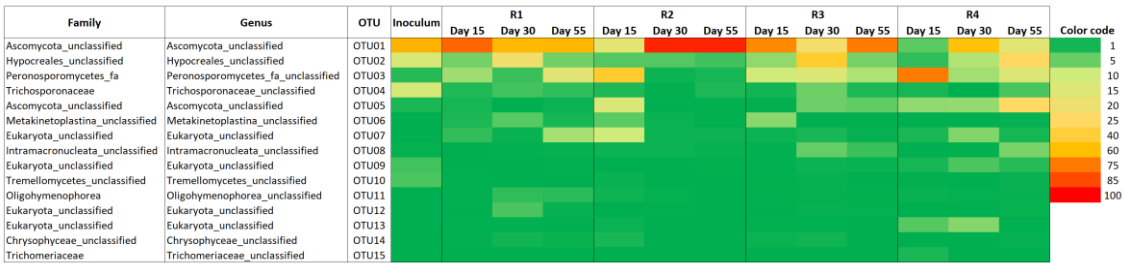

Figure 4. Heat map that showed the dominant eukaryotic OTUs with more than 1 % of total relative abundance.

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, prokaryotic community was more diverse and had more species richness than the eukaryotic community, following a common trend described by Muñoz-Palazon et al. (2019). 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 the 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 pair 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 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 with 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 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 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 abundance were notably higher on all operational days in the R4, but it was irrelevant in the rest of 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 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 it high representation in the inoculum sample and its posterior depletion in the rest of the samples as 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 a preservation of similarity between samples were two unclassified Eukaryote (OTU12 and OTU13) and OTU11, corresponding with a ciliate of the *Oligohymenophorea* family, which did not have the importance and dominance as reported by some authors (Chan et al., 2021; Muñoz-Palazon et al., 2019).

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 Figure 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 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 with *Taibaiella*, OTU31 from the *Spirosomaceae* family, OTU49 belonging to the *Bdellovibrio* genus, OTU54, a microorganism from *env.OPS_17* genus and OTU68 affiliated with *Ferruginibacter*. Some of these bacteria have been reported as hydrolyser microorganism for acetate and other substrates, for example OTU27, OTU 54, OTU55 and OTU68 (Sarvajith et al., 2018; Szabó et al., 2017; Zhang et al., 2019a). This agrees with our results due to simple

hydrolysis of acetate correspond 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 the Figure 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 (Zhao et al., 2013), 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, the R3 bioreactor decrease 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 experimentational 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.

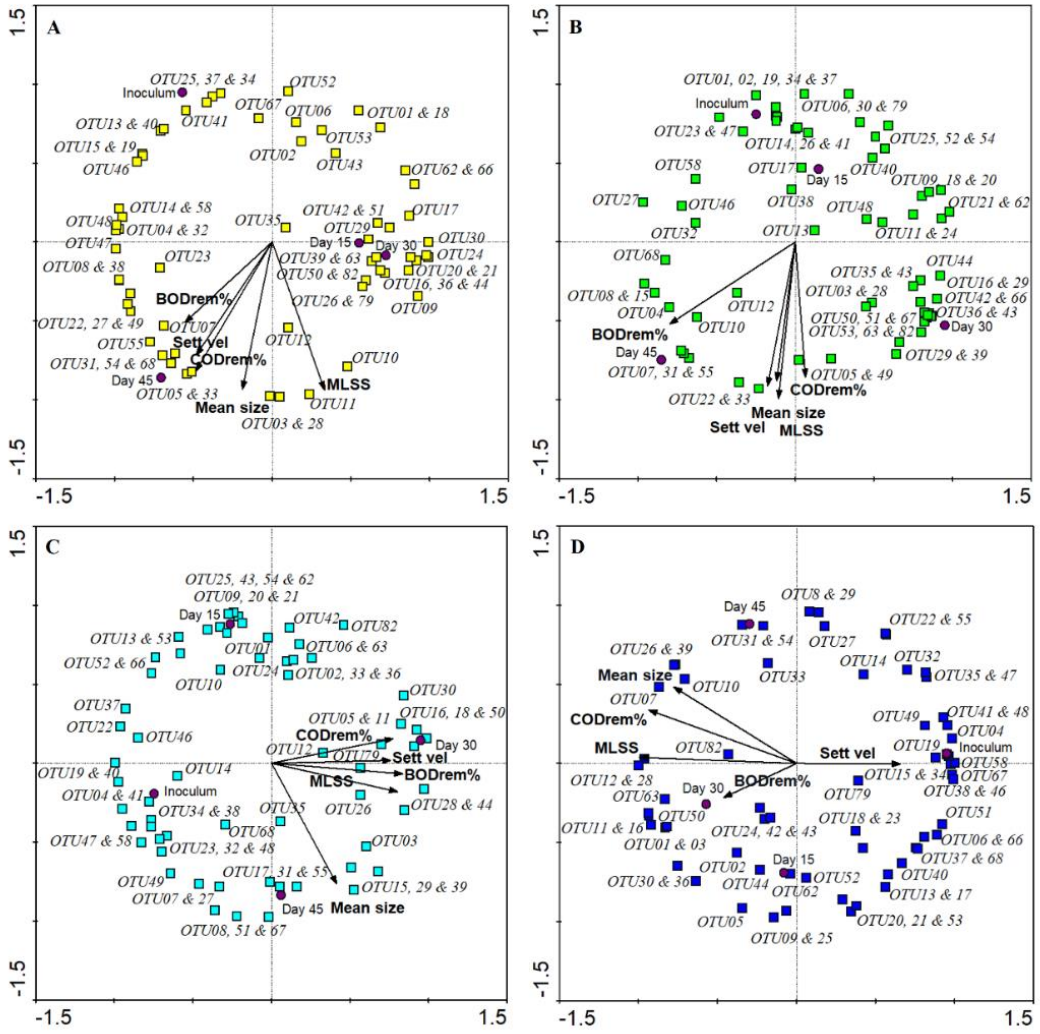

Figure 5. Multivariate redundancy analysis of the most abundant prokaryotes with physicochemical parameters (logarithm) for the continuous-flow reactors R1(A), R2(B), R3 (C) and R4 (D). Operational days correspond with periods of time: Inoculum: from day 0 to day14; Day 15: from day 15 to day 29; Day 30: from day 30 to day 44; and Day 45: from day 45 to day 55.

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 of 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 (corresponded 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 destabilization of granular biomass in the R4 bioreactor.

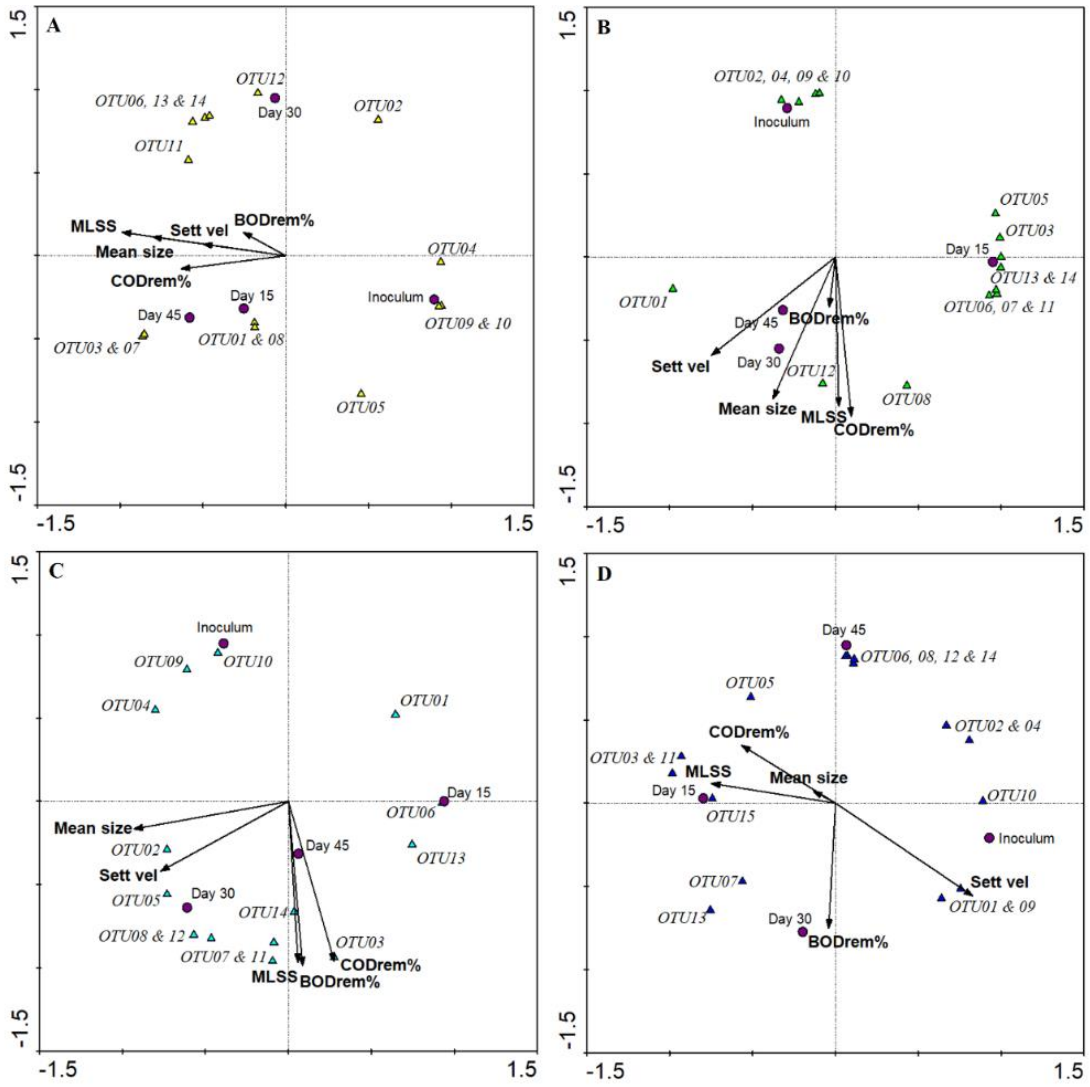

Figure 6. Multivariate redundancy analysis of the most abundant eukaryotes with physicochemical parameters (logarithm) for continuous-flow reactors R1(A), R2(B), R3(C) and R4(D). Operational days correspond with periods of time: Inoculum: from day 0 to day14; Day 15: from day 15 to day 29; Day 30: from day 30 to day 44; and Day 45: from day 45 to day 55.

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 the 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 continuous-flow reactor operational conditions. However, other continuous-flow reactor designs, especially the R4 configuration, were not capable of maintaining stable granules, giving rise to effluents with evident turbidity as a result of the presence of biomass not retained in the bioreactors. The R1 configuration at 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 stablish 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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Supplementary information

Figure S1: Appearance of granules for each CFR. It can be observed that R1 presented the most defined granules and the clearer water, while the rest of the bioreactors possessed a more disintegrated granules and more turbid water.

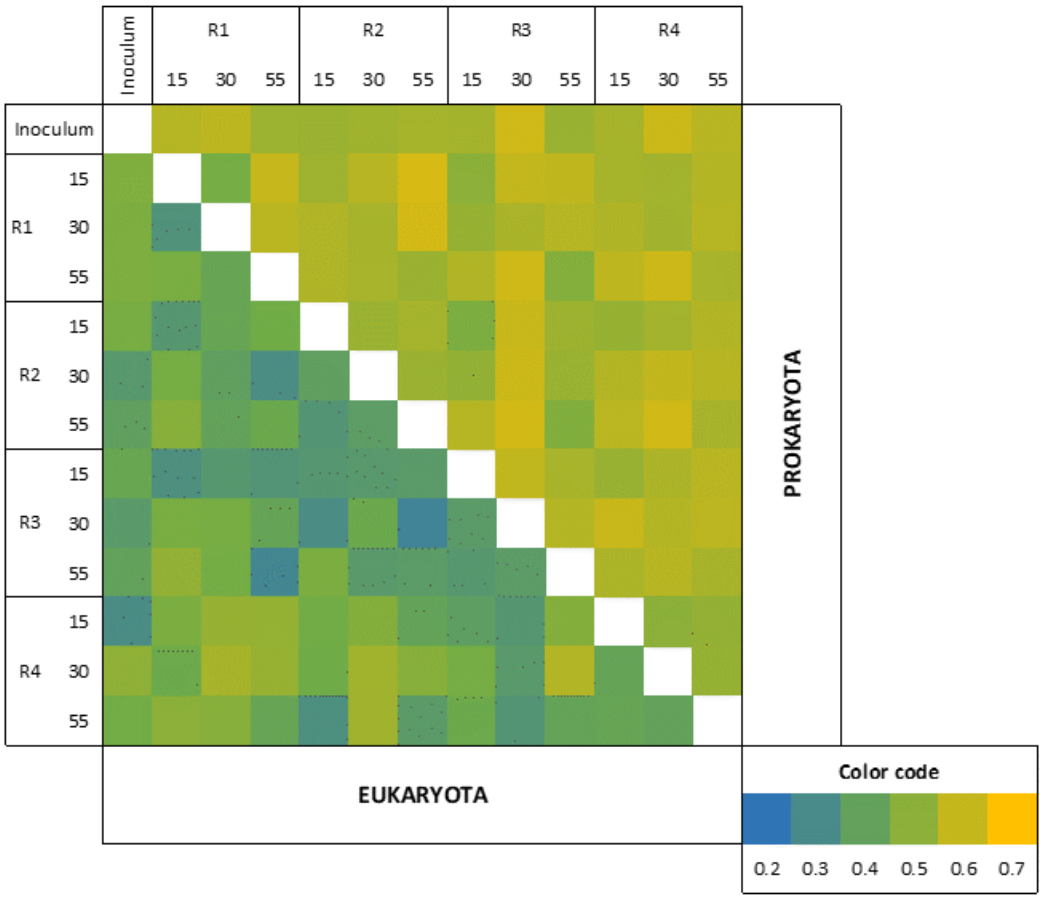

Figure S2. β-diversity (Whittaker index) of prokaryote community and eukaryote community.

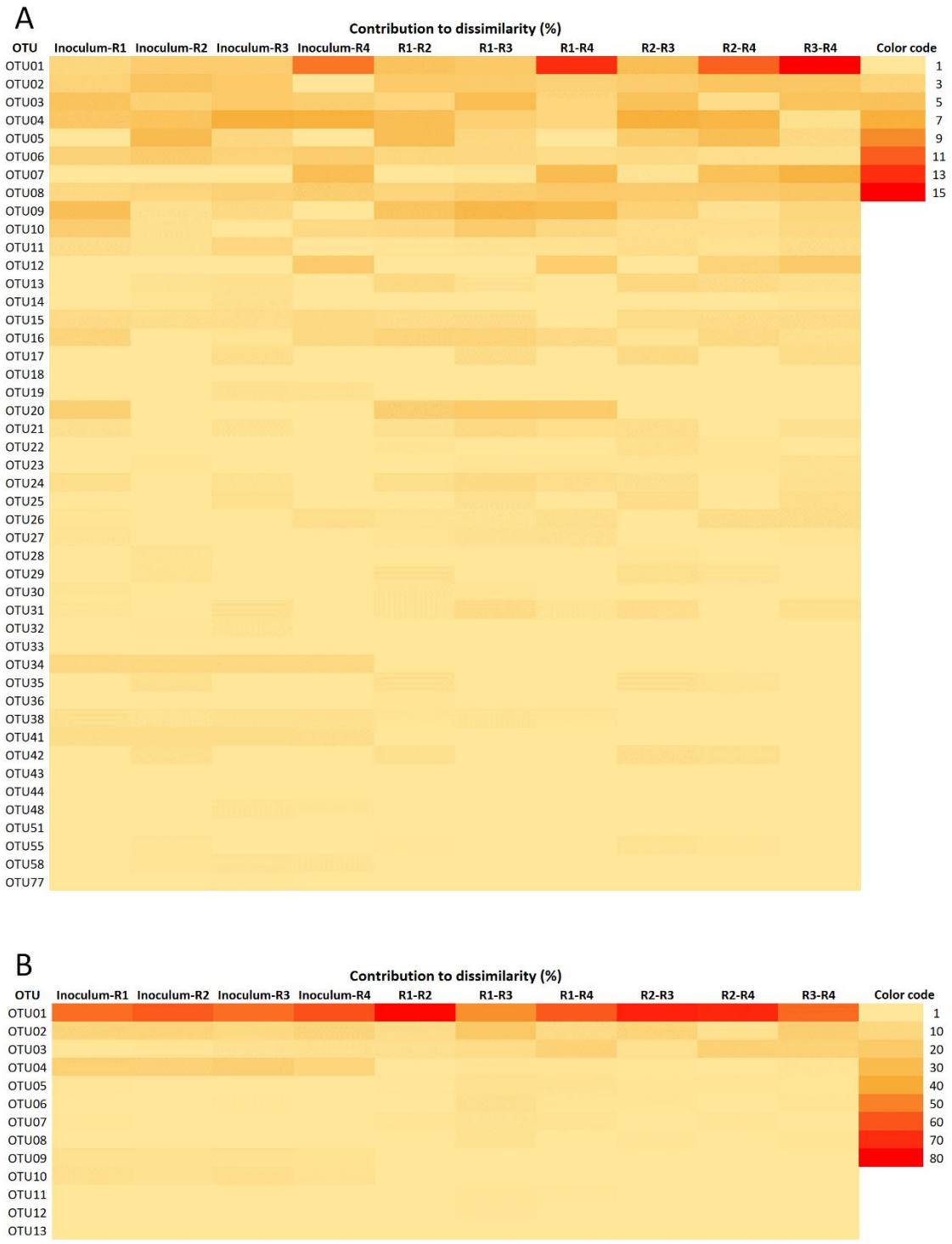

Figure S3. Similarity Percentages analysis for prokaryotic (A) and eukaryotic (B) community.

Quantification of target genes

The quantification of target genes by qPCR is presented in Figure S4. For the bacterial 16S rRNA gene, the number of copies per gram of biomass in the inoculum was 5.1×10^{10}. The operation of each reactor caused a slight decrease in contrast with the seed granular sludge but in the same order of magnitude. For the bacterial 16S rRNA gene, statistically significant differences were not found for biomass from the different reactors operated with diverse baffles (*p*-value of 0.6873 for Bray-Curtis PERMANOVA), so the use of different configurations did not affect the number of copies of bacterial 16S rRNA gene. On the other hand, for archaeal 16S rRNA gene quantification, a slight decrease was produced in all CFRs between the beginning and the end of the experiment. The R1 bioreactor experienced a loss of two orders of magnitude at day 30, but its number of copies for the archaeal 16S rRNA gene per gram was recovered at the end of the experiment. In terms of copies of the fungal 18S rRNA gene, oscillations and diverse patterns were observed. For the R1 bioreactor, differences were not found between the inoculum and day 15. However, at day 30, two orders of magnitude were lost, from 5.1×10^8 copies·g^{-1} to 6.6×10^6 copies·g^{-1}, but at day 55, this gene recovered one order of magnitude. There was a strong depletion of the fungal 18S rRNA gene for R4 at day 15, which came down from 5.1×10^8 to 1.0×10^6 copies·g^{-1}. However, the number of copies gradually recovered until reaching 1.7×10^8 copies·g^{-1}. In terms of the bacterial ammonia monooxygenase (*amoA*) gene, responsible for the oxidation of NH_4^+ to NO_2^-, a very low number of copies was found in all CFRs. Indeed, most data were almost at the same level as the detection limit of the equipment, so further research should be done to investigate this metabolic activity. Finally, the nitrous oxide reductase clade I (*nosZ I*) had a very slight decrease for all reactors at day 55 relative to the inoculum, from 1.4×10^9 copies·g^{-1} to 4.9×10^8, 4.0×10^8, 3.1×10^8 and 2.6×10^8 copies·g^{-1} for the R1, R2, R3 and R4 bioreactors, respectively, highlighting a previous reduction in the R1 bioreactor at day 30 (5.4×10^7 copies·g^{-1}), possibly produced by the strong proliferation of denitrifying *Acinetobacter* microorganisms at day 30 in the R1 bioreactor.

Figure S4. Absolute quantification of target genes represented as the logarithm of the number of copies per gram of granule; the studied genes were the bacterial 16S rRNA, archaeal 16S rRNA, fungal 18S rRNA, *amoA* and *nosZ I*.

Principal component analysis

PCA was carried out to cluster the samples according to the microbial community. PCAs from the prokaryotic and eukaryotic communities are shown in Figure S5. The prokaryotic community PCA highlighted the effect caused by the changes in the operational mode (from batch to continuous-flow operation) in the prokaryotic community, as it was possible to observe the inoculum sample secluded and distanced from the rest of the samples. Moreover, it revealed three clusters, one of them composed by day 55 from the R1, R2 and R3 bioreactors, another cluster formed by day 30 and 55 from the R4 configuration, and finally, a third cluster was composed by day 15 and 30 from all reactors, especially for the R1 and R2 bioreactors (except day 30 of the R4 design). Within this large cluster, how days 15 and 30 from the R1 reactor and the same samples from the R2 configuration were clearly defined as sub-groups, indicating the effect produced by the configuration and design of each reactor. The longest distances were observed between the inoculum and R4 reactor samples (days 15, 30 and 55). Despite the dominant OTUs of a prokaryotic community of R4 being the most similar to the inoculum, the evenness of these phylotypes was responsible for the changes, as could be corroborated in the community study and α-diversity (Table S2), highlighting day 30. In terms of eukaryotic community, PCA revealed a similar spatial location for the R1, R2 and R3 bioreactors, while R4 was displaced in other coordinates, despite having a distance between samples equivalent to the R1 configuration. The R2 and R3 bioreactors had shorter distances concerning the inoculum than the R1 and R4 reactors.

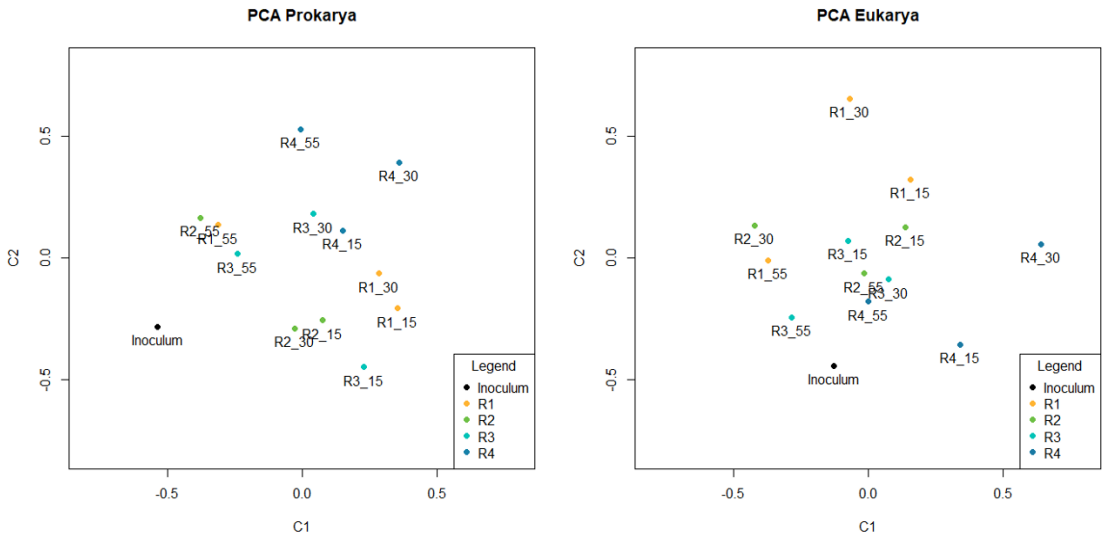

Figure S5. Principal component analysis for the prokaryotic and eukaryotic samples.

Table S1. α-diversity for prokaryotic community.

		Simpson	Shannon-Wienner	Pielou's Evenness	Chao-1
Inoculum		0.9688	4.338	0.07289	1663
Day 15	R1	0.9561	3.813	0.04612	1634
	R2	0.9485	3.774	0.04330	1691
	R3	0.9641	4.003	0.04853	1920
	R4	0.9538	3.911	0.05364	1691
Day 30	R1	0.9582	3.909	0.05566	1597
	R2	0.9755	4.329	0.07587	1839
	R3	0.9468	3.692	0.06954	1157
	R4	0.8785	3.158	0.02593	1502
Day 55	R1	0.9635	4.058	0.06599	1406
	R2	0.9440	3.714	0.04596	1460
	R3	0.9565	3.955	0.05355	1568
	R4	0.9455	3.648	0.04483	1449

Table S2. α-diversity for eukaryotic community.

		Simpson	Shannon-Wienner	Pielou's Evenness	Chao-1
Inoculum		0.5637	1.2560	0.08166	49.88
Day 15	R1	0.3654	0.8991	0.07228	43.17
	R2	0.7409	1.7700	0.20960	55.50
	R3	0.4646	1.0460	0.07693	59.00
	R4	0.4377	1.1600	0.08392	41.11
Day 30	R1	0.5776	1.3210	0.09140	71.00
	R2	0.1424	0.4020	0.03114	51.46
	R3	0.7261	1.6430	0.17230	37.00
	R4	0.6439	1.5560	0.15810	43.00
Day 55	R1	0.5790	1.3680	0.08730	50.14
	R2	0.1422	0.4041	0.04539	42.17
	R3	0.4313	1.0510	0.06500	82.25
	R4	0.8133	1.9940	0.21610	37.50

CHAPTER 2

Elucidating the role of organic loading rate on the performance and microbial dynamics of a novel continuous-flow aerobic granular sludge reactor

CHAPTER 2

Elucidating the role of organic loading rate on the performance and microbial dynamics of a novel continuous-flow aerobic granular sludge reactor

Abstract

Aerobic granular sludge technology operated in continuous-flow reactors enables the upgrading of existing conventional wastewater treatment plants. The viability of applying this technology at full-scale increases with the simplicity of the bioreactor design. Hence, in contrast to previous research carried out in complex configurations, this study is based on evaluating the capacity of a novel single-chamber continuous-flow bioreactor to operate at different loads found in domestic sewage, highlighting that this configuration combines and joins the advantages of activated sludge and granular sludge technologies. At organic loading rates ranging from 0.45 to 1.85 kg $COD \cdot m^{-3} \cdot d^{-1}$, the reactors achieved high performance, removing >80 % of the chemical oxygen demand, regardless influent characteristics. The trend of ammonium removal rates was risen over operational time. Moreover, the granular size and biomass concentration increased with the organic loading rate. Changes in the influent did not compromise the structure of the granules. Microbiota studies showed a high diversity in communities at lower organic loading rates, whereas a strong microbial competition was detected at high loading rates, linking with lower evenness. Phylotypes belonging to *Hypocreales* played a key role in mature granules.

Keywords: aerobic granular sludge; continuous-flow reactor; microbial ecology; organic loading rate; single chamber.

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1. Introduction

The AGS technology is a biological technology for wastewater treatment in which microorganisms are grouped in a dense spherical biofilm without carrier material (Franca et al., 2018). The compact granular biomass and the excretion of extracellular polymeric substances (EPS) allow to withstand the effects of toxic compounds and changing loading rates (Hamza et al., 2018). Moreover, this technology has excellent settling properties which, combined with the hydrophobic surface of granules, enables the separation of the biomass from the aqueous phase in the biological reactor, resulting in a more compact bioreactor (Franca et al., 2018). In addition, the high compactness hinders the mass transfer of O_2 and nutrients, creating different redox conditions depending on granule zones and, thus, allowing the presence of high microbial diversity and metabolic versatility. Subsequently, a granule would have the same niches than the oxic, anoxic and anaerobic chambers of other technologies for nutrient removal (Franca et al., 2018; Winkler et al., 2018). This advantage, together with the high sludge retention time, promotes the growth of slow-growing microorganisms, allowing the simultaneous removal of different pollutants such as organic matter, nitrogen, phosphorus and recalcitrant pollutants (Franca et al., 2018). For these reasons, the AGS requires approximately 20 %–50 % less energy than conventional activated sludge (CAS), its ecological footprint is 25 %–75 % lower than that of CAS and, in addition, it produces both capital and operational cost savings (Pronk et al., 2017). Due to these benefits, the AGS technology represents an optimal alternative to treat urban wastewater, industrial wastewater and even polluted drinking water (Hurtado-Martinez et al., 2021; Kent et al., 2018).

Traditionally, AGS has been operated in sequential batch reactors (SBRs) to facilitate granule development because the selection pressure exerted by the discharge stage promotes the washout of light flocs, which may negatively interfere with water treatment (Silva et al., 2023). However, current investigations are focused on the development of continuous-flow reactors (CFRs), to reduce construction and operation costs, to simplify maintenance of the technology, to adjust to existing infrastructures and to treat large flows in a more practical mode (Kent et al., 2018; Silva et al., 2023). This constitutes a challenge because the granules should be retained within the bioreactor but, at the same time, filamentous flocs must be removed. For that, several continuous-flow designs have been proposed. Some of them are based on serial multiple chambers,

submerged membranes, clarifiers with returning of granules or hybrid sequential batch and continuous-flow reactors (Rosa-Masegosa et al., 2021). However, these designs are complex systems, their operation is difficult and, moreover, they could contribute to the granule disintegration (Samaei et al., 2023); hence, an optimal CFR design is required to achieve simpler CFR configuration for full-scale implementation. In this sense, Rosa-Masegosa et al. (2023) have developed a new simple CFR for AGS consisting in a single-chamber bioreactor without the need for granule recirculation, which retained and maintained stable granules, and removed light flocs while achieving an organic matter removal rate of above 95 %. This novel design must be evaluated to verify its possibility as a potential option for full-scale AGS-CFR implementation under a wide range of organic matter load.

The organic loading rate (OLR) is one of the key parameters that affect the formation and stability of AGS (Iorhemen and Liu, 2021). It is considered in the wastewater treatment process design and is used as an indicator to assess the treatment capacity of the reactor. Studies have reported that AGS in SBRs could operate in a range from approximately 0.3 to 15 kg $COD \cdot m^{-3} \cdot d^{-1}$ (Chen et al., 2019a). A low OLR promotes the formation of small and compact granules, but the granulation period is longer, and the growth of filamentous microorganisms could produce granular disaggregation. At high OLRs, the start-up period is considerably reduced, the granular biomass is larger and less compact, but it can disintegrate and lead to the collapse of the system (Iorhemen and Liu, 2021). According to Peyong et al. (2012), domestic wastewater is considered as low-strength wastewater, containing a range of BOD_5 from 110 to 350 mg $O_2 \cdot L^{-1}$ (Tchobanoglous et al., 2003). The treatment of low-OLR wastewater by CAS gives rises to the weakening of floc formation, generating settleability problems which lead to the deterioration of effluent quality in terms of suspended solids and organic matter (Miyake et al., 2023). By contrast, granular biomass can maintain the stability more satisfactorily than CAS when a decrement in wastewater strength is produced, driven by the production of EPS (Chen et al., 2019a).

The simple design and operation of the new AGS-CFR (Gonzalez-Martinez et al., 2024) described by Rosa-Masegosa et al. (2023) make it a potential candidate for full-scale implementation. The aim of the present study was to investigate and evaluate its response in terms of granular stability, organic matter removal performance and microbial diversity when the bioreactor is exposed to different OLRs of domestic wastewater.

2. Materials and methods

2.1. Bioreactor configuration and start-up

Four single-chamber CFRs were used to determine the responses of the bioreactors to different OLR in terms of carbon and nitrogen removal and granular properties. Each bioreactor had a working volume of 6 L, following the selected design described by Rosa-Masegosa et al. (2023), which consists of a single column of 72 cm in height and 10 cm in inner diameter, and at the top of the reactor there is a settler to avoid the loss of compact biomass during effluent discard (Fig. 1).

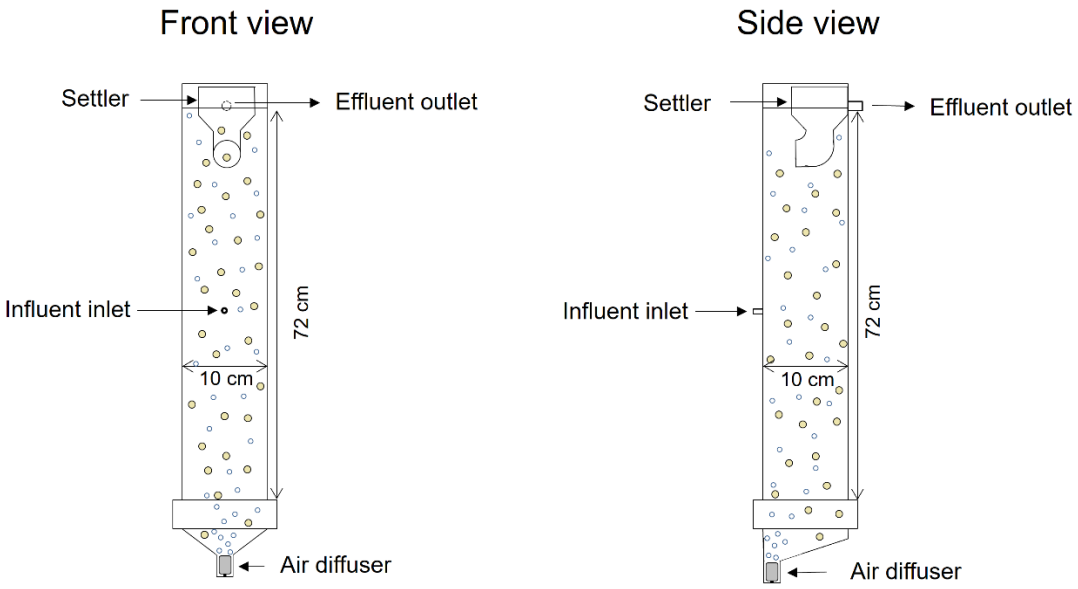

Figure 1. Schematic diagram of the single-chamber AGS-CFR configuration used in this work.

The bioreactors were operated with a hydraulic retention time (HRT) of 6 h for 60 days. Aeration was supplied through a diffuser placed at the bottom of the reactors. The influent was synthetic medium simulating urban wastewater (Table S1), and it was introduced in the bioreactors at mid-height by mean of Watson–Marlow peristaltic pumps (United Kingdom). To study the typical range of BOD_5 in raw wastewater, each bioreactor had a different concentration of $CH_3COONa{\cdot}3H_2O$ as carbon source, to simulate 400 mg $O_2{\cdot}L^{-1}$ of BOD_5, 300 mg $O_2{\cdot}L^{-1}$ of BOD_5, 200 mg $O_2{\cdot}L^{-1}$ of BOD_5 and 100 mg $O_2{\cdot}L^{-1}$ of BOD_5 for R1, R2, R3 and R4, respectively. The COD values obtained were 460 mg $O_2{\cdot}L^{-1}$, 350 mg $O_2{\cdot}L^{-1}$, 225 mg $O_2{\cdot}L^{-1}$ and 113 mg $O_2{\cdot}L^{-1}$ for R1, R2, R3

and R4, respectively, corresponding with 1.85 kg COD·m^{-3}·d^{-1} for R1, 1.40 kg COD·m^{-3}·d^{-1} for R2, 0.90 kg COD·m^{-3}·d^{-1} for R3 and 0.45 kg COD·m^{-3}·d^{-1} for R4 of OLR, conducted by the Eq (1):

$$OLR = \frac{Q \cdot C}{V} \quad (1)$$

The bioreactors were inoculated with 250 mL of granular biomass from a CFR system in steady state, acclimated to a BOD_5 of 300 mg O_2·L^{-1}.

2.2. Physicochemical analyses

Periodically, influent and effluent samples of each bioreactor were evaluated. Ammonium, nitrate and nitrite ions were measured using a Metrohm ion chromatograph. The organic matter was determined through the COD and BOD_5, according to the American Public Health Association (APHA) methods (2017). The mean size and settling velocity of the granules were measured following Muñoz-Palazon et al. (2023c). In addition, mixed liquor suspended solids (MLSS) of each bioreactor were determined in triplicate according to APHA methods (American Public Health Association, 2017).

2.3. Biological analyses

2.3.1. Sample collection and DNA extraction

Biomass samples were collected periodically and processed following Muñoz-Palazon et al. (2018). The DNA extracted of each sample was suspended in 150 µL of deionised water and kept at -20ºC.

2.3.2. Absolute quantification of target genes

Quantitative polymerase chain reaction (qPCR) was performed to quantify the absolute abundances of three target genes using the QuantStudio-3 Real-Time PCR system (Applied Biosystems). Total *Bacteria* and *Archaea* were estimated by bacterial and archaeal 16S rRNA genes, whereas total *Fungi* were determined by means of fungal 18S rRNA genes. The reactions, calibration curves, primers and qPCR conditions are described in Correa-Galeote et al. (2021a).

2.3.3. Next-generation sequencing

Illumina MiSeq was performed for prokaryotes and eukaryotes using the following primers: for prokaryotes, 16S rRNA Pro341 forward 5'-CCTACGGGNBGCASCAG-3' and 16S rRNA Pro805 reverse 5'-GACTACNVGGGTATCTAATCC-3'; for eukaryotes, 18S rRNA Euk1391F 5'-GTACACACCGCCCGTC-3' and 18S rRNA EukB 5'-TGATCCTTCTGCAGGTTCACCTAC-3'. Bioinformatic analysis was performed following Rosa-Masegosa et al. (2023).

2.4. Statistical and ecological analyses

The data obtained by the bioinformatics pipeline were used to carry out the biostatistical and ecological analyses.

First, α-diversity (Simpson, Shannon-Wiener, Pielou's Evenness and Chao-1 indices) was calculated for prokaryotic and eukaryotic communities using the PAST 3.14 software. Permutational multivariate analysis of variance (PERMANOVA) was performed in the PAST 3.14 software to determine statistically significant differences between samples. The similarity percentages analysis (SIMPER) was calculated under Bray-Curtis algorithm by PAST 3.14 software, for OTUs with a minimum of 1 % of relative abundance for prokaryotes and 0.5 % for eukaryotes. Principal component analysis (PCA) was conducted using OTUtable transformed to centered logarithm to avoid zero values using bioconductor ALDEx2 and robCompositions packages implemented in R software, with the aim to compare the microbial communities over time and among different bioreactor conditions. The relationship between dominant operational taxonomic units (OTUs) and physicochemical parameters was evaluated by multivariate redundancy analysis (RDA). For that purpose, the parameters were transformed to the log (x + 1). The computation was executed using CANOCO 4.5, following Muñoz-Palazon et al. (2018).

3. Results and discussion

3.1. Effects of organic loading rate on granular sludge and performance ratios

Nowadays, several AGS bioreactors operate in continuous mode, however, most of them have multiple chambers or require sludge return system, hindering the operation

and the granular stability (Yan et al., 2021). In this research, the bioreactor has a single chamber, simplifying their use at full scale. The experiment was performed in two stages, the start-up stage was the period before day 24, and the steady-state stage was the period from day 24 until the end of the experiment. The granular characteristics were evaluated by mean size, settling velocity and biomass concentration (Fig. 2 and Fig. S1). In terms of mean size (Fig. 2A), at the steady state, the bioreactors followed a tendency directly proportional to the OLR. In this way, R1 was the bioreactor with the largest granular biomass, followed by R2 and R3, which did not statistically differ ($p > 0.05$); the R4 granules were the smallest ones. The size of the R1 bioreactor granules decreased from 9.0 to 7.5 mm during the first week of operation. Then, mean size stabilised at 11.9 ± 1.0 mm, agreeing with the value reported by Rosa-Masegosa et al. (2023). For R2 and R3 bioreactors, after some fluctuations, the granular size reached values of 9.7 ± 0.6 and 9.2 ± 1.0 mm, respectively, during the first stage of experimentation. Despite the similar mean size between R2 and R3 at the start-up stage, the R3 conditions promoted granules characterised by filamentous structures clearly visible on the biomass surface, whereas the R2 biomass developed strong and compact structures (Fig. S2). In the steady state, the filamentous of granules of bioreactor R3 were reduced, possibly by the secretion of EPS (Sun et al., 2021b). The lower OLR available in the R4 bioreactor resulted in a mean granule size of 7.2 ± 1.2 mm, and the granular surface had a regular shape, possibly caused by reducing negative charges of the cell surface at low carbon concentrations (Li et al., 2022). A similar pattern was found by other authors (Cui et al., 2015; Muñoz-Palazon et al., 2023c), who reported that a higher OLR produces longer granules, whereas low carbon concentrations induce a smaller granular size.

Regarding the settling velocity (Fig. 2B), the values also depended on the OLR. For the R1, R2 and R3 bioreactors, the settling velocity increased from 76 $m \cdot h^{-1}$ to more than 100 $m \cdot h^{-1}$ at day 12, whereas the R4 bioreactor had the lowest values (between 60 and 70 $m \cdot h^{-1}$). After this period, the differences among the first three reactors were more significant. Moreover, all bioreactors, including R4, suffered a slow and progressive decrease of settleability, with mean values of 80.0 ± 11.9 $m \cdot h^{-1}$, 71.6 ± 5.1 $m \cdot h^{-1}$, 56.6 ± 8.1 $m \cdot h^{-1}$ and 45.5 ± 8.3 $m \cdot h^{-1}$ for R1, R2, R3 and R4, respectively. Based on the results, OLR had a direct effect on the compactness, stability and density of the granules and, ultimately, on the granular settling velocity (Cydzik-Kwiatkowska et al., 2016). The studied configuration improved the settleability and the compactness of granules in

comparison with previous studies operated in SBR, which were not able to withstand granular integrity with OLRs closed to 2 kg $COD \cdot m^{-3} \cdot d^{-1}$(Silva et al., 2022).

Regarding the biomass concentration (Fig. 2C), all bioreactors had a granular biomass concentration of 0.04 $g \cdot L^{-1}$ at the beginning of the experiment. Throughout the experiment, there was relationship between the ORL and the biomass concentration in the bioreactors. In this way, R1, the bioreactor that supported 1.85 kg $COD \cdot m^{-3} \cdot d^{-1}$, achieved an average biomass concentration of 5.0 ± 0.3 $g \cdot L^{-1}$ during the steady state. Reactor R2 (withstanding 1.40 kg $COD \cdot m^{-3} \cdot d^{-1}$) had an average concentration of 2.6 ± 0.8 $g \cdot L^{-1}$ from day 24, although at the end of the experiment, biomass concentration decreased to 2 $g \cdot L^{-1}$. Reactor R3, with 0.90 kg $COD \cdot m^{-3} \cdot d^{-1}$, stabilised its biomass concentration at around 1.6 ± 0.6 $g \cdot L^{-1}$. For R4, with 0.45 kg $COD \cdot m^{-3} \cdot d^{-1}$ of OLR, a biomass concentration of 0.4 ± 0.2 $g \cdot L^{-1}$ was achieved during the steady-state stage. These results suggest that an increase in OLR produces a notable growth of granular biomass, as previously reported by Rosman et al. (2014).

The results highlight the relevance of OLR for the exploitation and operation of bioprocesses, specifically in AGS-CFRs, because it is directly correlated with the morphological properties. In this research, the obtained data revealed how changes in OLR significantly modified the size and settlement of granules ($p < 0.05$), but the configuration of this novel technology was able to withstand the typical OLR found in urban wastewater, without compromising the resilience of the granules. This has previously been reported for AGS operated in sequential batch cycles, where filamentous flocs are washed out during the discard period (Iorhemen and Liu, 2021; Muñoz-Palazon et al., 2023c). The success of this simpler bioreactor relies upon the capability of treating a wide range of OLR, retaining quality granular biomass in structural terms, without the need of granules recirculation or complex design and operation.

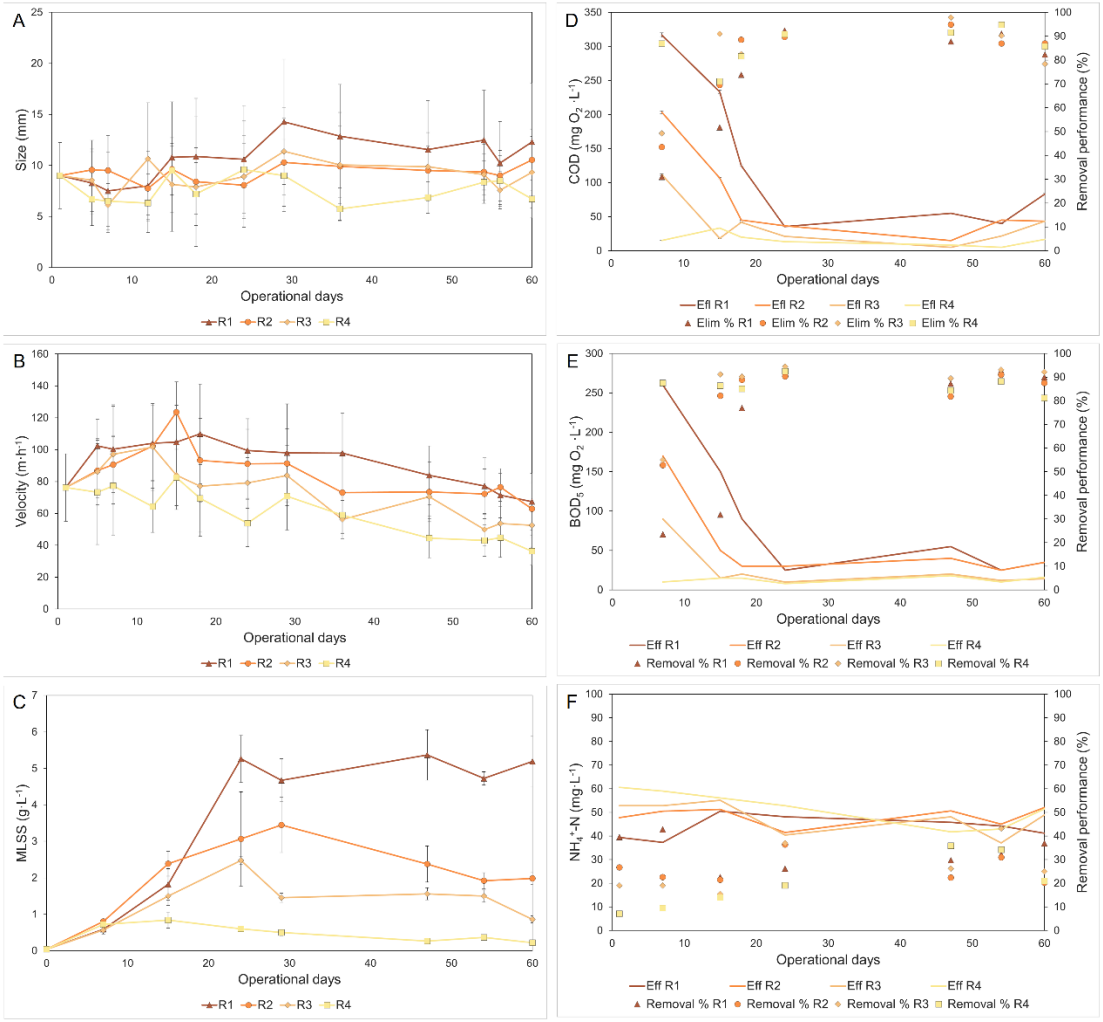

Figure 2. Effect of OLR on A) mean granule size; B) granule settling velocity; C) biomass concentration; D) COD removal; E) BOD_5 removal and F) NH_4^+-N removal.

The removal of organic matter and ammonium was analysed to evaluate the chemical performance of each bioreactor in relation to the OLR. In terms of COD (Fig. 2D), reactor R1 progressively reduced the concentration in the effluent. At day 18, this bioreactor met the discharge requirements of the European legislation (Directive 91/271/EEC), with an effluent concentration of 125 mg $O_2 \cdot L^{-1}$, coinciding with the stipulated limit according to the European legislation (Directive 91/271/EEC). From day 24, R1 reached stabilisation, with average values of 53.5 ± 21.6 mg $O_2 \cdot L^{-1}$ in the effluent and a removal rate of 88.4 ± 4.5 %, with a maximum of 92.4 %. Reactor R2 followed the same pattern, with a gradual reduction in the effluent COD. At day 15, the effluent concentration met the discharge requirements, with 106.7 mg $O_2 \cdot L^{-1}$, and during the

steady state, the mean values were 35.0 ± 13.8 mg $O_2 \cdot L^{-1}$ in the effluent, with a COD removal rate of 89.6 ± 3.7 %, reaching a maximum of 94.9 %. The effluent values of reactors R3 and R4 met the discharge requirements throughout the controlled experiment. The mean values of R3 during the stable stage were 22.8 ± 15.7 mg $O_2 \cdot L^{-1}$ of COD concentration in the effluent and a removal rate of 89.3 ± 8.1 %, with a maximum of 97.8 %. Reactor R4 showed an effluent concentration of 10.8 ± 5.2 mg $O_2 \cdot L^{-1}$ and a COD removal rate of 90.7 ± 3.7 %, reaching a maximum of 94.7 %. These results were similar to those obtained in previous AGS studies in SBR and CFR configurations (Kent et al., 2018; Muñoz-Palazon et al., 2020b).

In terms of BOD_5 (Fig. 2E), the trends were similar to those of COD. During the start-up stage, the effluent BOD_5 was progressively reduced, except for R4, which was stable due to the low influent concentration (100 mg $O_2 \cdot L^{-1}$). Bioreactor R1 had an average effluent concentration in the steady state of 35.0 ± 14.1 mg $O_2 \cdot L^{-1}$, achieving 25 mg $O_2 \cdot L^{-1}$, the level for water discharge specified by the European regulations (Directive 91/271/EEC), from day 24. The mean BOD_5 removal rate was 91.0 ± 3.0 %. For R2, the mean values were 87.7 ± 4.2 %, also achieving 25 mg $O_2 \cdot L^{-1}$ at day 54. The R3 effluent met the European requirements from day 15 because the effluent average BOD_5 concentration was 14.0 ± 4.3 mg $O_2 \cdot L^{-1}$, with a removal rate of 92.3 ± 2.1 %. Finally, the R4 effluent met the requirements from the beginning, with a mean of 13.0 ± 4.8 mg $O_2 \cdot L^{-1}$ during the steady-state stage and a removal rate of 86.5 ± 4.8 %. The effluents of bioreactors R1 and R2 only met the discharge requirements punctually; however, the percentage removed was sufficient to fulfil the conditions before emitting the effluent. Moreover, no statistically significant differences ($p > 0.05$) were detected for the removal rates of the bioreactors at steady state. In this sense, irrespective of the OLR, the biomass concentration in each bioreactor was adjusted to achieve high organic matter removal rates, according to the OLR treated.

Regarding the NH_4^+-N removal (Fig. 2F), there were no statistically significant differences between the bioreactors at steady state ($p > 0.05$). In steady state, the mean values of NH_4^+-N in the effluent were similar for all bioreactors, below 45 mg$\cdot L^{-1}$, independently of the OLR treated. The removal ratios for R1, R2, R3 and R4 were 31.3 ± 4.5 %, 27.5 ± 7.5 %, 32.8 ± 8.8 % and 27.5 ± 8.7 %, respectively. Therefore, an HRT of 6 h was not sufficient for complete ammonium oxidation. A longer HRT could increase the ammonium removal capacity, as reported by Wan et al. (2013a), who achieved an

effluent with 5 $mg \cdot L^{-1}$ of NH_4^+-N with an HRT of 12 h. In addition, the nitrate and nitrite values in the effluent were below 0.55 $mg \cdot L^{-1}$ and below the detection limit, respectively, indicting efficient denitrification. These results highlight that the organic matter concentration was sufficiently high for heterotrophic denitrification to occur.

3.2. Quantification of target genes

The absolute quantification of target genes was performed for archaeal and bacterial 16S and fungal 18S rRNA genes to describe the effect of the OLR on the granular sludge in CFRs (Fig. S3).

The bacterial target gene in the granules used as inoculums had a magnitude order of 10^{10} copies·g of granule^{-1}, which remained in the same magnitude order regardless of the OLR of bioreactors. Reactor R1 did not show any change in the number of bacterial copies throughout the operation, similar to reactor R4. However, reactors R2 and R3 experienced a decrease in the number of copies by an order of magnitude, with R2 affected from day 47 and R3 from day 60. The archaeal populations were significantly impacted, revealing competitive disadvantages over time. The inoculum samples had 10^6 copies·gram of granule^{-1}, a value significantly lower than that of the bacterial copies, which is typical in biotechnological approaches with organic carbon since bacteria usually have competitive advantages over archaea in terms of carbon uptake. By day 7, the number of archaeal copies increased by an order of magnitude, but this increase was not stable over time, except for the granules in R1, where we observed stable values of archaeal copies in the range of 10^6 to 10^7. This suggest that the OLR allowed the coexistence of archaea and bacteria because both had access to the substrates. However, for the remaining bioreactors, there was no clear archaeal dynamic, with decreases by up to two orders of magnitude. The results of the qPCR of the total *Fungi* in the granular sludge used as inoculum indicate the essential role of fungi in the mature granules, with values higher than 10^8 18S rRNA copies·gram of granule^{-1} (Gonzalez-Martinez et al., 2018). The granules of R1 consistently showed a stable copy number above nine orders of magnitude. Fungi are optimal consumers and degrader of organic matter and form efficient consortia with bacteria in several natural and biotechnological environments (Robles-Morales et al., 2021). The average values of fungal copies in R2 and R3 remained stable, ranging from 10^8 to 10^9; for R4, the lowest number of fungal copies was observed,

with a value off 3.59 x 10^7 per gram of granule at day 24, although initial levels recovered afterwards.

3.3. Microbial community dynamics

3.3.1. Dynamics of the prokaryotic community

The prokaryotic community of the bioreactors was represented by 69 OTUs with a relative abundance of >1.5 % (Fig. 3). At the superkingdom level, all predominant OTUs belonged to *Bacteria*. The inoculum sample was dominated mainly by OTU018, which was affiliated with the *JGI_0000069-P22* order, with a total relative abundance of 8.5 %. This order belongs to the *Gracilibacteria* class, which is linked to the production of EPS and sludge granulation (Jin et al., 2022). Other OTUs with high representation in the inoculum sample were OTU003, affiliated with the *Verrucomicrobiae* class, and OTU001, from the *Pseudomonas* genus. The genus *Pseudomonas* accelerates granulation and participates in nutrient remotion (Han et al., 2022). Other dominant OTUs with relative abundances of more than 3 % were OTU007, taxonomically affiliated with the *Xanthobacteraceae* family, which can remove ammonium and nitrate via partial denitrification and anammox (Hurtado-Martinez et al., 2021), OTU009, which belongs to the *Corynebacterium* genus, related to nitrogen removal and with the capacity to treat toxic and high-strength wastewater (Han et al., 2022; Liu et al., 2018; Muñoz-Palazon et al., 2020b); OTU002, from the *Leucobacter* genus, which, according to Hurtado-Martinez et al. (2021), is linked to granulation, OTU021, affiliated with the *Burkholderiaceae* family, and OTU036 of the genus *Flavobacterium*. Because of its EPS production capacity, *Flavobacterium* can assist in granule formation (Cydzik-Kwiatkowska and Zielińska, 2016). It is also involved in the removal of organic matter, P and N (Chen et al., 2016; Liang et al., 2022; Meng et al., 2020). During the experimental period, each OLR tested produced different changes in the prokaryotic community. At the beginning of the experiment with R1, some OTUs had an increased relative abundance, namely OTU001, OTU002, OTU003 and OTU016. Of these, OTU016 is affiliated with the *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* genus, involved in denitrification (Rosa-Masegosa et al., 2023). At day 24, OTU006 and OTU010, of the family *Rhodocyclaceae* and the genus *Proteiniphilum*, respectively, dominated the community, together with OTU001, OTU002, OTU003, OTU025 (*Dysgonomonadaceae*

family) and OTU015 (*Pseudomonas* genus). The *Rhodocyclaceae* family has both aerobic and anaerobic strains, which produce an excess of EPS, thereby contributing to granular strength (Lv et al., 2014). Their proliferation at high OLR (Chen et al., 2019b) might explain their high numbers in R1 and R2. After that day, the total abundances of OTU002, OTU006, OTU010, OTU015 and OTU025 were progressively reduced until the end of the experiment; this stage was dominated by OTU001, OTU002, OTU003 and OTU004. Of these, OTU004 is taxonomically affiliated with the *Spirosomaceae* family, associated with BOD_5 removal, OTU005, from the *Chitinophagaceae* family, and OTU014 of the genus *Brevundimonas*, with a high EPS excretion ability (Rosa-Masegosa et al., 2023). Globally, OTU001, OTU002 and OTU003, dominated at the end of the experiment, along with other phylotypes such as OTU004 and OTU014. In contrast, the dominant OTUs in the inoculum, such as OTU007, OTU009, OTU018, OTU021 and OTU036, suffered a strong decrease in their relative abundances over time. The R2 bioreactor operation strongly selected OTU001 at the beginning of the experiment until it reached 14.6 % of the total relative abundance. Other OTUs that were codominant with OTU001 at day 7 were OTU002, OTU003, OTU005, OTU006 and OTU009. After that, the same OTUs dominated the community, together with OTU010, OTU015 and, punctually, OTU046, belonging to the *Flavobacterium* genus. At the end of the experiment, OTU001 was still dominant, but there were changes in the community because previous dominant OTUs were replaced by other phylotypes related to OTU004, OTU008, which was affiliated with the high-versatile taxon NS9 marine group from the *Flavobacteriales* order and related with granular stability (Chen et al., 2019a), OTU011 from the *Burkholderiaceae* family, OTU024 from the *Sericytochromatia* class, OTU028 of the genus *Solitalea*, family *Sphingobacteriaceae*, which can degrade ammonia and organic matter (Lv et al., 2014), and OTU045, affiliated with the EPS-producing genus *Bdellovibrio* (Wan et al., 2013b). In steady state, most microorganisms were related with granular stability and contaminant remotion, as observed for R1. The R3 bioreactor did not exert a strong selection of the microbial population until the end of the experiment. At day 7, the community was similar to the inoculum sample, although OTU018, OTU021 and OTU036 showed reduced relative abundances, allowing the domination of OTU001, OTU003 and OTU009, followed by OTU002, OTU005 and OTU006. After that, previous OTUs co-dominated with OTU004, OTU007, OTU008 and OTU015. During the steady-state stage, OTU004 and OTU008 were the OTUs with the highest relative abundances, followed by OTU024, OTU047, affiliated with the *SM2D12* family (*Rickettsiales* order),

OTU048, belonging to the *SWB02* genus (*Hyphomonadaceae* family) and, to a lesser extent, OTU005, OTU011, OTU012 and OTU020 from the order *OPB56* (*Ignavibacteria* class). Notably, all dominant OTUs in the inoculum almost disappeared at the end of the experiment, indicating the effect of OLR changes in the bioprocess. Finally, the R4 bioreactor, showed a pattern similar to that of R3 because at the end of the experiment, all dominant OTUs found in the inoculum sample were drastically reduced. At the beginning of the experiment, the prokaryotic community was similar to that in the inoculum. From days 15 to 24, there was a selection of OTU002, OTU004, OTU005, OTU007, OTU008 and OTU0019 (affiliated with the *Rhizobiaceae* family). After day 24, there were changes in the prokaryotic community, when non-dominant OTUs in the inoculum sample were selected. Therefore, the *Rhizobiaceae* family, represented by OTU016, OTU019, OTU022 and OTU049, showed a gradually increasing abundance throughout the experiment, reaching a total relative abundance of 9.9 %. This indicates that this family is advantaged by the low access to organic matter. The genus *Bdellovibrio* (OTU059 and OTU045) and the family *Burkholderiaceae* also dominated at the end of the experiment, together with OTU005, OTU012, OTU020 and OTU033 (*SM1A02* genus), reported as an anammox strain (Liang et al., 2022).

The prokaryotic dynamics highlight the key effect of OLR in microbiota and metabolic pathways because R1 and R2 had similar trends related to the dominant phylotypes, such as the genus *Pseudomonas* and the families *Spirosomaceae, Dysgonomonadaceae* and *Rhodocyclaceae*. The bacterial populations of R3 and R4, under lower OLR, were represented by the *SM1A02* genus and the families *Rhodobacteraceae*, *Sphingobacteriaceae* and *Rhizobiaceae*, the order *OPB56*, the class *Sericytochromatia* and the NS9-marine group (OTU008). The dynamics of microbial community demonstrate how the dominant phylotypes were selected to adapt to the operational conditions, without significant modifications in the physicochemical performance.

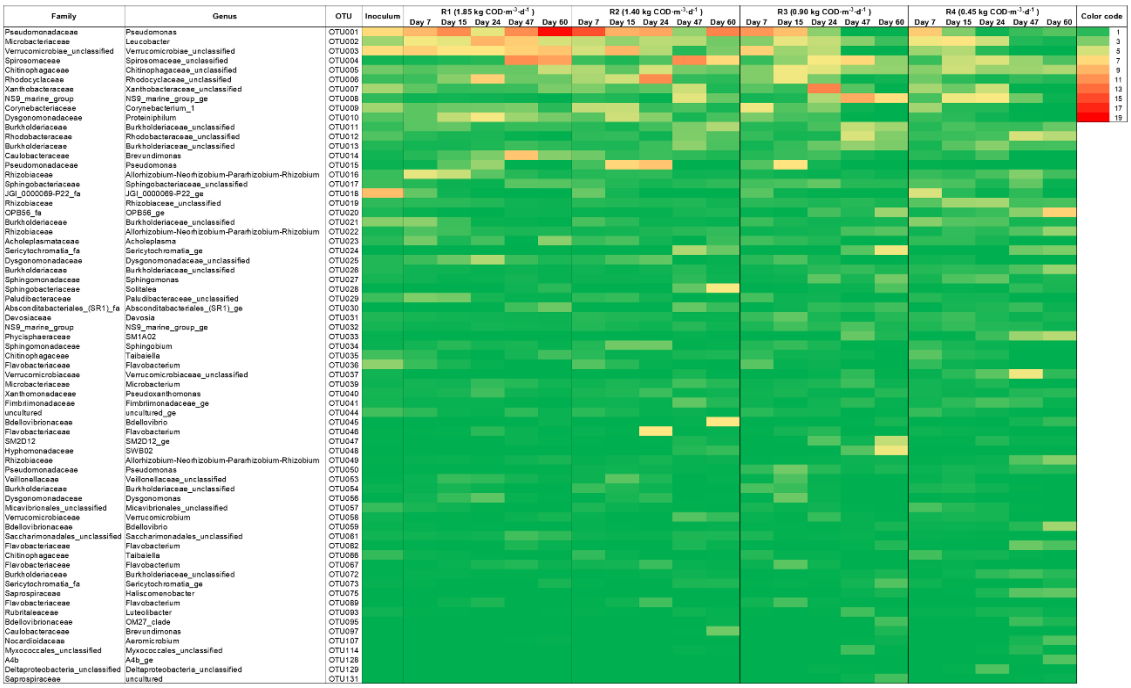

Figure 3. Heat map of prokaryotic operational taxonomic units with a total abundance of >1.5 %.

3.3.2. Dynamics of the eukaryotic community

The eukaryotic community was characterised by 29 OTUs with a relative abundance of >1 % (Fig. 4). The inoculum was dominated by OTU02, affiliated with an unclassified fungus from the *Ascomycota* phylum, with a relative abundance of 69.3 %. In addition, OTU01, belonging to the *Hypocreales* order, OTU03 and OTU04 from the *Ascomycota* phylum and OTU05, affiliated with the *Peronosporomycetes* class, were highly represented in the inoculum. The phylum *Ascomycota* is a large fungal group with the ability to decompose several types of pollutants and large contribution to biomass aggregation (Maza-Márquez et al., 2016). The order *Hypocreales* is an important group in biofilms (Li et al., 2020b), whereas the *Peronosporomycetes* group, affiliated with the *Oomycota* phylum, plays a role in decomposition and contains pathogens of other eukaryotes (Thines, 2018). After the inoculation of the bioreactors, the eukaryotic communities in the granular biomass revealed diverse strategies. In R1, OTU002 suffered a reduction in relative abundance, especially after day 15, and nearly disappeared at the end of the experiment; OTU03 and OTU05 also were not dominant in this bioreactor. At the steady state, OTU01 was dominant together with OTU07, affiliated with the class

Oligohymenophorea. According to Chan et al. (2021), the ciliate group of *Oligohymenophorea* plays an important role in controlling the microbial community, especially in the initial steps of granular formation, because they can consume particulate organic matter in suspension, from the growth of biomass (microorganisms) or the disintegration of biomass. At the beginning of the operation of R2, OTU01, OTU02 and OTU04 dominated the community. However, from day 24, OTU02 had competitive disadvantages against OTU001, which experienced a sudden growth and was the dominant OTU until the end of the experiment. From days 47 to 60, other OTUs proliferated, such as OTU03, OTU04 and OTU08, belonging to the plant-pathogenic genus *Microbotryum*, OTU06 and OTU12 (unclassified eukaryote), OTU07 and OTU13, affiliated with the *Oligohymenophorea* class, and two OTUs belonging to non-classified eukaryote (OTU10 and OTU16). In R3, OTU02 was the most dominant OTU at day 7, followed by OTU01, OTU03, OTU04 and OTU05. However, OTU01 had a slower growth in R3 than in bioreactors R1 and R2. Subsequently, OTU01, OTU03, OTU05 and OTU07 increased their relative abundances. At the end of the experiment, OTU01 was one of the main OTUs, together with OTU03, OTU07, OTU08 and OTU09, which belonged to an unclassified eukaryote, and OTU10. In the R4, OTU01, OTU02, OTU03, OTU04 and OTU05 dominated at the beginning of the experiment. After that, changes in dominance were noticed until day 47, when the eukaryotic community was most stable and dominated by OTU01, OTU03 and OTU08.

The population pattern of the eukaryotic communities differed from that of the prokaryotes. Mainly, OTU01 was dominant in all reactors at the end of the experiment, but its growth was benefited by the organic matter availability. Thus, the dominant phylotypes in reactors R2, R3 and R4 were similar in the steady state, whereas the greatest differences were found in R1, revealing the ubiquitous occurrence of *Hypocreales.* OLR $\leq$ 0.90 kg $COD \cdot m^{-3} \cdot d^{-1}$ promoted the coexistence of a greater number of phylotypes, increasing the eukaryotic diversity.

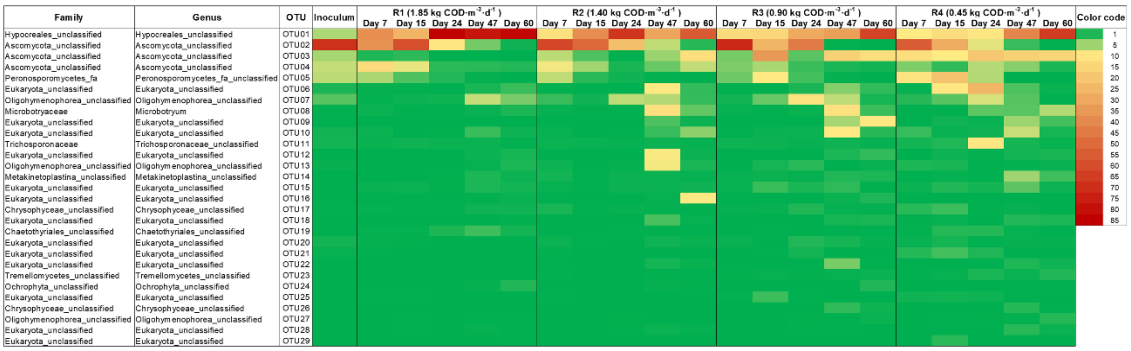

Figure 4. Heat map of eukaryotic operational taxonomic units with a total abundance of >1 %.

3.4. Microbial diversity and similarity analysis

The α-diversity indices were calculated for the prokaryotic and eukaryotic communities (Table S2). In terms of the prokaryotic community, the Shannon diversity index showed a high diversity for all samples. The statistically significantly greater diversity was related to the low OLR. Evenness, calculated by Pielou's index, revealed that a high concentration of organic matter modulated the selection of specific microorganisms, indicating that the bacterial community became less uniform. The Simpson index corroborated these results as there was a significant increase with a decrease in the OLR, indicating a higher diversity at low organic matter levels, as also reported by Hua et al. (2020) and Muñoz-Palazon et al. (2023c). Concerning the Chao-1 index for species richness, it showed no significant differences among the different bioreactors. A slight drop in species richness was observed over time, but without any statistically significant differences ($p > 0.05$) between day 7, which had a greater species richness, and the remaining experimental period. In general terms, higher OLR induced a lower diversity and evenness due to the competitive advantages of few phylotypes to consume organic matter more rapidly. This trend had been widely reported for biological water systems under high OLR and/or in the presence of toxics (Hua et al., 2020; Szabó et al., 2017).

Related to the eukaryotic community, the Shannon index revealed a lower diversity for the eukaryotic than the prokaryotic community. The R3 bioreactor had a statistically significantly higher diversity than R1 ($p < 0.05$). R4 had also higher diversity ($p < 0.05$) than bioreactors R1 and R2. Pielou's index indicated that the eukaryotic

community had a low evenness, although it was more uniform in the bioreactor under the lowest OLR. With reference to the Simpson diversity index, the R4 bioreactor revealed the greatest diversity. The values of the Chao-1 index did not significantly differ among the bioreactors.

The SIMPER analysis for prokaryotes (Fig. S4) showed how OTU018 and OTU036, belonging to the *JGI_0000069-P22* order and the *Flavobacterium* genus, differed drastically between samples. They were in the inoculum sample, but they could not compete under the new conditions, suffering a depletion of their relative abundance. OTU001, affiliated with *Pseudomonas*, contributed to the differences between R1 and R2 vs. R3 and R4, principally by its dynamic during the operation, whereas this OTU did not effectively contribute to differentiate R3 from R4 (3 %). R2 was differentiated from the rest of bioreactors by OTU006, OTU015 and OTU028, because these phylotypes were very representative in R2' granules, whereas in the rest of bioreactors, appearance of these OTUs was punctual. OTU007 and OTU008 acquired a high relative abundance (more than 10 %) in R3, whereas in R2 and R4 their maximum relative abundance was 5 %, and in R1 these OTUs could not proliferate. The SIMPER results showed that contribution to dissimilarity given by dominant OTUs were more equal, this trend was specially remarked in R3 and R4. Similarity data between bioreactors corroborated the community studies which demonstrated similar phylotypes for higher OLRs (R1 and R2) and other characteristic OTUs were found at lower OLRs (R3 and R4).

In terms of dissimilarity of eukaryotic community (Fig. S5), SIMPER analysis exhibited less plasticity than in prokaryotic populations, because the number of dominant OTUs that contributed to differentiate the bioreactors was lower. OTU03, belonging to *Ascomycota*, contributed to differentiate the R1 community at high OLR against the rest of bioreactors, because their relative abundance was lower than 1 %, whereas R2, R3 and R4 acquired values up to 19 %. In addition, it was observed a gradual progression in the contribution to dissimilarity from R2 to R4. OTU05 and OTU06 contributed to great differences in R4 against R1, R2 and R3 bioreactors. Both OTUs had high relevance in R4 until day 24, but their abundance was limited in the rest of bioreactors. OTU01 taxonomically affiliated with *Ascomycota* had a high abundance in the initial periods of experimentation in R1, highlighting its competitive advantage in this reactor at high OLR. Also, it was present in the other bioreactors, but on the contrary, in R1 and R2 proliferated at the end of experimentation.

3.5. Principal component analysis

Based on the PCA results (Fig. 5), the prokaryotic communities of different bioreactors were gradually distanced depending on the OLR (Fig. 5A). For the start-up stage, all samples were grouped in a cluster due to the same origin of inoculum. Results showed that during the steady state, OLR produced differences in prokaryotic community. Moreover, for R1, R2 and R3 bioreactors, samples of day 47 and 60 showed larger distances between them, even more in reactors fed with higher OLR. It can be noticed that higher OLRs promoted a more rapid change in prokaryotes dynamics, possibly driven by the competence of dominant phylotypes for substrate, highlighting the samples from day 47 and 60 of R1, whereas in R4 these samples showed high similarity, supporting the results obtained by the evenness and diversity indices, which allowed the community to achieve an earlier balance in microbiota of R4 than in the rest of bioreactors, as shown in Table S2.

In contrast, PCA of eukaryotic communities (Fig. 5B) revealed that differences in populations were less pronounced along the different treatments. However, a first cluster was formed by samples of inoculum, day 7 and day 15, whereas samples of day 24 began to differ by operational conditions, excepting for the bioreactor R3. The eukaryotic community of mature granules at day 47 and day 60 pointed out larger distances from the inoculum, although the differentiating effect of OLR was less patent. Distances between samples were larger at lower OLR, corresponding to a slower stability at those loading rates, agreeing with Figure 4.

Briefly, the prokaryotic community revealed higher modifications in terms of abundance under high OLR, whereas dynamics of eukaryotic community showed the opposite trend.

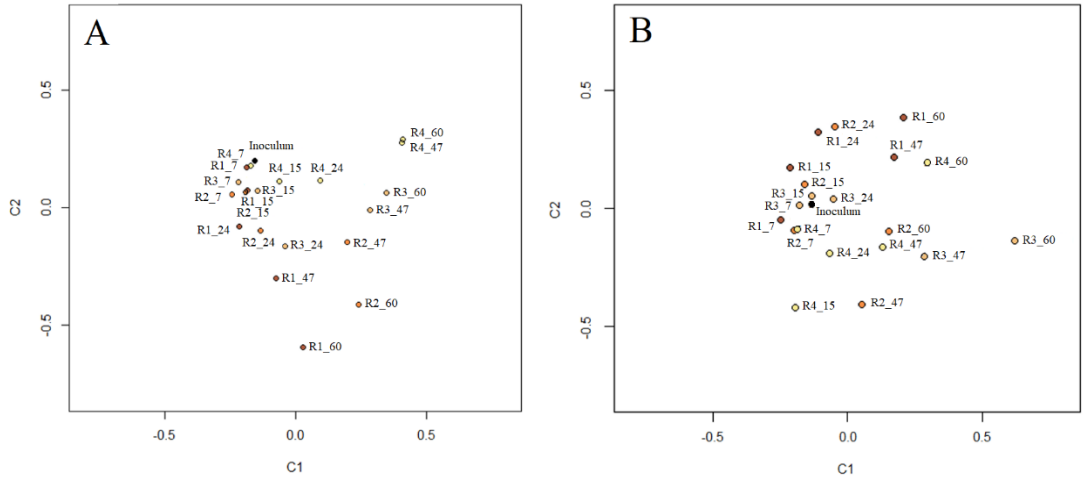

Figure 5. Principal component analysis for the prokaryotic (A) and the eukaryotic communities.

3.6. Multivariate redundancy analyses

The RDAs were calculated to reveal the linkage between the dominant OTUs of the prokaryotic and eukaryotic communities and the physicochemical parameters (COD removal ratio, BOD_5 removal ratio, ammonium removal ratio, mean size, settling velocity and MLSS) over the operational time of each system.

Figure 6 shows the RDAs for the dominant prokaryotic OTUs and the operational parameters. For R1, organic matter removal (measured as COD and BOD_5 removal), MLSS and mean size were negatively correlated with the initial period of the experiment, whereas the correlation was positive with day 47 (Fig. 6A). These parameters were strongly correlated with OTU014 and OTU067, corresponding to the genera *Brevundimonas* and *Flavobacterium*, respectively. Specifically, *Flavobacterium* was positively related to COD removal as it plays an important role in the decomposition of organic matter in general and acetate in particular, as reported by Muñoz-Palazon et al. (2023c). Further, settling velocity was positively correlated with OTU008, OTU034, OTU041 and OTU054, belonging to the NS9 marine group, the *Sphingobium* genus and the families *Fimbriimonadaceae* and the *Burkholderiaceae*, respectively. The NS9 marine group was positively correlated with the composition of the EPS excreted, in particular the extracellular protein/extracellular polysaccharide (PN/PS) ratio, which is an indicator of granulation level and, thus, related with the settling velocity (Chen et al.,

2019a). A similar role has been reported for the *Sphingobium* genus. According to Gómez-Acata et al. (2018), phylotypes belonging to *Sphingobium* are linked to high granular aggregation. All these phylotypes were strongly and positively linked with day 15, when the biomass reactor achieved a better settleability. Furthermore, the settling velocity was negatively correlated with the final period of the experiment, which is attributed to the loss of density at high organic loads (Iorhemen and Liu, 2021). The RDA for the prokaryotic community of the R2 bioreactor is shown in Figure 6B. Further, OTU022, OTU049 and OTU072 were positively correlated with organic matter remotion and the MLSS. These parameters were negatively correlated with the starting period of the experiment. The NH_4^+ removal was correlated with day 24 and OTU046 and OTU089, both belonging to the *Flavobacterium* genus, which has the capacity to remove N (Meng et al., 2020). Mean granule size was strongly and positively correlated with OTU059, from the *Bdellovibrio* genus, whereas settling velocity had a positive linkage with day 15 and OTU003, OTU031 and OTU050, affiliated with the *Verrucomicrobiae* class and the genera *Devosia* and *Pseudomonas*. The RDA shown in Figure 6C demonstrates that, for R3, COD, BOD_5 and NH_4^+ removal, mean granule size and MLSS were positively correlated with day 47 of the experiment and negatively with the start-up stage. The positive linkage of NH_4^+ removal with several phylotypes explains the high ammonia oxidation from days 47 to 54. On the contrary, settling velocity had a negative correlation with the last period of the experiment and was linked to the class *Verrucomicrobiae* (OTU003). For R4, settling velocity and MLSS were negatively correlated with the steady-state stage, and the OTUs with a strong linkage were OTU003, OTU021, OTU029 and OTU061 (Fig. 6D). For R4, in contrast to the other bioreactors, MLSS and BOD_5 removal were not positively correlated with the final period of the experiment, possibly caused by the absence of sufficient substrate for biomass growth, consistent with results obtained Rosman et al. (2014). In general terms, organic matter removal and MLSS were negatively correlated with the start-up period for reactor R1, R2 and R3, whereas this relationship did not follow the same tendency for R4 bioreactor. Generally, settling velocity was positively correlated with day 15, with a strong linkage with the class *Verrucomicrobiae* (OTU003) for R2, R3 and R4 bioreactors.

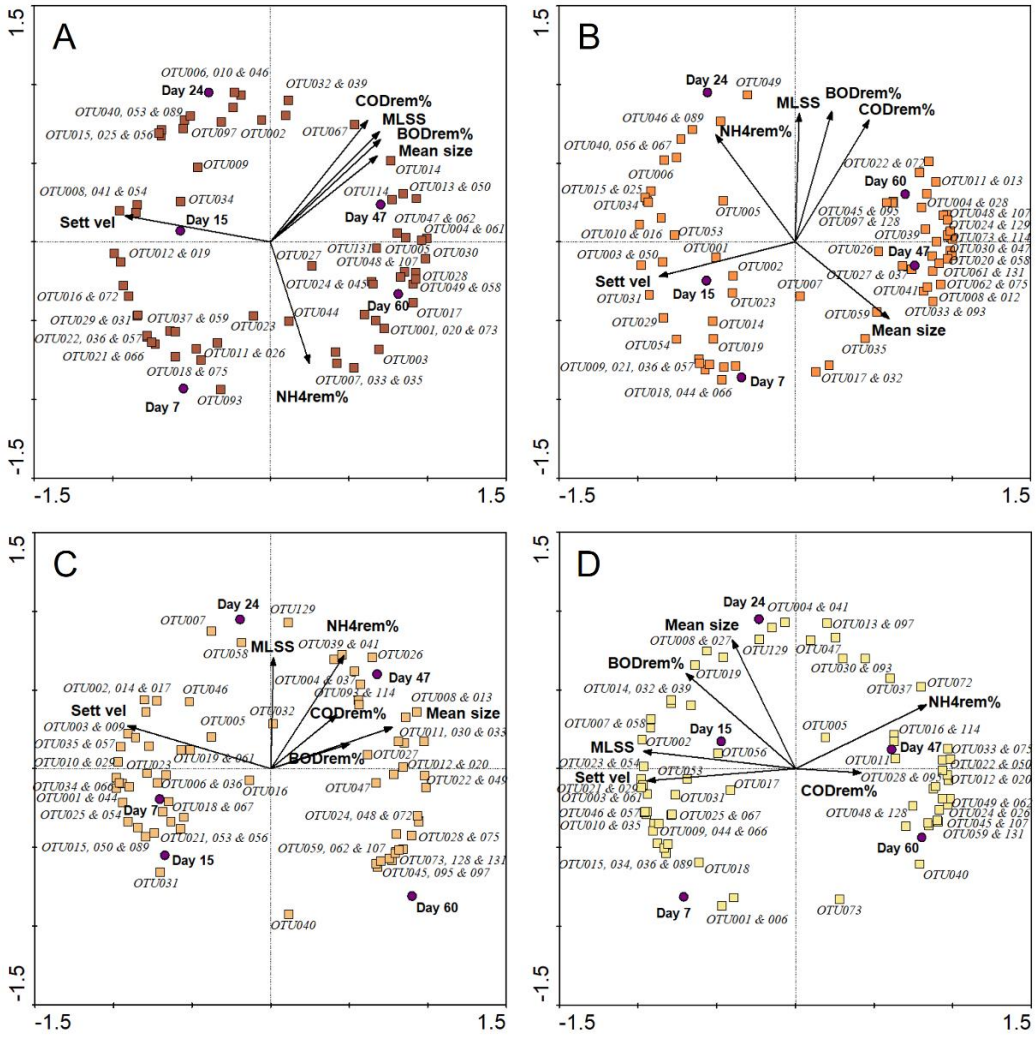

Figure 6. Multivariate redundancy analysis of the dominant prokaryotic community and physicochemical parameters for the different bioreactors: R1 (A); R2 (B); R3 (C); R4 (D).

Figure 7 shows the RDAs of the dominant eukaryotic OTUs and the physicochemical parameters. The linkage of R1 with physicochemical performance (Fig. 7A) showed that the organic matter removal and the MLSS had a strong and positive correlation with OTU01, belonging to the *Hypocreales* order. This phylotype plays an essential role in biofilm conformation and maintenance (Li et al., 2020b). Settling velocity had a positive linkage with OTU11, from the *Trichosporonaceae* family, which facilitates the formation of denser granules (Gonzalez-Martinez et al., 2018). The RDA for the R2 bioreactor (Fig. 7B) highlights how mean size, organic matter removal and MLSS are positively linked to the steady-state stage, whereas NH_4^+ removal and settling

velocity are correlated with day 24. For R3 (Fig. 7C), RDA showed how the organic matter and NH_4^+ removal rates, together with mean granule size, were correlated with day 47. More specifically, the BOD_5 removal rate was positively linked to OTU03, an unclassified *Ascomycota*. Moreover, settling velocity had a positive linkage with days 15 and 24, as well as OTU04 and OTU05. Finally, in R4, the eukaryotic community showed a pattern similar to that of the prokaryotic community in terms of physicochemical parameters (Fig. 7D). The factors MLSS, mean size and BOD_5 removal were negatively correlated with the final period, in contrast to NH_4^+ and COD removal. The NH_4^+ removal rate was strongly correlated with OTU08 (*Microbotryum* genus), and settling velocity was related to OTU02. Summarising, organic matter removal and MLSS were positively correlated to the steady state, except for R4 bioreactor.

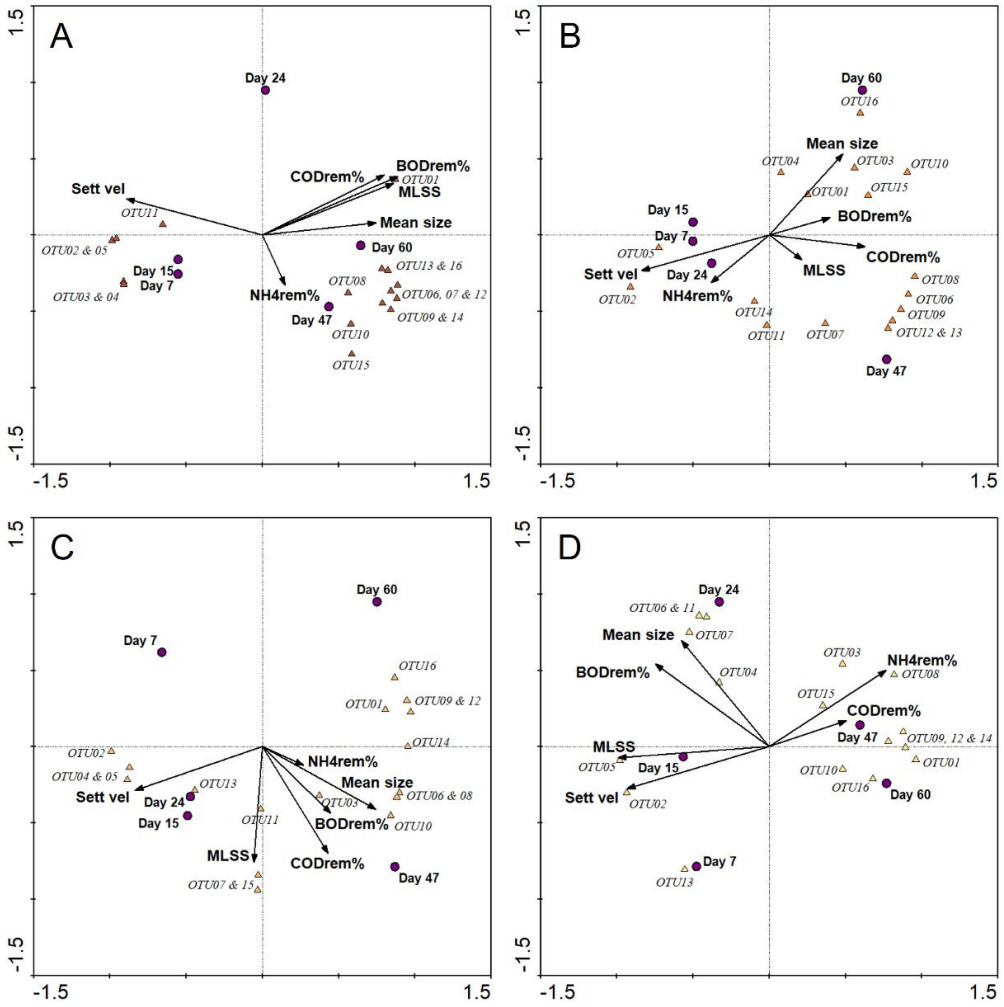

Figure 7. Multivariate redundancy analysis of the dominant eukaryotic community and physicochemical parameters for the different bioreactors: R1 (A); R2 (B); R3 (C); R4 (D).

4. Conclusions

This study demonstrated the successful implementation of the novel single-chamber design of AGS-CFR under a wide range of OLRs of urban wastewater treatment. The results showed the COD removal above 80 % for all OLR levels and the trend of ammonium removal ratio was progressively increasing over experimentational time, independently of influent characteristics. Granular size and biomass concentration were positively correlated with OLR. The granular microbiomes were modulated also by OLR levels. The genus *Pseudomonas* and the family *Spirosomaceae* were codominant at 1.85 and 1.40 kg $COD \cdot m^{-3} \cdot d^{-1}$, whereas lower OLR (0.90 – 0.45 kg $COD \cdot m^{-3} \cdot d^{-1}$) enriched the *OPB56* order and the families *Rhodobacteraceae* and *Rhizobiaceae*. The *Hypocreales* order was the ubiquitous eukaryotic phylotype in all reactors, but specific *Ascomycota* phylotypes were OLR-dependent.

Acknowledgments

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Supplementary information

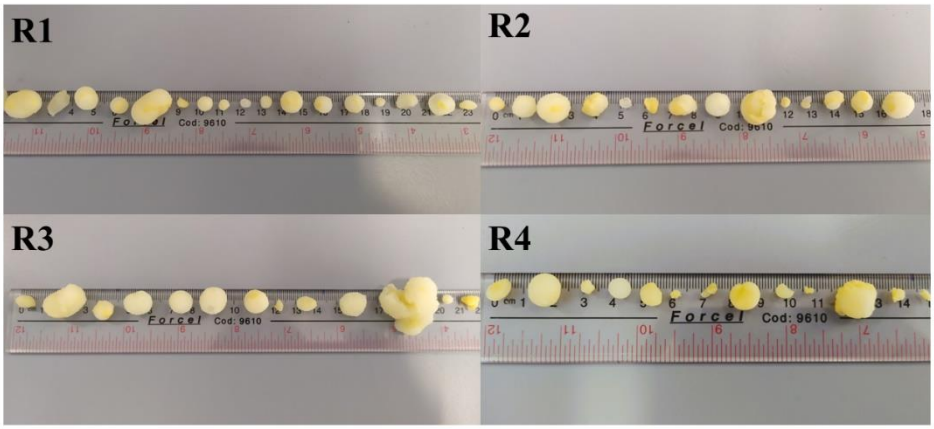

Figure S1. Digital images of granular sludge of bioreactors R1, R2, R3 and R4.

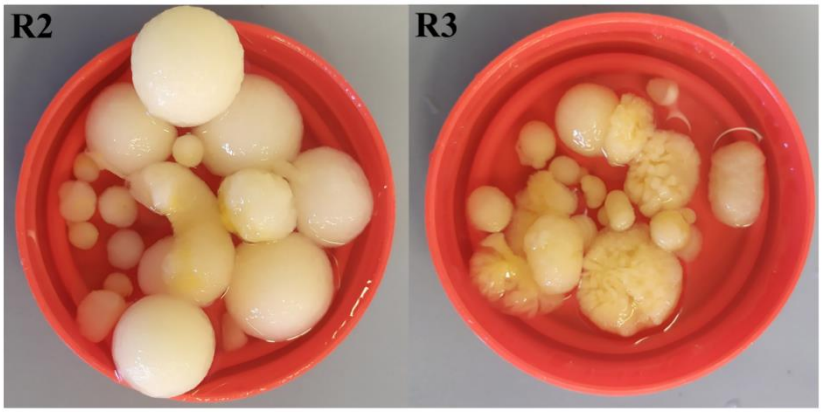

Figure S2. Digital images of granules cultivated in R2 and R3 bioreactor during the adaptation.

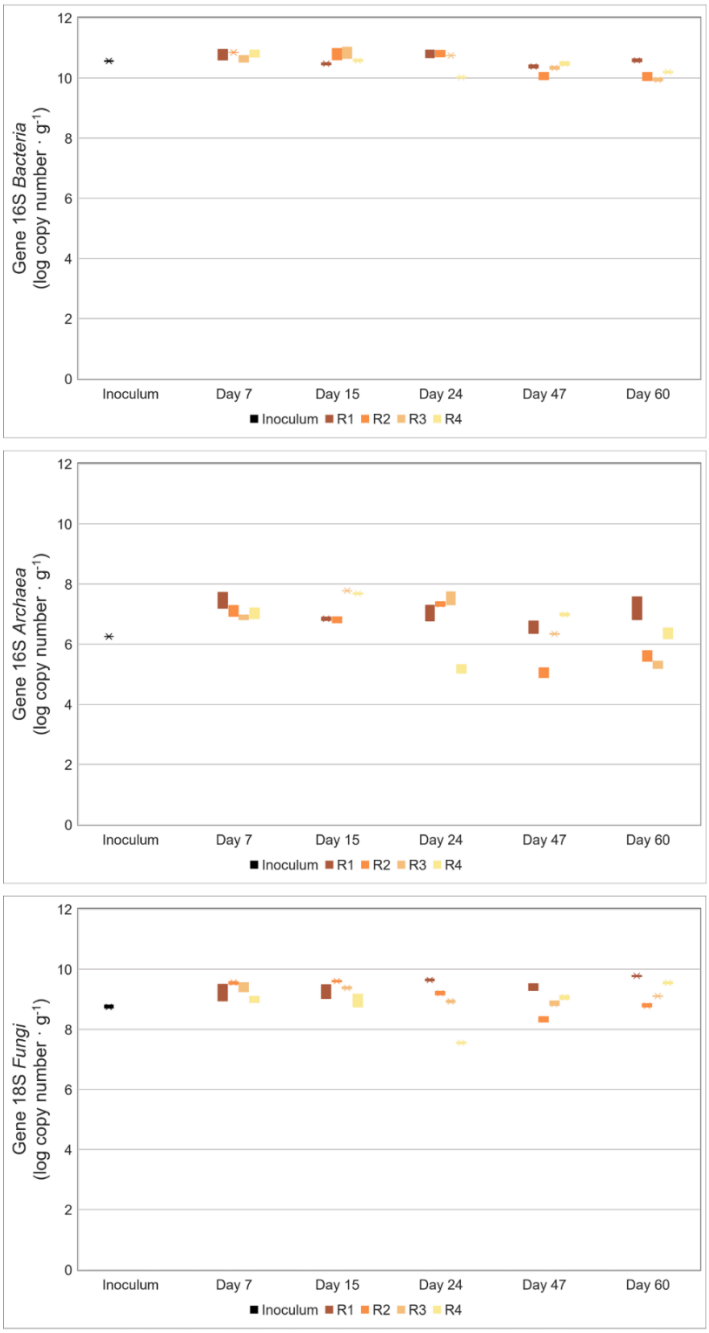

Figure S3. Gene copy numbers (log No. copies·g of granule^{-1}) for total *Bacteria*, total *Archaea* and total *Fungi*.

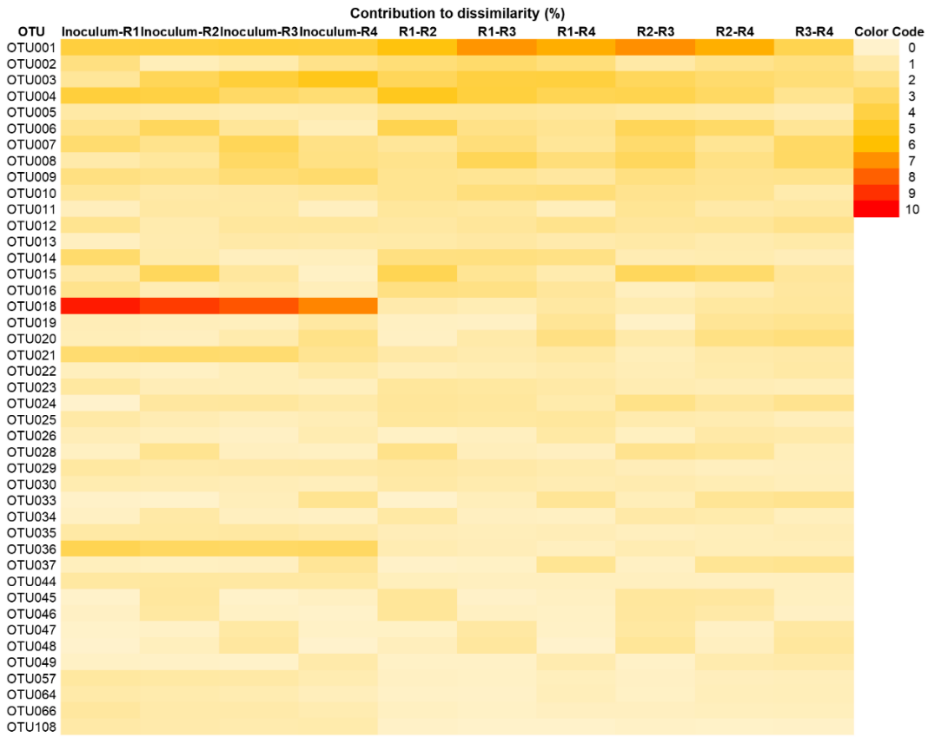

Figure S4. Similarity percentage analysis for prokaryotic community.

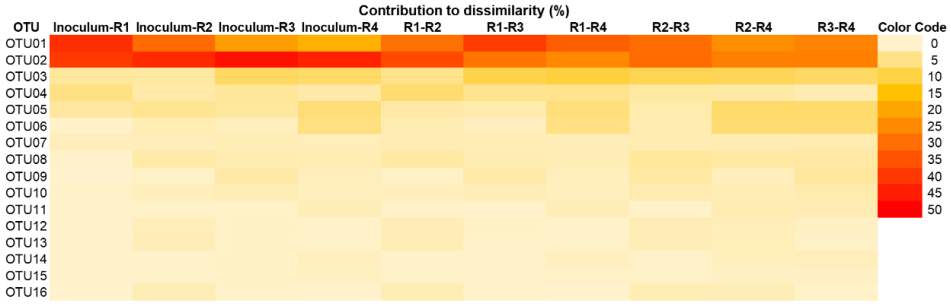

Figure S5. Similarity percentage analysis for eukaryotic community.

Table S1. Synthetic wastewater composition.

	$CH_3COONa•3H_2O$ ($g•L^{-1}$)	**NH_4Cl ($g•L^{-1}$)**	**$MgSO_4•7H_2O$ ($g•L^{-1}$)**	**K_2HPO_4 ($g•L^{-1}$)**	**KCl ($g•L^{-1}$)**	**KH_2PO_4 ($g•L^{-1}$)**
R1	1.050	0.250	0.100	0.085	0.040	0.030
R2	0.787	0.250	0.100	0.085	0.040	0.030
R3	0.525	0.250	0.100	0.085	0.040	0.030
R4	0.262	0.250	0.100	0.085	0.040	0.030

Table S2. α-diversity for prokaryotic and eukaryotic communities.

	Reactor	Operational days	Shannon	Pielou's Evenness	Simpson	Chao-1
Prokaryotic community	Inoculum		4,442	0,06075	0,9718	2407
	R1	7	4,504	0,06725	0,972	2607
		15	4,419	0,06555	0,9685	2404
		24	4,313	0,05354	0,9671	2472
		47	3,981	0,04182	0,9506	2276
		60	3,809	0,03513	0,9375	2374
	R2	7	4,322	0,05004	0,9622	2620
		15	4,294	0,05677	0,9654	2438
		24	3,982	0,04503	0,9554	2088
		47	4,364	0,06334	0,9692	2202
		60	4,318	0,05589	0,965	2162
	R3	7	4,466	0,06069	0,9706	2732
		15	4,332	0,06422	0,9703	2085
		24	4,33	0,0612	0,9665	2359
		47	4,471	0,07669	0,972	1896
		60	4,447	0,07471	0,9753	1776
	R4	7	4,542	0,06349	0,9759	2950
		15	4,606	0,07762	0,9783	2377
		24	4,673	0,08412	0,9795	2130
		47	4,582	0,07752	0,9801	1935
		60	4,52	0,07369	0,9773	1817
Eukaryotic community	Inoculum		2,59	0,07088	0,8489	242,1
	R1	7	2,784	0,0767	0,9047	286,6
		15	2,487	0,06013	0,8527	281,4
		24	2,126	0,04873	0,7596	231,9
		47	2,355	0,06126	0,8278	223,8
		60	2,001	0,04228	0,7315	273,3
	R2	7	2,524	0,07214	0,84	242,2
		15	2,478	0,06273	0,848	255,4
		24	2,036	0,05394	0,7387	197,7
		47	3,426	0,1379	0,9357	290,5
		60	2,905	0,0777	0,885	306,3
	R3	7	2,589	0,0736	0,8595	287,9
		15	2,462	0,07065	0,7991	238,1
		24	2,882	0,1026	0,8673	216
		47	3,517	0,1573	0,9446	243,6
		60	3,092	0,08839	0,8935	304,1
	R4	7	2,872	0,08796	0,8941	315,4
		15	3,53	0,1459	0,9514	385,9
		24	3,572	0,1625	0,9475	279,1
		47	3,439	0,1534	0,9446	238,7
		60	3,086	0,1028	0,9076	280,5

CHAPTER 3

Impact of hydraulic retention time on a novel single-chamber aerobic granular continuous-flow reactor treating wastewater: physicochemical and microbial characterisation

CHAPTER 3

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Abstract

This research provided novel insights into the effect of hydraulic retention time on the key operational parameters, degradation performance, biomass characteristics and microbial dynamics of a novel single-chamber continuous-flow reactor for aerobic granular sludge technology to assess the feasibility of this compact design for full-scale implementation. For that, four bioreactors treated the same simulated urban wastewater under hydraulic retention times of 8, 6, 4 and 2 hours. Results revealed that the bioreactor operated at 2 hours of hydraulic retention time showed disadvantages in terms of physicochemical performance. Bioreactors operated at 8, 6 and 4 hours of hydraulic retention time achieved stable granules and a higher removal performance of organic matter. However, the most environmentally effective hydraulic retention time was 4 hours, featuring the optimal granular stability, allowing for the treatment of larger volume of water in shorter time. Microbial communities were modulated according to the operational conditions, although the eukaryotic order *Hypocreales*, the prokaryotic family *Spirosomaceae* and the *Brevundimonas* genus were ubiquitous in all bioreactors, but the family *Chitinophagaceae* was microbial core of granules from bioreactors with optimal hydraulic retention time. The bioreactor operated at the shortest hydraulic retention time exhibited the highest changes in microbial dynamics.

Keywords: aerobic granular sludge, continuous-flow reactor, hydraulic retention time, microbial succession, single-chamber bioreactor

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1. Introduction

Aerobic granular sludge represents a promising alternative technology for wastewater treatment, resulting in reduced energy requirements and decreased carbon footprint (Yu et al., 2024; Yu and Wang, 2024). The formation of granular biomass is promoted by the hydrodynamic shear force of the system and the production of extracellular polymeric substances (EPS), which enable the aggregation of microorganisms into compact spheres (Liu et al., 2022). This dense granular structure offers several advantages, such as resistance to stressful conditions and high effectiveness in separating biomass from treated water, maintaining high biomass retention in the reactor, consequently, reducing sludge production and recirculation needs. Additionally, the compact conformation creates different redox conditions within a granule, promoting diverse metabolic pathways within each niche to simultaneously remove organic matter, nitrogen, phosphorus and other pollutants (Hamza et al., 2022; Samaei et al., 2023). In the event of sludge purging, anaerobic digestion can stabilise the sludge, achieving yields similar to those for activated sludge (Zaghloul et al., 2023). Furthermore, EPS extracted from biomass could be used in industrial manufacturing processes, reducing sludge management costs and promoting a circular economy (Feng et al., 2021).

Sequential batch reactors (SBRs) have typically been the operational mode for AGS technology, as their high selection pressure reduces fluffy flocs and promotes feast-famine conditions, enhancing granular stability. However, SBRs are not optimal for full-scale urban wastewater treatment plants (WWTPs) because most WWTPs are configured to operate in continuous-flow mode (Samaei et al., 2023). Continuous-flow reactors (CFRs) for AGS technology can be adapted to existing WWTP infrastructure, treat higher wastewater volumes and reduce capital, operational and maintenance costs (Kent et al., 2018). Despite these advantages, operating AGS in CFRs presents challenges, primarily in determining suitable selection pressure to maintain granular integrity (Samaei et al., 2023). Numerous configurations of CFRs have been developed, but most exhibit structural complexity, involving multiple chambers or granule recirculation mechanisms, which render them suboptimal designs for full-scale wastewater applications (Rosa-Masegosa et al., 2021; Yan et al., 2021).

In an effort to develop a more suitable CFR, Rosa-Masegosa et al. (2023) described a successful single-chamber CFR configuration for AGS technology that does not require biomass recirculation, but rather uses an internal settler-baffle at the water

output. Within this key piece, hydrodynamic shear force ceases, and dense biomass that rise up through the baffle does not achieve the output level, remaining within the bioreactor, whereas water and light flocs are easily withdrawn. Only a few authors have reported the development of novel single-chamber CFRs for AGS, but operational issues arising from the hydraulic features of these designs include the release of floccular biomass on effluent discharges (Li et al., 2020a; Song et al., 2022b). However, the simpler design presented in this research enhances scalability, operation and maintenance, as well as mitigating the granular damage caused by recirculation or by low hydraulic shear force, without requiring any supporting carrier used in previous CFR configurations with single or several chambers (Li et al., 2020a; Song et al., 2022b; Yan et al., 2021).

Hydraulic retention time (HRT) is a crucial operational parameter in designing and operating WWTPs because it affects biomass stability and production, bioreactor performance and treatment capacity (Iorhemen and Liu, 2021; Rosman et al., 2014). It is also an important driving factor for granulation (Berzio et al., 2023). HRT is closely linked to the biomass selection pressure of AGS technology, significantly impacting the type and quantity of microorganisms, which is essential for maintaining granular stability through shear stress, especially in CFRs. In this sense, a long HRT allows for the development of slow-growing microorganisms but can lead to fluffier structures that disrupt the microbial balance. Moreover, this scenario could result in unnecessary energy consumption and operational costs, without significant improvement in treatment efficiency. Conversely, a short HRT promotes the washout of light flocs, enriching the bioreactor with dense granules but potentially removing granular biomass and reducing effluent quality due to increase of solids and incomplete biodegradation of pollutants (Kent et al., 2018; Tchobanoglous et al., 2003; Wang et al., 2021).

HRT is regulated according to the operational requirements of the WWTP, so a competitive bioreactor design should allow operation across a wide range of HRTs, having into account that shorter HRTs are more desired to achieve a more sustainable technology. This study aimed to investigate the influence of HRT on the novel AGS-CFR system to determine the optimal range for achieving high-quality effluent and to understand how different HRTs affect microbiome dynamics and pollutant biodegradation processes, with the goal of evaluating the feasibility of this novel design for full-scale implementation.

2. Material and methods

2.1. Reactors set-up and operation

This study was performed using four bioreactors, following the stablished design from Rosa-Masegosa et al. (2023), consisting in a single column of 72 cm in height and 10 cm in internal diameter. Within it, coupled to the effluent outlet, was a small baffle that allowed effluent discharge and the washout of fluffy flocs, avoiding the loss of denser biomass (Fig. S1). The function of this baffle was to inhibit the upward movement of dense granules towards the outlet, achieved through the curvature and height of the settler-baffle configuration. Air flow was introduced as fine bubbles through a diffuser positioned at the bottom of each bioreactor, with a flow rate of 3.5 $L \cdot min^{-1}$, corresponding to up-flow of 0.75 $cm \cdot s^{-1}$. To assess the effect of HRT on the performance and microbial communities of the new CFR technology, HRTs of 8, 6, 4 and 2 h were employed for the R1, R2, R3 and R4 bioreactors, respectively, corresponding to 0.73, 0.98, 1.46 and 2.93 kg $COD \cdot m^{-3} \cdot d^{-1}$. The HRTs selected were informed by prior research on AGS carried out by Wan et al. (2014a) and Li et al. (2016a). For 103 days, the bioreactors were fed with synthetic medium simulating urban wastewater, composed of 0.5246 $g \cdot L^{-1}$ $CH_3COONa \cdot 3H_2O$, 0.25 $g \cdot L^{-1}$ NH_4Cl, 0.04 $g \cdot L^{-1}$ KCl, 0.1 $g \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$, 0.085 $g \cdot L^{-1}$ K_2HPO_4 and 0.03 $g \cdot L^{-1}$ KH_2PO_4. This simulating wastewater resulted in an influent with 244 ± 25 mg $O_2 \cdot L^{-1}$ of COD, 189 ± 27 mg $O_2 \cdot L^{-1}$ of BOD_5, 66 ± 1 mg $N \cdot L^{-1}$, 17 ± 2 mg $P \cdot L^{-1}$, and a pH in the range 7.9 to 8.2. Each bioreactor was inoculated with 1.05 L (17.5 % of the total volume of bioreactors) of one-month-stored granules that were previously operated in CFRs. The temperature during the experiment was 18.3 ±1.2 ºC.

2.2. Physicochemical wastewater analyses and granular characteristics

Organic matter, nitrogen and phosphorus were periodically measured from influent and effluent samples. Ammonium, nitrate, nitrite, and phosphate ions were quantified using a Metrohm ion chromatograph (Switzerland). For this purpose, the influent and effluent samples were filtered through a membrane filter with a pore size of 0.22 μm. Chemical oxygen demand (COD) and biological oxygen demand at day 5 (BOD_5) analyses were performed according to the Standard Methods for the Examination of Water and Wastewater of the American Public Health Association (APHA) (2017). Granules were periodically (twice a week) characterised by their mean size and settling

velocity in a representative sample set. To minimize disruption to the homeostasis of the reactors, each sample consisted of 20 ± 5 pieces to avoid influencing biomass concentration. Measurements were performed using a magnifying glass Nikon SMZ800 with an ocular micrometer, following a modified method based on Muñoz-Palazon et al. (2023c). The biomass concentration within the bioreactors was determined by measuring mixed liquor suspended solids (MLSS) in triplicate (American Public Health Association, 2017).

2.3. Carbon mass balance

To perform a carbon mass balance in the system, two primary reactions must be considered. The first belongs to the removal of organic matter, which is oxidised to carbon dioxide (CO_2) through biological processes. This reaction can be described by the following chemical equation for oxidation:

$$C_nH_aO_b + ¼ /(4n + a – 2b)\ O_2 \rightarrow nCO_2 + (a/2)\ H_2O$$

In this equation, the second term represents the amount of oxygen required for the oxidation of organic matter, which correlates to the COD. Since the synthetic medium used in the experiments consists of a known carbon source (acetate), the stoichiometry for the oxidation reaction of the organic matter in the wastewater is given by:

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

The reaction governing the transformation of substrate into new biomass was also determined. Although the precise stoichiometry for this biomass formation is not explicitly known, it can be experimentally quantified by measuring the increase in mixed liquor volatile suspended solids (MLVSS). Assuming that new microbial cells can be represented by the empirical formula $C_5H_7NO_2$, the oxygen equivalent (COD) for biomass generation can be approximated at 1.42 grams of COD per gram of MLVSS (Hoover and Porges, 1952), as described by the following reaction:

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

The oxygen demand per unit of biomass is calculated as:

$$(\Delta O_2/\Delta C_5H_7NO_2) = (5 \times 32\ g/mol\ O2) / (113\ g/mol\ C_5H_7NO_2) = 1.42\ g\ O_2/g\ MLVSS$$

By applying a global mass balance to the system, the amount of CO_2 generated in each of the four reactors can be estimated based on COD analyses of both influent and effluent samples, along with MLVSS measurements.

2.4. Biological analyses

2.4.1. Biological sample collection and DNA extraction

Following the methodology of Muñoz-Palazon et al. (2022b), biomass samples from each bioreactor were collected periodically, centrifuged at 5,000 rpm for 20 min at 4 º C, and the supernatant was discarded. Pellets were then stored at -20 º C. DNA extraction from the samples was performed using the FastDNA SPIN Kit for Soil (MP Biomedicals, USA), following the manufacturer's instructions. DNA from each sample was eluted in a final volume of 150 µL of deionised water and stored at -20°C.

2.4.2. Absolute quantification of target genes and sequencing

The DNA extracted was used to quantify the number of copies of bacterial and archaeal 16S rRNA genes, the fungal 18S rRNA gene and the bacterial ammonia monooxygenase (*amoA)* gene in order to estimate the abundance of total *Bacteria*, *Archaea*, *Fungi,* and ammonia-oxidising bacteria (AOB). The *C.* Accumulibacter 16S rRNA gene was used as a proxy for polyphosphate-accumulating organisms (PAOs). Quantitative polymerase chain reactions (qPCRs) were performed using a QuantStudio-3 Real-Time PCR system (Applied Biosystems), following the mixture solution, pair of primers and cycling conditions described by Pérez-Bou et al. (2024). Additionally, nucleic acids were used for next-generation sequencing in order to identify the *Prokaryota* and *Eukaryota* communities through Illumina MiSeq platform, according to the details described by Rosa-Masegosa et al. (2024a).

2.5. Statistical and ecological analyses

Simpson, Shannon-Wiener, Pielou's Evenness and Chao-1 indices for α-diversity, as well as the Whittaker index to capture the β-diversity, were calculated using PAST 3.14 software. Statistically significant differences among samples were determined by

permutational multivariate analysis of variance (PERMANOVA) under a Euclidean algorithm and 999 bootstrap, which were also conducted using PAST 3.14 software. Similarity percentages analysis (SIMPER) was performed under the Bray-Curtis algorithm using PAST 3.14 software to determine the contribution of dominant operational taxonomic units (OTUs) to the dissimilarities between biological samples. OTUs accounting for less than 1 % of dissimilarity across all compared samples were discarded.

To assess the significant differences in OTUs abundance between bioreactors, the expected effect size (EES) was calculated. OTU abundance data were adjusted to account for zero values and subsequently transformed using a centered log-ratio (CLR) transformation, facilitated by the generation of 128 Monte Carlo Dirichlet simulations, The CLR-transformed data were then utilised for EES computation, employing the ALDEx2 package implemented in the R environment, according to Rodriguez-Sanchez et al. (2018).

To cluster the microbial communities, principal component analysis (PCA) was conducted following the approach by González-Martínez et al. (2021). The OTU table was corrected to avoid zero-count issues calculated for Bayesian multiplicative replacement of zero value. The obtained OTU table was then transformed using CLR transformation. These corrected samples of the OTU table were used for singular value decomposition calculation, and the results were visualised through the PCA plot using the ALDEx2 package implemented in R software.

Multivariate redundancy analysis (RDA) was performed to establish the relationship between dominant OTUs (more than 1.5 % for prokaryote and more than 1 % for eukaryote) and physicochemical parameters. For this analysis, the data were transformed to the log(x + 1) and introduced into CANOCO 4.5 software, which computed the RDA using 499 unconstrained Monte-Carlo simulations under a full-permutation model (Rodriguez-Sanchez et al., 2019).

2.6. Prediction of functionality of microbial functional groups in prokaryotic populations.

The potential functions of the prokaryotic community were predicted using Functional Annotation of Prokaryotic Taxa (FAPROTAX) software version 1.2.7, following the protocol described by the tool developers (Louca et al., 2016). The numbers of OTUs involved in specific functions were calculated, and their relative abundances were determined. Based on this information, a table was constructed that included the temporal dynamics of each bioreactor, with functions representing more than 0.5 % of the total.

3. Results and discussion

3.1. Effects over system performance

3.1.1. Biomass concentration

The biomass characteristics and water quality were dependent on the different HRTs. One of the first detected changes was a substantial increase in terms of biomass concentration in R4 (2 h of HRT), which reached 5.8 $g \cdot L^{-1}$ by operational day 20, possibly caused by the high organic loading rate (OLR) (Fig. 1A). However, this triggered hydrodynamic issues due to the high MLSS concentration and large granule sizes (Fig. S2A). Consequently, biomass had to be periodically withdrawn from the reactor, resulting in unstable MLSS evolution for the R4 bioreactor throughout the experiment, maintaining an average MLSS concentration around 3.5 ± 1.4 $g \cdot L^{-1}$. For bioreactors R1 and R2, the average MLSS concentration was approximately 1.3 $g \cdot L^{-1}$ during the first 68 days, while for the R3 bioreactor it was 2.0 $g \cdot L^{-1}$. From day 70 onwards, there was an increase of MLSS in all bioreactors, with average values of 3.9, 4.2 and 4.8 $g \cdot L^{-1}$ for R1, R2 and R3 bioreactors, respectively, with peak values ranging from 4.5 to 6.3 $g \cdot L^{-1}$.

These progressive increasing trends in MLSS concentration corroborated that this single-chamber design challenges the idea that AGS systems operated in SBRs have superior biomass retention compared to CFRs, as reported by Kent et al. (2018). Furthermore, the results of this study pointed out that, even at an HRT of 2 h, the bioreactor configuration successfully retained granules, refuting previous results from others CFR designs with HRTs shorter than 3 h, which suffered biomass washout (Samaei

et al., 2023; Wan et al., 2014a). Generally, the experimental biomass concentration in the R4 bioreactor was statistically higher than in R1 and R2 bioreactors (*p*-values of 0.0151 and 0.0393, respectively), while no statistically significant differences were found between R4 and R3. It is worth noting that in R1 and R2 bioreactors, granules suffered an increase of filamentous microorganisms around day 45, whose washout caused a decrease of MLSS. Hou et al. (2021), reported that the proliferation of filamentous microorganisms in AGS is produced at low OLRs, corroborating our findings. Despite the potential negative impacts during punctual periods, the beneficial properties of filamentous microorganisms related to the formation of sludge aggregates could not be ignored, as reported (Song et al., 2022a).

These results suggested that among tested HRTs ranging from 8 to 2 h in the novel AGS-CFR configuration, the R3 bioreactor (4 h of HRT) achieved an optimal ratio between granular properties and OLR, resulting in greater biomass concentration and depollution performance.

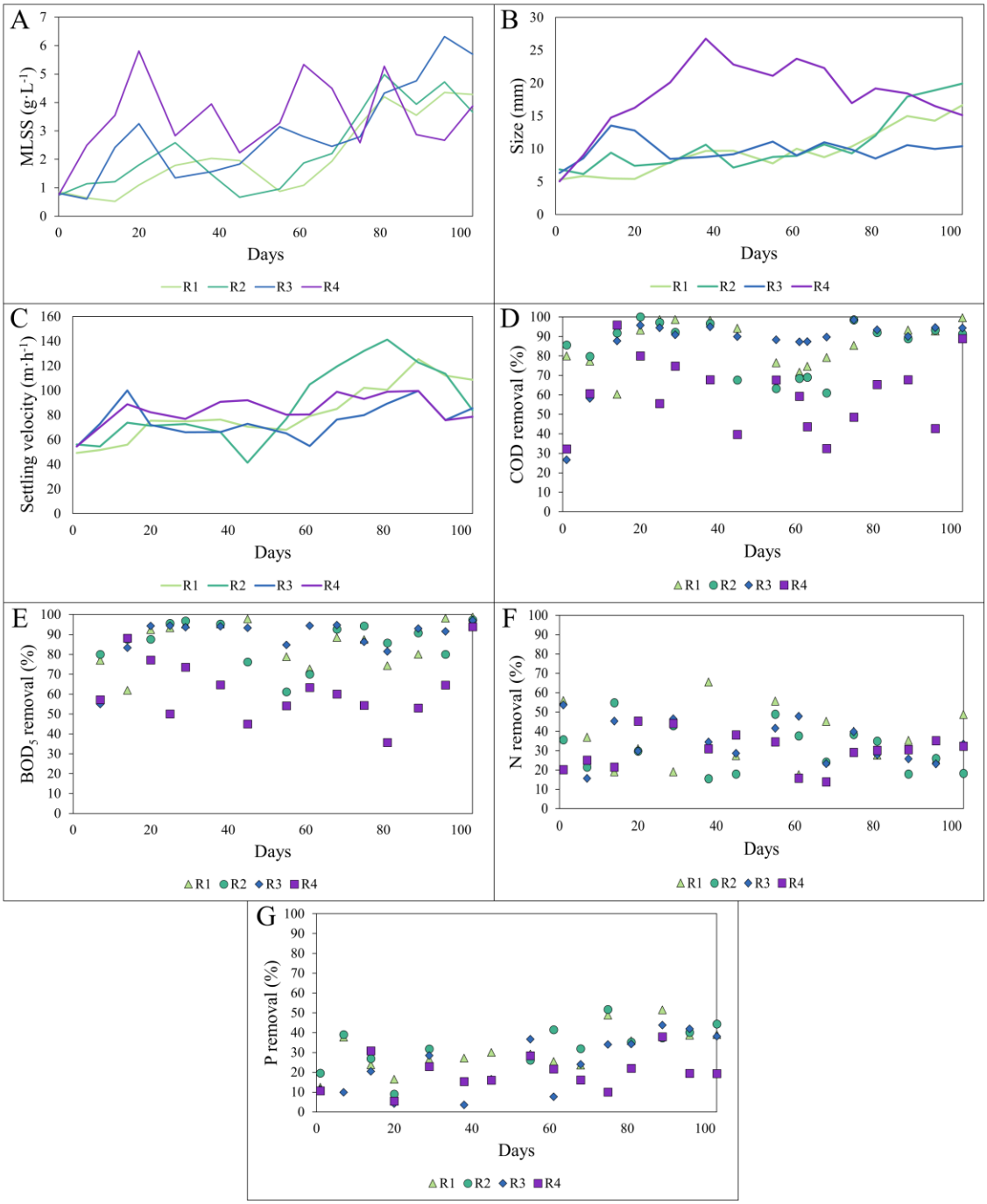

Figure 1. Granular characteristics and removal performance for each bioreactor: Biomass concentration (A); mean size of granules (B); mean settling velocity of granules (C); COD removal performance (D); BOD5 removal performance (E); nitrogen removal performance (including ammonium, nitrate, and nitrite) (F); and phosphorus removal performance (including phosphate) (G).

3.1.2. Granular size and settleability

In terms of granular size (Fig. 1B), the mean size of granules in the R4 bioreactor was statistically higher than in the R1 (*p*-value 0.0002), R2 (*p*-value 0.0005) and R3 (*p*-value 0.0002) bioreactors, averaging 17.9 ± 5.6 mm and reaching mean values as high as 26.8 ± 5.9 mm (Fig. S2B). In contrast, the average granular size remained stable around 10.0 mm until day 78 for R1, R2 and R3 bioreactors. Beyond this time point, the granular size of R1 and R2 bioreactors increased to 16.6 ± 4.9 and 19.9 ± 5.1 mm, respectively, while granules in the R3 bioreactor maintained a mean size close to 10.0 mm until the end of the experiment despite treating higher-strength wastewater.

Regarding settleability, there were no statistically significant differences between different HRTs, with velocity rates of 82 $m \cdot h^{-1}$, 89 $m \cdot h^{-1}$, 75 $m \cdot h^{-1}$ and 84 $m \cdot h^{-1}$ for R1, R2, R3 and R4 bioreactors, respectively (Fig. 1C). All bioreactors maintained adequate settling velocity to preserve granulation and not be washed out throughout the experiment. Generally, settling velocity is mutually dependent on the compaction and size of granules. The increase in MLSS may be associated with a higher density of granules already present in the bioreactors, or with the formation of new granules. Therefore, despite changes in granular size and settling velocity observed across all conditions evaluated, the results suggested that R4, operating at 2 h of HRT, may not represent the optimal model for the exploitation of AGS-CFR, whereas R3 bioreactor, had granules with great physical characteristics in terms of biomass retention and granular stability.

3.1.3. Organic matter removal

For COD (Fig. 1D), the removal ratio was generally above 75 % for all reactors, except R4. Effluent of R1 and R2 bioreactors met the discharge requirements (125 mg $O_2 \cdot L^{-1}$, according to the European legislation (Directive 91/271/EEC)) from the beginning of the experiment. R3 achieved values below the discharge limit during the second week, with concentrations lower than 40 mg $O_2 \cdot L^{-1}$ from day 14 (Fig. S3). These results suggest that this novel design at 4 h of HRT can reach equivalent COD removal performance similar to other CFRs operated with comparable or longer HRTs, despite those studies use multiple chambers and complex configurations as reported by Berzio et al. (2023) and Sun et al. (2019). The biomass withdrawal carried in the R4 bioreactor caused fluctuations in COD removal performance, which affected the effluent quality and prevent it from

meeting discharge requirements on a few specific days, such as days 45 and 68. The results for R4 related to biomass characteristics and biodegradation performances indicate that it may not be practical at full-scale operations. Furthermore, the R4 trend must consider the high OLR, which exceeds that of most studies carried out in CFRs (Berzio et al., 2023; Li et al., 2020a; Song et al., 2022b; Sun et al., 2019).

The same pattern was observed for BOD_5 (Fig. 1E), with R1, R2 and R3 achieving removal efficiencies close to 90 %, complying with European wastewater discharge standard (Directive 91/271/EEC), except for a few days between days 40 and 60 for R1 and R2 (Fig. S3), whose period coincided with a decrease in biomass concentration through the washout of floccular biomass during the water discharge. R3 bioreactor showed the most stable values, continuously reaching the discharge limits. In contrast, BOD_5 removal for the bioreactor operated at 2 h of HRT did not meet the quality requirements from environmental discharge, with average values close to 62 %, even with an episode of removal performance below 40 % on day 80. The R4 oscillations for organic matter removal were caused by biomass withdrawal whose aim was maintaining circular movement and hydrodynamic shear force. These results of COD and BOD_5 highlighted the efficient exploitation of the novel AGS-CFR at 4 h of HRT.

3.1.4. Carbon mass balance

The carbon mass balance was conducted for each bioreactor over 20-day intervals (Table S1). The results indicated that bioreactor R1 exhibited the lowest carbon removal. In contrast, bioreactor R4 achieved the highest carbon removal, with average COD and BOD_5 removal rates of 10.82 and 8.58 g $O_2 \cdot d^{-1}$, respectively. These findings aligned with the expected influence of HRT. However, in systems with shorter HRT, such as R4 (2 h), the OLR is so elevated that there is a risk of process instability, leading to inefficient organic matter removal and biomass washout (Li et al., 2016a; Wan et al., 2014a). Despite this, R4 reached the highest MLSS concentration, as our previous results have shown. Although R4 demonstrated excellent carbon removal performance, the overall results suggest that, from an operational standpoint, it may not represent a viable HRT for treating influent with these characteristics, due to the excessive biomass growth and failure to meet the standard quality parameters for the discharge of treated water. A portion of the consumed carbon was assimilated into new biomass, as indicated by the MLVSS measurements, while the remainder was released as CO_2 gas through the microbial oxidation of organic matter. Significant variations were observed in biomass production,

as indicated by MLVSS, across the different conditions. The results suggested that the bioreactor operated at 4 h of HRT (R3) represented an excellent HRT from the organic carbon removal and granular stability points of view in this novel single AGS-CFR configuration.

3.1.5. Nitrogen removal

For nitrogen removal (Fig. 1F) of ammonium, nitrate and nitrite, no statistically significant differences were distinguished between different HRTs, with a mean removal performance of 37 %, 31 %, 34 % and 30 % for R1, R2, R3 and R4 bioreactors, respectively, probably due to nitrogen assimilation and nitrification-denitrification processes. The average effluent concentration was 41.7 $mg \cdot L^{-1}$, 45.6 $mg \cdot L^{-1}$, 43.2 $mg \cdot L^{-1}$ and 46.3 $mg \cdot L^{-1}$, respectively (Fig. S3). However, results suggest that ammonia oxidation was enhanced by the longest HRT, possibly due to the lower availability of organic matter and the proliferation of nitrifying microorganisms. In contrast, nitrification was affected by the shortening of HRT; it is well known that an insufficient HRT increases the risk of inefficient nutrient removal (Cydzik-Kwiatkowska and Wojnowska-Baryła, 2015). Additionally, results indicate that, nitrification is the limiting process for nitrogen removal under the studied conditions, due to the low nitrate and nitrite levels observed in the effluent (Fig. S3).

3.1.6. Phosphorus removal

For phosphorus removal (Fig. 1G), in terms of phosphate, the bioreactors had an average performance of 31 %, 33 %, 24 % and 20 % for R1, R2, R3 and R4, respectively. As expected from the results, bioreactors at 8 and 6 h of HRT had statistically greater P removal than the bioreactor at 2 h of HRT, with *p*-values of 0.0047 and 0.0079, respectively. All bioreactors showed a trend of increasing P removal capacity, especially R1, R2 and R3. Results suggest that longer HRTs enhance P removal performance, possibly due to the greater activity of PAOs, which have slow growth that is promoted by a longer HRT. Short HRTs might not provide enough time to convert organic matter into polyhydroxyalkanoates (PHAs) for PAOs under anaerobic conditions. During the subsequent aerobic conditions, PAOs could only use their stored PHAs as energy and carbon sources to support biomass growth, glycogen replenishment and phosphate uptake. Thus, P removal efficiency in the continuous-flow system declined with short HRTs (Li et al., 2016a).

It is important to highlight that observing denitrification and phosphate removal processes in an aerobic single-chamber continuous-flow system is uncommon in wastewater treatment (Tchobanoglous et al., 2003), and therefore, achieving higher removal performance of these nutrients under these conditions will be a future challenge.

3.2. Absolute quantification of target genes

The absolute quantification of archaeal, bacterial and *C.* Accumulibacter 16S rRNA genes, as well as fungal 18S rRNA and *amoA* genes, is available in Figure S4. In the inoculum sample, the 16S rRNA bacterial gene had 1.3×10^{10} copies per gram of granule, a tendency maintained independently of time and treatment. However, a slight decrease pattern was observed in R1, and fluctuation over operational time, along with a reduction of one order of magnitude in the R2 bioreactor on day 68, were noted. For the 16S rRNA archaeal gene, the inoculum sample had a copy number of 4.7×10^{6}. Under different conditions, the copy number of 16S rRNA archaeal gene was slightly lower than the inoculum. Despite oscillations, the bioreactor at 2 h of HRT promoted the gradual proliferation of *Archaea*, while a decreasing tendency was observed in R1, R2 and R3 bioreactors over operational time, a fact reported in a AGS operated in SBR by Muñoz-Palazon et al. (2018). The results indicate that the archaeal populations, being four orders of magnitude less abundant, had a competitive disadvantage against *Bacteria* domain, as reported Sun et al. (2018). At the beginning of the experiment, the copy number for 18S rRNA fungal gene was 6.0×10^{8} per gram of biomass. The bioreactor with 4 h of HRT maintained its values throughout the experiment, whereas the rest of bioreactors experienced a slight reduction of fungal copies until day 68. In this context, the absolute abundance of fungi population in R3 may be associated with the granular compactness and the stability of physiochemical performance. This study reports for first time the key roles of fungi in AGS-CFR technology concerning the stability of granular properties, challenging previous research that suggested fungi solely serve as colonization structure for bacteria, while their proliferation inducing granular instability (Liang et al., 2024a). By day 103, the fungal copies were recovered, especially in the bioreactor R2, which showed the greatest increase, achieving 4.2×10^{9} gene copies, similar to the levels obtained for the16S rRNA bacterial gene.

The bacterial *amoA* gene involved in ammonia oxidation was also evaluated. The inoculum had 1.8×10^8 copies of *amoA* per gram of granule. Regardless of the treatment studied, the number of copies of this gene generally tended to rise, indicating an increase in the potential of nitrification of all treatments. Despite the greater copy number of *C.* Accumulibacter 16S rRNA gene (average of 1.6×10^7, 2.0×10^7, 2.8×10^7 and 5.7×10^7 for R1, R2, R3 and R4 bioreactors, respectively) compared to other studies, the P removal rate was not significantly higher (Amorim et al., 2018; Pérez-Bou et al., 2024). These results suggest that PAO activity is influenced not only by the abundance of these microorganisms but also by the operational parameters and aerobic/anaerobic conditions (Fan et al., 2019).

3.3. Microbial community dynamics

3.3.1. Prokaryotic community dynamics

For the prokaryotic community, 58 OTUs were dominant, with more than 1.5 % of relative abundance (Fig. 2), and all of them belonged to the *Bacteria* domain. The majority of OTUs in the inoculum sample were OTU01 (7.78 % of relative abundance), OTU04 (3.80 %), OTU05 (5.10 %), OTU49 (3.38 %) and OTU51 (5.60 %), corresponding to the *Spirosomaceae* family, the *Leucobacter* genus, the *Chitinophagaceae* family, and the *Macellibacteroides* and the *Paludibacter* genera, respectively. The *Spirosomaceae* and the *Chitinophagaceae* families have been found to be key part of the granular core (Suhonen et al., 2023), and the *Paludibacter* and the *Macellibacteroides* genera have been reported to increase their relative abundance in stored granules (Zhang et al., 2019a), which is consistent with the origin of the inoculum. The *Leucobacter* genus has been noted for having negative superficial charges that facilitate granulation processes and is also associated with pollutant removal and settleability (Gonzalez-Martinez et al., 2017).

After inoculation of the stored granular biomass, the HRT induced different prokaryotic community dynamics. Most OTUs showed two differentiated periods: an "initial period" from day 14 and day 38 and a "steady-state period" between day 68 and day 103. OTU49 and OTU51, phylotypes dominant in the inoculum sample, reduced their relative abundance to less than 1 %, in all reactors tested demonstrating the inability to proliferate competitively, possibly due to the aerobic conditions, low substrate affinity or

displacement of the physical niche. OTU01, despite some oscillations, maintained its dominance throughout the experiment in the R1, R2 and R3 bioreactors. However, in the R4 bioreactor (2 h of HRT), the total relative abundance of OTU01 was depleted by day 38 of operation. OTU04 and OTU05 maintained their relative abundance until day 38, with exception of R4, which produced an early reduction of OTU05.

Other OTUs increased in abundance during operation, particularly OTU02, affiliated with the *Brevundimonas* genus, which was the dominant bacterium at the end of experimentation in all bioreactors. This genus is well known in the wastewater field for its potential role in EPS secretion, denitrification processes and solubilising phosphate in wastewaters (Pastore and Sforza, 2018; Song et al., 2022a). The proliferation of *Brevundimonas* genus could be linked to the progressive increase in phosphate performance (Fig. 1G). In addition, some studies have described the enrichment of the *Brevundimonas* genus with progressively shorter HRTs and higher OLRs in granular and activated sludge systems (Lusinier et al., 2021; Rosa-Masegosa et al., 2024a).

R1, R2 and R3 bioreactors followed a similar pattern for OTU10, OTU11, OTU12 and OTU14 dynamics. OTU10, belonging to the *Proteiniphilum* genus, was dominant at the end of experimentational period in the R1, R2 and R3 bioreactors, as was OTU14, which is taxonomically affiliated with the *Saccharimonadales* order. The presence of these phylotypes influenced the compactness of granules, as the *Proteiniphilum* genus is known as an acetate utiliser with a high tolerance to ammonia in anaerobic niches, and the *Saccharimonadales* order has denitrifying activity (Feng et al., 2023; Meunier et al., 2016; Yu et al., 2024). The *Chitinophagaceae* family (OTU05) could contribute as well to the granular stability found in R1, R2 and R3 bioreactors, due to its relevant role in the granular core (Suhonen et al., 2023).

OTU11, a member of the *Hydrogenophaga* genus, showed higher relative abundance during the initial period of operation regardless of HRT, while OTU12, belonging to *Taibaiella* genus, was more prevalent on days 38 and 68, mainly in reactors with HRTs ranging from 8 to 4 h. Therefore, the lowest representation of this genus in the communities studied was detected in R4. The proliferation of *Taibaiella* genus in microbial communities of attached biofilms was also promoted by longer HRTs, as corroborated Tiwari et al. (2019), whereas Zhang et al. (2022a) reported its proliferation at high food-to-microorganism ratio. The *Taibaiella* genus plays a role in the granular core and is related to degradation of the organic compounds (Paulo et al., 2021; Suhonen

et al., 2023). The presence of the *Hydrogenophaga* genus in the initial period of the experiment could be related to the maintenance and maturation of granules, as this genus is associated with EPS production (Gomeiz et al., 2023).

OTU17 and OTU18, belonging to the NS9 marine group and the *Xanthomonadales* order, represented more than 4 % of the total relative abundance on day 14 for R1 and R2 bioreactors. In contrast, the same OTUs had a relative abundance below 1.5 % on day 14 for bioreactors with 4 and 2 h of HRT, corresponding with greater OLRs, as reported Rosa-Masegosa et al. (2024a) for the NS9 marine group. The prevalence of the *Xanthomonadales* order could be due to their usual participation in substrates decomposition during the last stages, suggesting that longer HRTs facilitated their growth (Orlova et al., 2023). Additionally, both phylotypes are involved in the nitrogen cycle, as the *Xanthomonadales* can carry out the autotrophic utilisation of NH_4^+ as the sole nitrogen source in low-dissolved oxygen nitrification process under low OLR (Fitzgerald et al., 2015; Zhang et al., 2024a).

OTU03, belonging to the *Pseudomonas* genus, showed strong representation during the initial period in bioreactors with 6, 4 and 2 h of HRT, while R1 (8 h of HRT) did not stimulate its growth. OTU31, affiliated with *Comamonas*, was favoured in the R3 and R4 bioreactors, achieving relative abundances of 1.9 % and 3.1 %, respectively. *Comamonas,* belonging to the *Comamonadaceae* family, is known by the high versatility in wastewater treatment because genus proliferate under high OLR (Simona et al., 2022) and also described as PAO involved in biological phosphorus removal (Zhang et al., 2024b).

Finally, the bioreactor with the shortest HRT promoted the growth of OTU06 (*Rhodocyclaceae* family), OTU07 (*Rhizobiaceae* family), OTU20 (*Flavobacterium* genus) and OTU25 (*Weeksellaceae* family), which could all have high substrate affinity and potentially displace others phylotypes from the ecological niche. The *Flavobacterium* genus play important role in the granular size and stability under high OLR in AGS (Chen et al., 2019b). The families *Rhodocyclaceae* and *Rhizobiaceae* and the *Flavobacterium* genus are known for EPS production or their role in granular stability (Gómez-Acata et al., 2018; Lv et al., 2014; Rosa-Masegosa et al., 2024b). Contrarily to our results, the *Rhizobiaceae* family was reported for proliferating at low OLRs (Rosa-Masegosa et al., 2024a). The population dynamics of the reactors was influenced by the HRT, with similar

communities for R1 and R2, greater differences with R3, and the most notable changes compared to R4, with the shortest HRT.

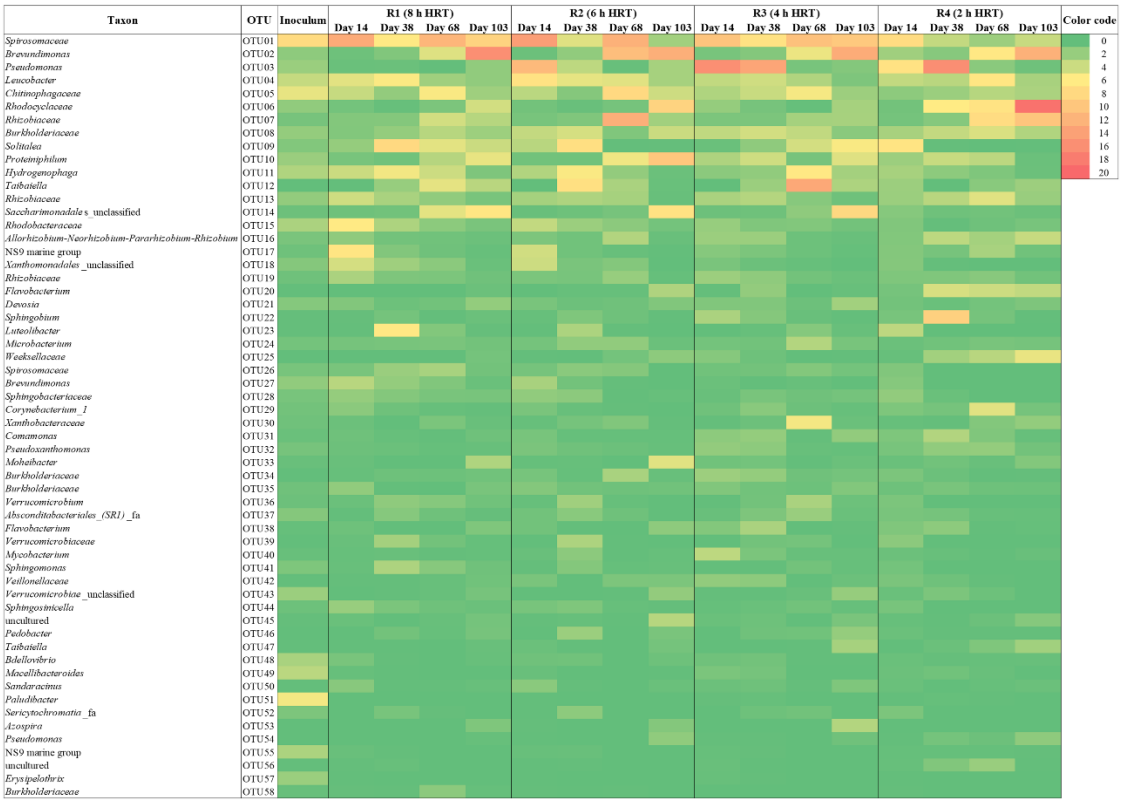

Figure 2. Heatmap of the prokaryotic community dynamics for all bioreactors over operational time with more than 1.50 % of relative abundance in at least one sample.

3.3.2. Eukaryotic community dynamics

Twenty-five OTUs dominated with more than 1 % of relative abundance for the eukaryotic communities (Fig. 3). The inoculum sample was represented by OTU01 (69.93 % of total relative abundance) of the *Hypocreales* fungi order, a typical order found in WWTPs sometimes associated with biofilms (Buratti et al., 2022; Li et al., 2020b), and OTU13 (16.75 %), belonging to *Microbotryum* genus. During the experimental period, the eukaryotic communities were modified depending on the different HRTs. Despite its oscillations, OTU01 was dominant for all bioreactors throughout the experimental period, revealing its competitiveness regardless of operational conditions or substrate availability. In fact, Trovão et al. (2022) describe species belonging to this order as exhibiting versatile strategies to exploit substrates, and their capability to survive under environmental stresses is highlighted.

Specifically, for the R1 bioreactor, OTU01 represented 77.80 % of the total relative abundance on day 14. In this bioreactor, there was an increase in the number of dominant eukaryotic phylotypes at day 38, particularly two unclassified eukaryotes and *Oligohymenophorea* with relative abundances of 39.54 %, 5.88 % and 9.55 %, respectively, but their representation was absent in the subsequent population scenarios in these granules. *Oligohymenophorea* belongs to a class of ciliates and plays an essential role in biofilm formation because it is associated with the control of microbial communities through the consumption of particulate matter (Chan et al., 2021). On day 68, OTU01, OTU04 (unclassified *Eukaryota*), OTU06 and OTU07 (belonging to the *Ascomycota* phylum) were the most representative phylotypes, with OTU04 surpassing OTU01 in terms of relative abundance. By the end of experimentation, new phylotypes gained relevance, modifying the evenness in the population. These included OTU01, OTU02 (belonging to the *Oligohymenophorea* class), OTU05 (affiliated with the taxon *Metakinetoplastina*) and OTU08 (unclassified *Eukaryota*). An interesting trend was observed in the eukaryotic population dynamics: only OTU01 was ubiquitous over the operational period with a high relative abundance above 10 %.

The R2 bioreactor followed a similar pattern, with OTU01 being the most abundant phylotype over time. However, there were slight variations linked with the enrichment of OTU09 (belonging to the *Ascomycota* phylum) and OTU14 (class *Oligohymenophorea*) on day 14. On day 38, the population showed the highest diversity across all experiments, with many OTUs acquiring values above 5 % of relative abundance, indicating the operational conditions that induced high competitiveness, even in phylotypes belonging to the same order such as *Oligohymenophorea*. From day 68 onwards, the trend was similar to R1, possibly caused by the coupling of phylotypes with the greatest advantages. By day 103, only 3 OTUs were dominant: OTU01 (59.70 %), OTU02 (27.70 %) and OTU03 (affiliated with the *Chrysophyceae* class), which increased its relative abundance to 7.18 %.

The eukaryotic population dynamics of the R3 bioreactor were like those of the R2 bioreactor, but the relative abundance pattern was different. Notably, the dominance of OTU01 was more stable over long-term operation, with values in the range of 18 % to 61 %, which did not allow the massive proliferation of other phylotypes, except on day 68. On day 103, the dominant OTUs were OTU01 and OTU02, following the patterns of

previously described reactors, but with the proliferation of *Metakinetoplastina*, as seen in R1.

In the R4 bioreactor, the population dynamics were uneven compared to the other reactors. In response to the shortest HRT, OTU06, followed by OTU01, were the dominant eukaryotic microorganism from day 14 to day 68. OTU06 is taxonomically affiliated with *Chaetothyriales* (*Ascomycota*), inhabits stressful environments and was detected in biofilms in the wastewater network (Dworkin et al., 2006; Li et al., 2020b). By the end of the experiment, the dominant OTUs were OTU01, OTU02 and OTU03 which shared the role in the community due to their similar relative abundance ranging from 20 % to 40 %. OTU05, affiliated with *Metakinetoplastina*, had a lower representation (6.57 %).

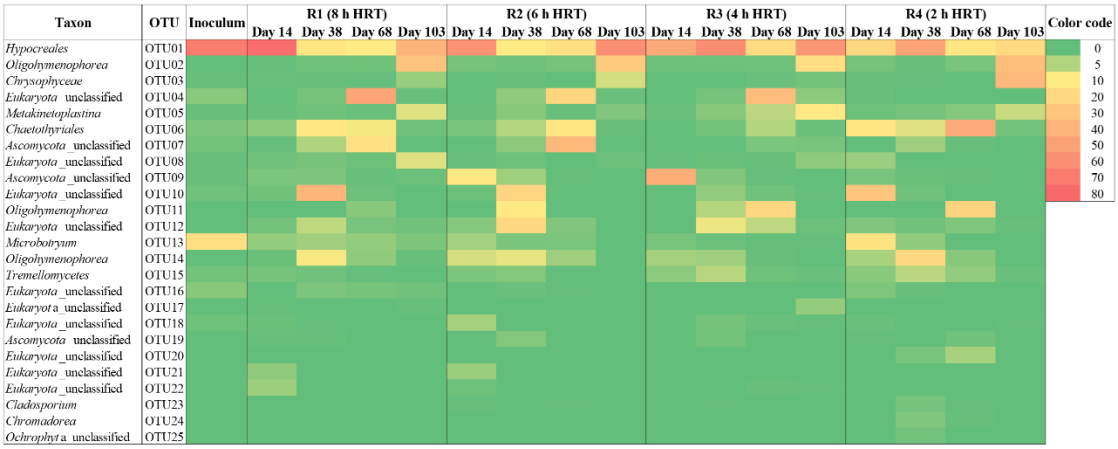

Figure 3. Heatmap of the eukaryotic community dynamics for all bioreactors over operational time with more than 1.00 % of relative abundance in at least one sample.

3.4. Prokaryotic ecological function prediction

The potential ecological functions were predicted using FAPROTAXv1.2.7 software. According to the results (Fig. 4), chemoheterotrophic metabolism, specifically aerobic chemoheterotrophy, was the main pathway for the organic matter degradation, associated with 37.4 %, 38.8 %, 39.1 % and 40.4 % of the community in bioreactors R1, R2, R3 and R4, respectively. This role was present in 17 dominant OTUs, including OTU03, OTU04, OTU11, OTU20, OTU21, OTU22, OTU24, OTU31, OTU36, OTU38, OTU39, OTU40, OTU41, OTU44, OTU46, OTU53 and OTU54. The

chemoheterotrophic metabolism of most of these taxa has been described by Dworkin et al. (2006), and the distribution of evenness was similar regardless HRT.

Although there were similarities in aerobic chemoheterotrophic metabolism across all bioreactors, fermentation activity was lower in R4 bioreactor compared to the other conditions tested, because low HRT trend to decrease the yields but other parameters as pH are essential to overview the process (Pau et al., 2024). The presence of potential ureolytic bacteria was observed, with mean values of 3.1 %, 2.8 %, 2.8 % and 1.9 % for the R1, R2, R3 and R4 bioreactors, respectively. Ureolytic bacteria, in addition to being involved in the nitrogen cycle, are associated with biomineral production, phosphate recovery and heavy metal removal, possibly linked to PAOs (Arias et al., 2017).

Functions related to the nitrogen cycle were directly influenced by the HRT, suggesting a higher capacity for nitrogen removal at longer HRTs (Li et al., 2013; Wan et al., 2014a). Figure 4 illustrates higher activities in nitrate respiration, nitrate fixation, nitrate reduction and nitrogen respiration from day 38 for HRTs of 8 and 6 h, while the bioreactor operating with a 4-hour HRT reached similar values by day 103. In contrast, the reactor with a 2-hour HRT did not achieve nitrogen abundance activities higher than 1 %. These findings indicate that HRT has a direct impact on nitrogen removal in aerobic granular systems.

Conversely, the biomass from bioreactors operated at shorter HRTs showed higher levels of human pathogens, animal parasites and symbionts.

Figure 4. Prokaryotic ecological function prediction for each biological sample.

3.5. Similarity percentages analysis

The SIMPER analysis determined the contribution of each OTU to the dissimilarity between samples. In terms of the prokaryotic community (Fig. S5), the OTU that contributed most to the dissimilarities between granules used as inoculum and the mature granules in each reactor was OTU51, a member of *Paludibacter*, a typical genus found in stored granules (Zhang et al., 2019a). This was followed by OTU48, OTU49, OTU55 and OTU57, affiliated with *Bdellovibrio*, *Macellibacteroides*, NS9 marine group

and *Erysipelothrix* taxa, respectively; these were characteristic phylotypes of the inoculum sample, but their competitive disadvantage in active granules led to their displacement.

The higher contribution to dissimilarities between the population dynamics of R1 and the rest of the bioreactors was driven by OTU01 and OTU02, as their relative abundances were considerably higher compared to the rest of the population, as corroborated in the studies of population dynamics (Fig. 2). OTU03, affiliated with *Pseudomonas*, was responsible for greater dissimilarities between R3 and the rest of the bioreactors due to its strong proliferation during start-up and on day 38. OTU06, affiliated with the *Rhodocyclaceae* family, contributed to the dissimilarities between R4 and R1, R2 and R3, as this bacterium was the most abundant on day 103 (19.05 %) and had a maximum representation of 8.59 % in the rest of the bioreactors.

The SIMPER conducted for eukaryotic communities (Fig. S5) demonstrated that OTU01, affiliated with *Hypocreales*, was the main contributor to differences between all reactors, with values above 10 %. OTU03, the dominant phylotype at the end of operation in all bioreactors, contributed mainly to differentiating bioreactor R3 from the rest of bioreactors. OTU02 was responsible for differences seen between R1 and R2 and the rest of bioreactors. R1, R2 and R3 had differences with more than 8 % of contribution compared to R4, caused by OTU04, which proliferated on day 68 in all reactors except in R4.

3.6. Diversity indices

The indices of α-diversity for the prokaryotic and eukaryotic communities are available in Table S2. For the prokaryotic community, the Simpson and Shannon-Wiener diversity indices indicate that all tested conditions led to a selection of microorganisms, resulting in a decrease in diversity, with the most pronounced effect observed in the R4 bioreactor. A reduction in Pielou's evenness was also noted across all reactors, particularly in R4. These results align with the population dynamics of prokaryotic organisms, showing that the relative abundance of OTUs shifted, leading to a smaller number of dominant OTUs occupying the main niches in the system. The Chao-1 index revealed two distinct scenarios: one for treatments with 8 and 6 h of HRT, and another for 4 and 2 h of

HRT. At 8 and 6 h, species richness gradually increased, whereas in the R3 and R4 bioreactors, richness initially increased but later decreased.

For the eukaryotic community, all indices confirmed a reduction in diversity, evenness, and species richness across all reactors over operational time, with a more pronounced trend in bioreactors with lower HRTs. These results suggest that this condition leads to stronger selection pressures on microorganisms, thereby reducing their metabolic capabilities, as corroborated by the functional prediction of microbial ecology.

The Whittaker index was calculated for β-diversity of prokaryotic and eukaryotic communities. It was noted that the eukaryotic community was more homogeneous than the prokaryotic community. Regarding prokaryotes, a comparison between the inoculum and subsequent samples showed significant dissimilarities after day 38. After that day, strong changes were observed in all bioreactors, especially in R4, which exhibited substantial dissimilarities among samples before and after day 38. For the eukaryotic community, the Whittaker index indicated significant dissimilarities between R4 and the other bioreactors from day 38 until the end of the operation.

3.7. Expected effect size

The EES was calculated to assess the extent to which eukaryotic and prokaryotic OTUs from specific populations exhibited significant differences compared to other sample clusters. For the comparisons between reactors R1, R2, R3, and R4, certain OTUs appeared to contribute to the observed differences between reactors R1, R2, and R3 versus R4, with a similar pattern noted for both eukaryotic and prokaryotic communities.

For the comparative analysis of prokaryotic communities between reactors (Table S3), the highest number of OTUs with statistically significant differences between R1, R2, and R3 against R4 were 90, 27, and 28, respectively. Additionally, several OTUs exhibited significant differences between R1 and R3, totalling 65 taxa. In contrast, fewer significant differences were observed in the comparisons between R1 and R2 (11 OTUs) and R2 and R3 (10 OTUs). Furthermore, none of these OTUs represented dominant phylotypes (defined as >1.5% relative abundance). OTU26, affiliated with the *Spirosomaceae* family, exhibited significant differences in R4 compared to the other reactors, being exclusively present in the reactors with an HRT longer than 2 h. Other OTUs that displayed differences between R1 and R3 relative to R4 included OTU01,

OTU36, OTU44, OTU47, and OTU55, taxonomically classified as *Spirosomaceae*, *Verrumicrobium*, *Sphingosinicella*, *Taibaiella*, and NS9 marine group, respectively. These OTUs were dominant in R1 and R3 but not in R4, with the exception of *Taibaiella*, which was only observed in R4.

In eukaryotic populations, the number of significantly different OTUs was lower, consistent with the observed species richness (Table S4). The analysis indicated that the higher taxonomic groups displaying significant differences were R1 versus R4 and R3 versus R4, with three OTUs identified for each comparison. These findings suggest that the resilience of eukaryotic communities in granular environments may be greater than that of prokaryotic communities under stress conditions, as previously proposed by Simon et al., (2016). The communities of R2 and R3 showed no OTUs with significant differences, confirming the similarity between these populations. Only OTU04 exhibited significant differences between R1, R2, and R3 compared to R4, driven by its proliferation on day 68 in all bioreactors except R4. In contrast, OTU15, belonging to the *Tremellomycetes* class, showed significant differences between R1 and both R3 and R4, as well as between R2 and R4, due to its higher abundance at shorter HRT (2 and 4 h), respectively.

In summary, the results of this analysis suggest that gradual variations in HRT have minimal impact on community phylotype composition due to the resilience of eukaryotic microorganisms (Philippot et al., 2021). However, extreme changes in this parameter, such as HRT of 2 h versus 8 h, exert a more significant selective pressure on the community, likely due to substantial alterations in substrate availability and competition dynamics.

3.8. Principal component analysis

PCA of the prokaryotic communities (Fig. 5A) showed two differentiated clusters, indicating that bioreactors operated at 8, 6 and 4 h of HRT shared a more similar prokaryotic community composition. In contrast, the community from samples of the bioreactor operated at 2 h of HRT exhibited greater distances, even in its own samples over operational time. By day 103, samples from R1, R2 and R3 showed shorter distances between them. These results suggested that the prokaryotic community of R4 did not

achieve successful stabilisation of population, possibly due to biomass withdrawal and intense competition for the physical niche within the granules.

Figure 5B shows the PCA for the eukaryotic community, revealing progressive differentiation from the inoculum over the experimental time. Initially, at day 14, all treatments were closely clustered with the inoculum. However, by days 38, 68 and 103, increasing distances from the inoculum were observed. Samples from R1, R2 and R3 formed a cohesive cluster, yet they exhibited gradual differences according to the HRT. In contrast, samples from R4 at days 38, 68 and 103 formed a distinct cluster apart from the rest of bioreactors, indicating that the eukaryotic community in the R4 bioreactor was more differentiated compared to R1, R2 and R3 bioreactors. The strong selection of R4 eukaryotic dynamics could be drive by consumption of high organic load (up to 12.56 g COD $\cdot d^{-1}$) in short time frame (2 h).

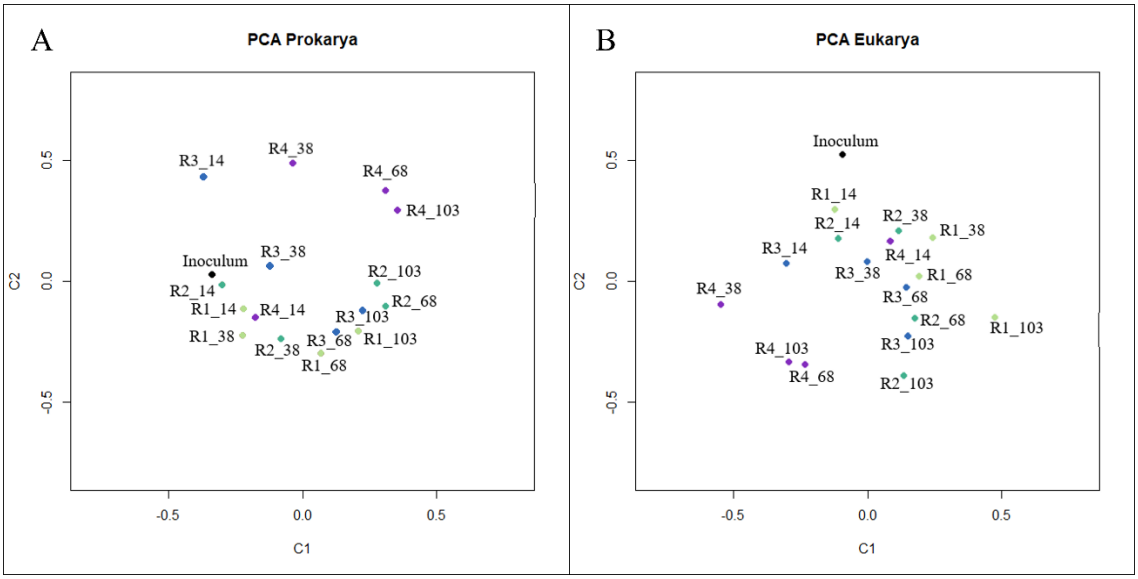

Figure 5. Principal component analysis for prokaryotic (A) and eukaryotic (B) communities.

3.9. Multivariate redundancy analysis

The RDAs illustrate the correlation between the microbial communities, physicochemical removal performance and biomass characteristics over the operational time of each treatment.

The RDAs for the dominant prokaryotic OTUs are displayed in Figure 6. For R1 (8 h of HRT), all the parameters (settling velocity, granular size, MLSS and organic matter, N and P removal) were positively correlated with the steady-state period of the

experiment (days 68 to 103). Mean granular size, settleability, biomass concentration and organic matter removal performance were strongly correlated with OTU07, OTU08 and OTU14, belonging to the *Rhizobiaceae* and *Burkholderiaceae* families and the *Saccharimonadales* order, respectively. OTU12, OTU34 and OTU46, from the *Taibaiella* genus, *Burkholderiaceae* family and *Pedobacter* genus, respectively, were linked to the P removal.

In the R2 bioreactor (6 h of HRT), phosphorus and BOD_5 removal, together with MLSS, settling velocity and granular size, were positively correlated with the later operational period, while the other parameters were negatively correlated with the steady-state period of the experiment. Specifically, BOD_5 removal and granular density were linked to the *Rhizobiaceae* family (OTU07), whereas mean size, MLSS and P removal performance were correlated with OTU02, OTU05, OTU10, OTU25 and OTU42, affiliated with the *Brevundimonas* genus, *Chitinophagaceae* family, *Proteiniphilum* genus, and *Weeksellaceae* and *Veillonellaceae* families, respectively. Some of these bacteria have a high capacity to secrete EPS, which promotes granular size and biomass concentration in the reactor (Song et al., 2022a). Conversely, OTU03, OTU08, OTU31, OTU49, OTU50, OTU51, OTU55, OTU57 and OTU58 were correlated with N and COD removal.

For the R3 bioreactor (4 h of HRT), MLSS, mean size, settleability, organic matter removal and P removal all had a positive correlation with the steady-state period of the experiment. Settling velocity, granular size and organic matter removal performance were positively correlated with OTU45 and OTU50. MLSS was positively linked to OTU54, whereas P removal was correlated with OTU01, OTU02, OTU09 and OTU12. Conversely, N removal was negatively correlated with the later period of the experiment but strongly correlated with OTU04.

The RDA plot of R4 bioreactor (2 h of HRT) showed a correlation of the settling velocity, mean size and MLSS with days 38 and 68 and were linked with OTU08, OTU13 and OTU56.

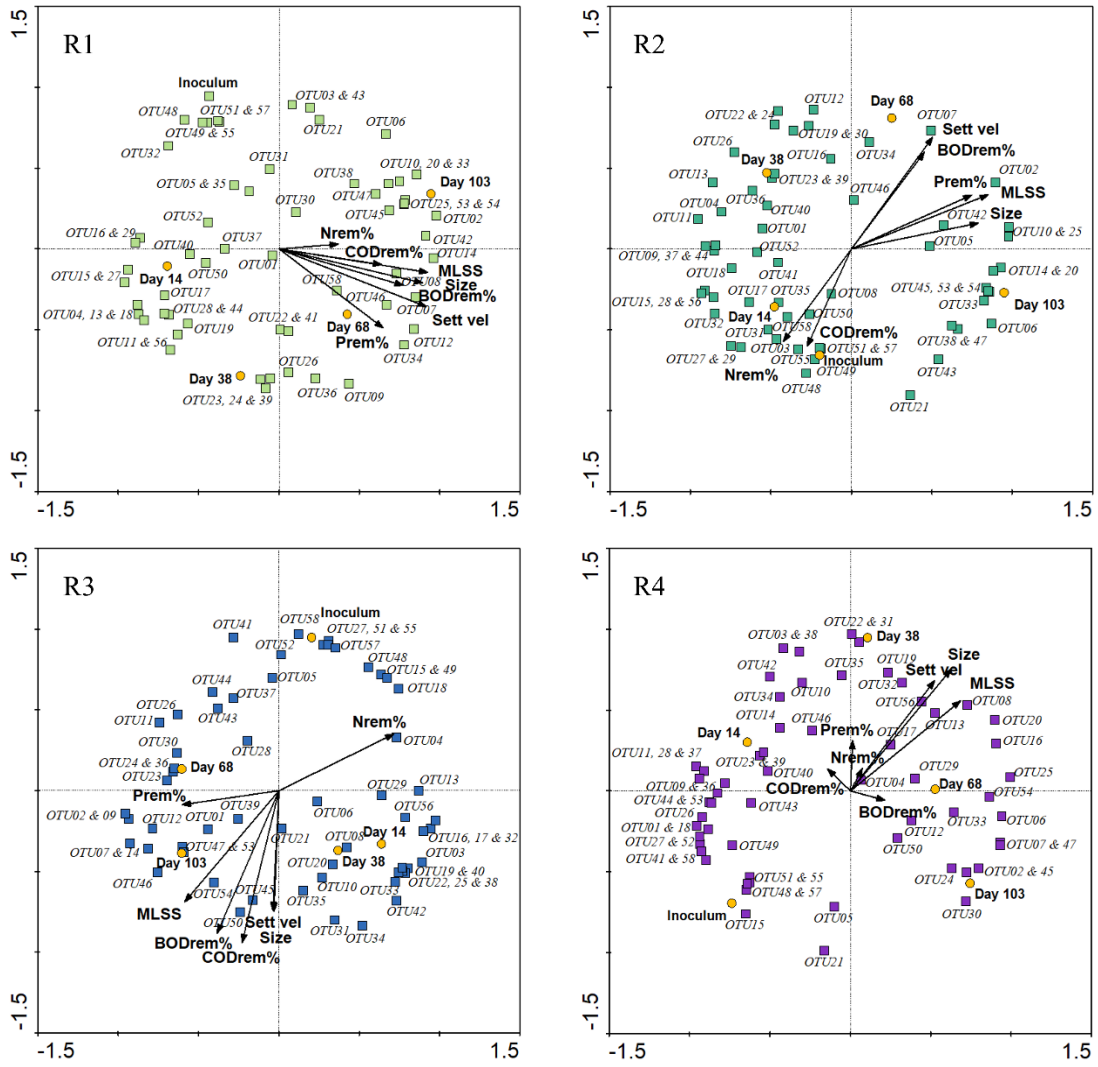

Figure 6. Multivariate redundancy analysis of the dominant prokaryotic community with physicochemical parameters for each bioreactor.

The RDAs of the dominant eukaryotic OTUs and their physicochemical parameters are shown in Figure 7. For the R1 bioreactor, organic matter, N and P removal, MLSS, settleability and granular size were negatively correlated with the initial period of the experiment (inoculum and day 14). Granular size and P removal were positively correlated with operational day 103, with a strong positive linkage to OTU02 and OTU03, taxonomically affiliated with *Oligohymenophorea* and *Chrysophyceae*, respectively. The role played by fungal phylotypes in the structural core of granules is essential for bacterial colonisation, as well as for the function of ciliates in the consumption of particulate matter, maintaining the spherical structure (Muñoz-Palazon et al., 2019).

The RDA of the eukaryotic community for the R2 bioreactor shows that COD removal was positively correlated with day 14, along with OTU18, OTU21 and OTU22. Mean size, MLSS and N and P removal were positively linked with the inoculum sample, day 103, and OTU02 and OTU03. Settling velocity was positively correlated with day 68.

For the R3 bioreactor, the nitrogen removal ratio and settling velocity were positively correlated with the inoculum sample and with OTU01, OTU13, OTU16 and OTU25. Granular size showed slight linkage to days 14 and 38, with a small established linkage between granular properties and the eukaryotic community. Organic matter removal was positively correlated with day 68 with OTU06, progressively increasing over operational time, acquiring high relevance in the system. MLSS and P removal were positively correlated with day 103, along with OTU02, OTU03, OTU08 and OTU17, where only *Oligohymenophorea* acquired values above 10 % of relative abundance (Fig. 3).

Finally, the RDA for the R4 bioreactor showed a strong correlation of biomass parameters (MLSS, settleability and mean size) with day 68 and OTU11 and OTU19. Organic matter and N removal were positively linked to the inoculum sample and day 103, with COD removal strongly correlated with OTU22.

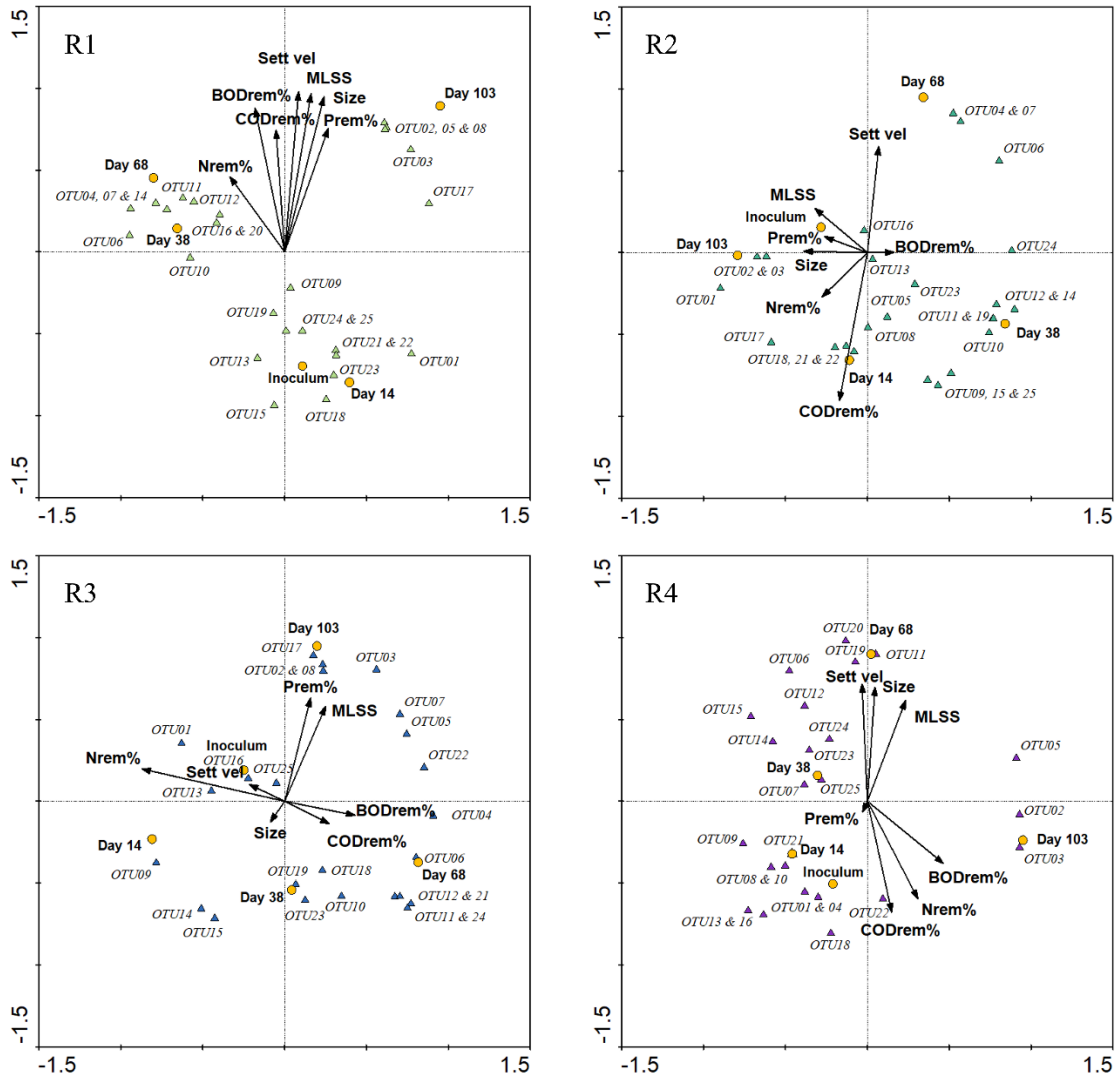

Figure 7. Multivariate redundancy analysis of the dominant eukaryotic community with physicochemical parameters for each bioreactor.

4. Conclusions

The effect of HRT on a novel single-chamber continuous-flow reactor for aerobic granular sludge technology was studied, with the aim to evaluate the feasibility of this design for full-scale implementation. The results indicate that the bioreactor operated at 2 hours of HRT had less favourable removal performance, MLSS and granular properties compared to the other bioreactors. Therefore, 2 hours was not an optimal hydraulic retention time for the exploitation of this novel AGS-CFR configuration. Results suggest that bioreactors operated at 8, 6 and 4 hours of HRT demonstrated success pollutants removal rates and more stable granular features for treating influents with these

characteristics, being favourable for the full-scale implementation and operation of this single-chamber AGS-CFR. Particularly, 4 hours of HRT was the most efficient and cost-effective condition in terms of remotion of higher organic loads maintaining the granular stability. Whereas focusing on nutrient removal, the longer HRTs (8 and 6 hours) offered more operational advantages.

The prokaryotic and eukaryotic communities were adapted to the different conditions, but the initial microbiota played a key role throughout the experiment. Notably, the CFR operated at 2 hours of HRT experienced the most pronounced changes in microbiota, while more similarities were found in the rest of bioreactors. These findings underscore the importance of optimising HRT to enhance the performance concerning discharge requirements for the implementation of AGS-CFR technology at full scale.

Acknowledgements

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Supplementary information

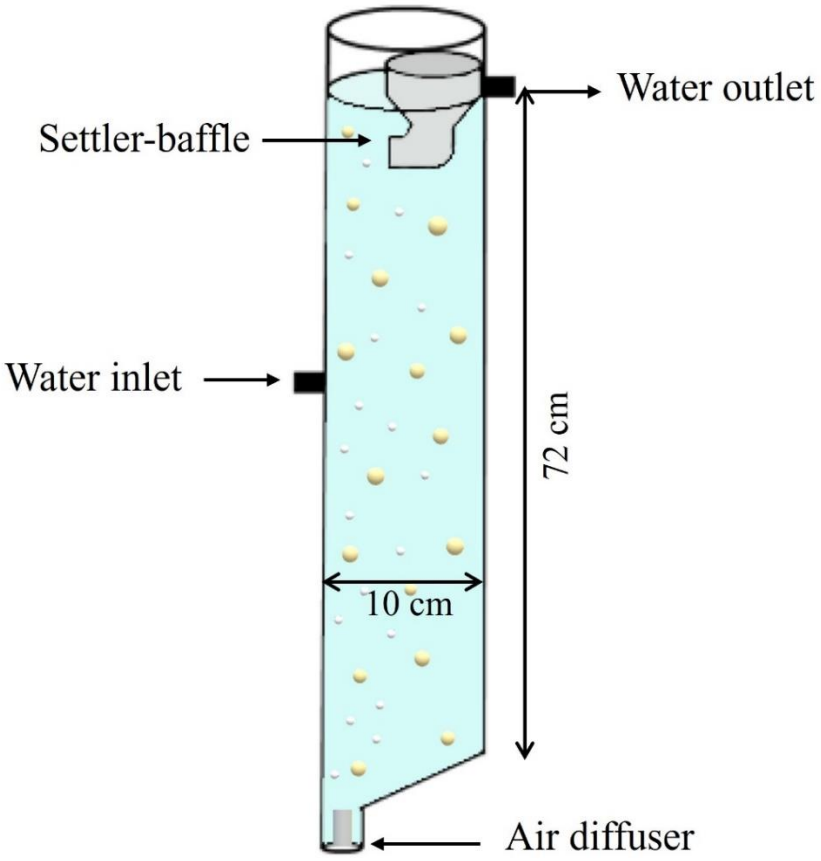

Fig. S1. Schematic diagram of the single-chamber AGS-CFR configuration used in this study.

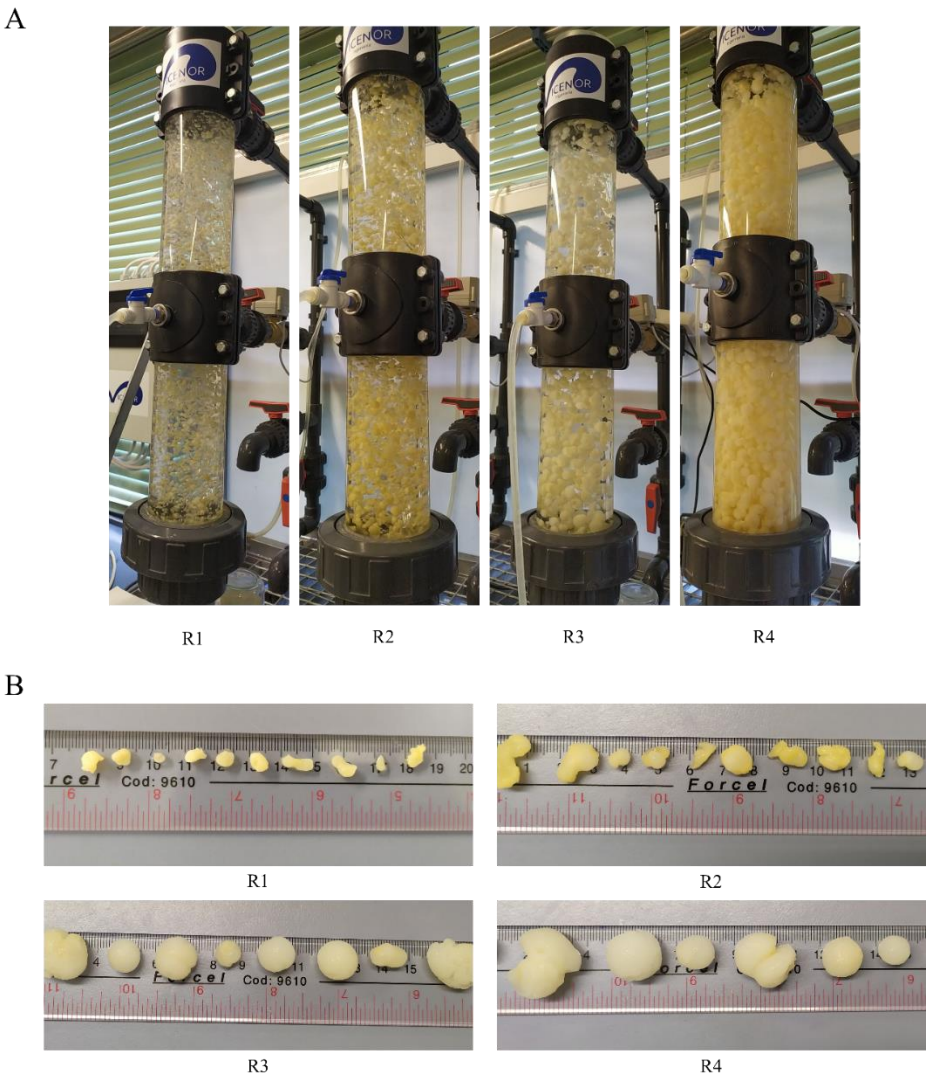

Fig. S2. Digital images of the four bioreactors where it is possible to appreciate the differences in terms of biomass concentration and granular size (A); digital images of granular sludge morphology (B).

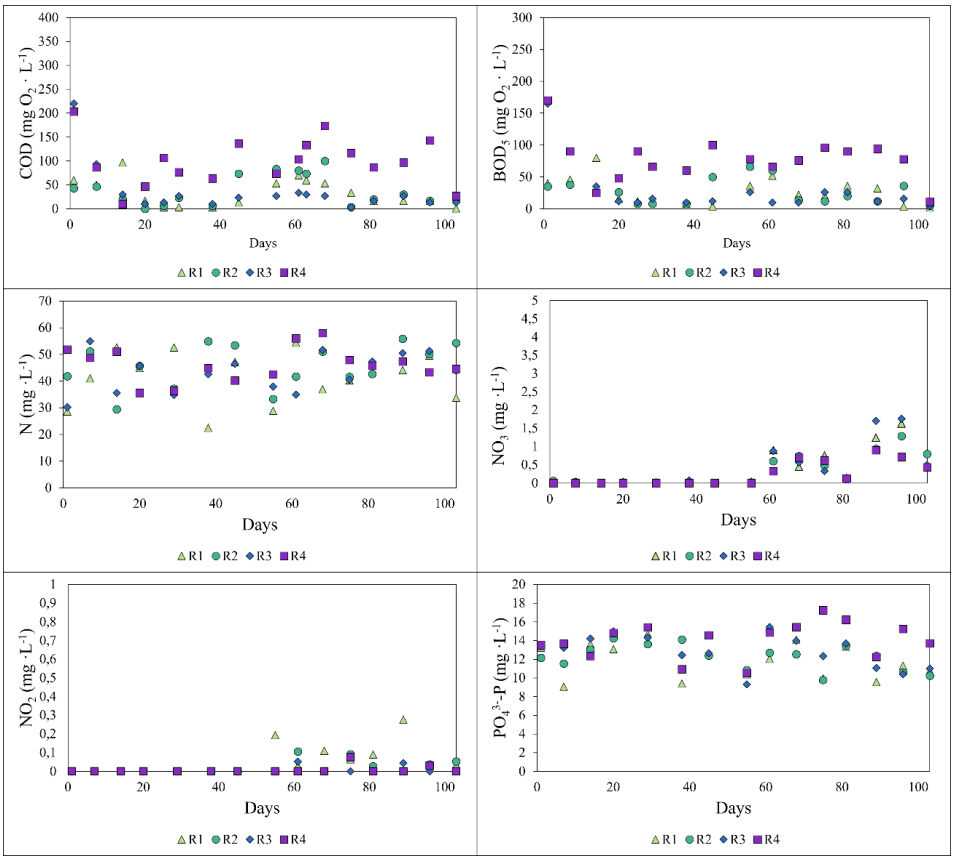

Fig. S3. Effluent concentration of COD, BOD_5, N (including ammonium, nitrate, and nitrite), NO_3, NO_2 and P (including phosphate) for each bioreactor.

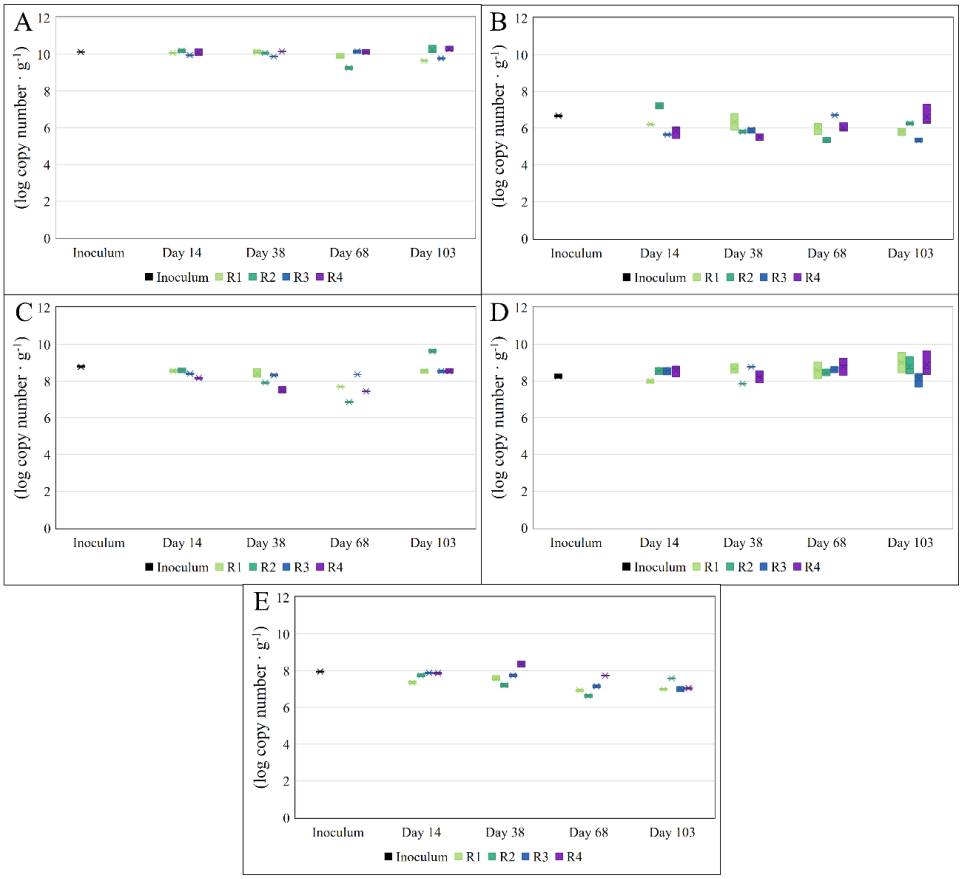

Fig. S4. Absolute quantification of genes: 16S rRNA of *Bacteria* (A); 16S rRNA of *Archaea* (B); 18S rRNA of *Fungi* (C); gene *amoA* (D); and 16S rRNA of *Candidatus* Accumulibacter (E).

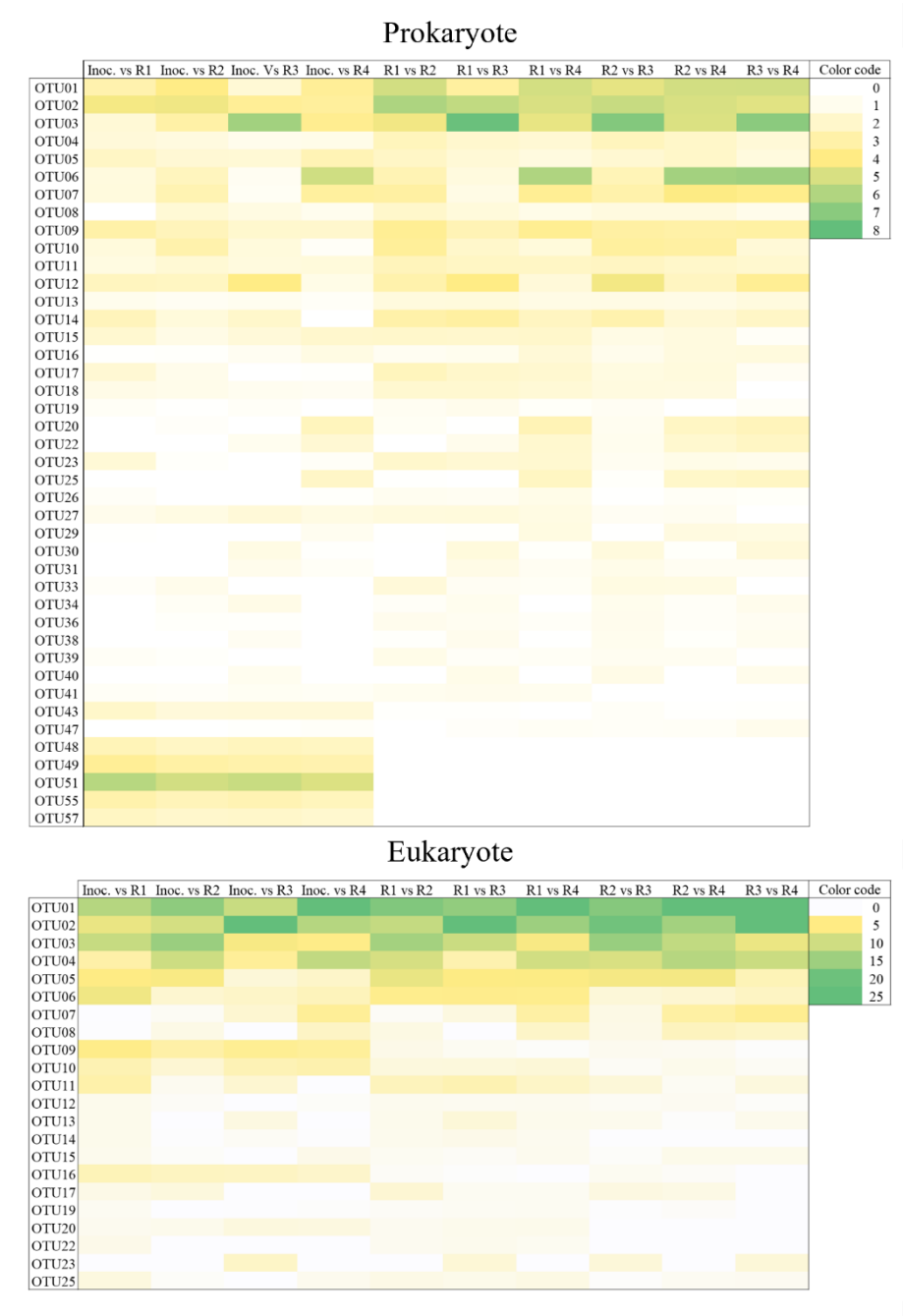

Fig. S5. Similarity percentage analysis for dominant prokaryotic and eukaryotic communities with more than 1 % of contribution at least in one sample group.

Table S1. Carbon mass balance of each reactor

BIOREACTOR	PERIOD	Q ($L \cdot d^{-1}$)	INFLUENT		EFFLUENT		REMOTION		COD ΔMLVSS ($g \cdot d^{-1}$)	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	Day 0-20	18	4,55	3,72	1,01	0,82	3,54	2,90	0,55	5,56
	Day 21-41	18	4,14	3,60	0,06	0,19	4,08	3,41	1,35	6,41
	Day 42-62	18	4,22	3,24	0,82	0,55	3,40	2,69	0,92	5,34
	Day 63-83	18	4,40	2,76	0,62	0,53	3,78	2,23	2,20	5,94
	Day 84-103	18	4,26	3,47	0,21	0,05	4,05	3,41	2,86	6,37
	Average		**4,31**	**3,36**	**0,54**	**0,43**	**3,77**	**2,93**	**1,57**	**5,93**
	SD		**0,16**	**0,38**	**0,40**	**0,31**	**0,30**	**0,50**	**0,94**	**0,48**
R2	Day 0-20	24	6,04	4,88	0,66	0,75	5,38	4,13	0,86	8,45
	Day 21-41	24	5,87	4,80	0,29	0,19	5,57	4,61	1,43	8,76
	Day 42-62	24	5,65	4,64	1,89	1,41	3,76	3,23	0,82	5,91
	Day 63-83	24	5,89	3,84	0,99	0,35	4,91	3,49	2,54	7,71
	Day 84-103	24	5,97	4,44	0,53	0,49	5,44	3,95	2,89	8,55
	Average		**5,89**	**4,52**	**0,87**	**0,64**	**5,01**	**3,88**	**1,71**	**7,88**
	SD		**0,15**	**0,42**	**0,62**	**0,48**	**0,74**	**0,54**	**0,96**	**1,17**
R3	Day 0-20	36	9,03	7,44	3,18	2,72	5,85	4,72	1,25	9,19
	Day 21-41	36	8,72	7,20	0,60	0,43	8,12	6,77	1,03	12,76
	Day 42-62	36	8,60	6,36	1,00	0,58	7,60	5,78	1,83	11,94
	Day 63-83	36	8,76	6,00	0,56	0,77	8,20	5,23	2,25	12,89
	Day 84-103	36	8,96	6,48	0,64	0,38	8,32	6,10	3,94	13,07
	Average		**8,81**	**6,70**	**1,20**	**0,97**	**7,62**	**5,72**	**2,06**	**11,97**
	SD		**0,18**	**0,60**	**1,12**	**0,99**	**1,03**	**0,79**	**1,16**	**1,61**
R4	Day 0-20	72	17,94	15,12	6,24	5,99	11,70	9,13	2,22	18,39
	Day 21-41	72	17,76	14,40	5,92	5,18	11,84	9,22	2,39	18,61
	Day 42-62	72	16,96	12,72	7,52	5,86	9,44	6,86	2,55	14,83
	Day 63-83	72	17,60	13,20	9,04	6,72	8,56	6,48	2,90	13,45
	Day 84-103	72	18,96	14,40	6,40	3,20	12,56	11,20	2,21	19,74
	Average		**17,84**	**13,97**	**7,02**	**5,39**	**10,82**	**8,58**	**2,45**	**17,00**
	SD		**0,73**	**0,98**	**1,28**	**1,34**	**1,72**	**1,93**	**0,29**	**2,71**

Table S2. α-diversity for prokaryotic and eukaryotic communities.

	Reactor	Days	Simpson	Shannon-Wiener	Pielou's Evenness	Chao-1
Prokaryotic community	Inoculum		0.9764	4.453	0.09251	1397
	R1	14	0.9600	4.031	0.06377	1413
		38	0.9707	4.281	0.08765	1353
		68	0.9631	4.068	0.06450	1788
		103	0.9499	3.962	0.05364	1718
	R2	14	0.9489	3.844	0.05190	1465
		38	0.9703	4.196	0.07102	1566
		68	0.9398	3.656	0.03626	1718
		103	0.9497	3.722	0.04570	1611
	R3	14	0.9518	3.984	0.05562	1877
		38	0.9623	4.128	0.06564	1646
		68	0.9491	3.742	0.04793	1491
		103	0.9531	3.829	0.05354	1399
	R4	14	0.9743	4.438	0.08397	1559
		38	0.9480	3.755	0.05775	1362
		68	0.9645	3.952	0.04954	1635
		103	0.9254	3.395	0.03816	1202
Eukaryotic community	Inoculum		0.9188	3.104	0.11090	260.9
	R1	14	0.8896	2.802	0.09579	215.0
		38	0.9411	3.463	0.17630	222.6
		68	0.8962	2.984	0.11040	252.3
		103	0.8743	2.754	0.05902	325.6
	R2	14	0.8239	2.517	0.07747	205.0
		38	0.9270	3.340	0.17640	195.7
		68	0.7389	2.021	0.03910	240.4
		103	0.8181	2.252	0.04777	304.0
	R3	14	0.7548	2.083	0.04985	244.2
		38	0.7741	2.332	0.05393	244.0
		68	0.8208	2.701	0.07966	278.6
		103	0.8634	2.652	0.08868	240.6
	R4	14	0.9293	3.331	0.16740	245.2
		38	0.6597	1.530	0.04086	139.4
		68	0.7139	1.926	0.04013	244.8
		103	0.8034	2.135	0.07168	168.6

Table S3. Expected effect size for prokaryotic community.

OTU	Taxon	R1vsR2	R1vsR3	R1vsR4	R2vsR3	R2vsR4	R3vsR4
OTU01	*Spirosomaceae*			-1.269			-1.422
OTU03	*Pseudomonas*		1.307	1.199			
OTU08	*Burkholderiaceae*			1.333			
OTU09	*Solitalea*			-1.194			
OTU16	*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*			1.442			
OTU18	*Xanthomonadales* unclassified			-1.047			
OTU20	*Flavobacterium*			2.003		1.089	
OTU26	*Spirosomaceae*			-1.481		-1.001	-1.417
OTU27	*Brevundimonas*			-1.263			
OTU31	*Comamonas*					1.084	
OTU32	*Pseudoxanthomonas*			2.083		1.229	
OTU34	*Burkholderiaceae*		1.362				-1.202
OTU36	*Verrucomicrobium*			-1.356			-1.197
OTU39	*Verrucomicrobiaceae*			-1.097			
OTU40	*Mycobacterium*		1.021				
OTU41	*Sphingomonas*			-1.422			
OTU44	*Sphingosinicella*			-1.204		-1.007	-1.059
OTU47	*Taibaiella*			-1.264			-1.146
OTU55	NS9 marine group			-1.099			-1.063
OTU56	uncultured			1.444			
Total number of OTUs with significant difference		11	65	90	10	27	28

Table S4. Expected effect size for eukaryotic community.

OTU	Taxon	R1vsR2	R1vsR3	R1vsR4	R2vsR3	R2vsR4	R3vsR4
OTU01	*Hypocreales*						-1.18082
OTU04	*Eukaryota* unclassified			-1.02188		-1.05016	-1.28875
OTU09	*Ascomycota*_unclassified			-1.17816			
OTU13	*Microbotryum*		-1.46931				
OTU15	*Tremellomycetes* unclassified		1.499324	2.263393		1.099968	
OTU17	*Eukaryota*_unclassified	-1.28529					
Total number of OTUs with significant difference		1	2	3	0	2	3

V - GENERAL DISCUSSION

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Aerobic granular sludge is recognised as an effective and advantageous technology for wastewater treatment. However, the main challenge is maintaining granular stability during long-term operation. To address this, AGS systems have been traditionally operated in sequential batch reactors. SBRs enable a fast and efficient selection of dense biomass, washing out excess of flocs that may compete with granular sludge and destabilise the system. Additionally, SBR operation enhances granular structure and stability through the feast-famine conditions. However, SBRs present operational drawbacks because most existing wastewater treatment infrastructures were designed for continuous-flow system, which are not compatible with batch-mode operation required for AGS-SBR. This mismatch can lead to inefficiencies in retrofitting or adapting these facilities for sequential treatment processes. For this reason, CFRs for AGS were developed to facilitate treatment at higher flow-rates in a more practical mode. CFRs also enhance a simpler control and maintenance, and reduce construction and operation costs, compared to SBRs (Kent et al., 2018; Silva et al., 2023). Nevertheless, the transition to CFRs still requires substantial modifications to accommodate the continuous-flow design, which presents challenges because the operational benefits may be offset by the need for more complex process control especially in maintaining compact biomass while preventing the washout of flocs. Additionally, long-term stability of the system could be compromised potentially limiting the application of CFRs in WWTP, as reported by several authors (Kent et al., 2018; Xu et al., 2022; Yan et al., 2021).

After reviewing existing AGS-CFRs configurations for wastewater treatment, it was concluded that no configuration has yet been proposed that offers simplicity in both design and operation. Most of them rely on biomass recirculation, which cause biomass damage, or involve complexity for full-scale implementation (Rosa-Masegosa et al., 2021; Yan et al., 2021). In detail, previous designs tend to have a complicated construction, operation and maintenance because they are based on multiple chambers (Chen et al., 2024; Li et al., 2015), complex configurations such as those used by Li et al. (2020c) or Sun et al. (2021a), bioreactors that require sludge return (Cofré et al., 2018; Li et al., 2016d; Sun et al., 2019), or hybrid systems combining SBR and CFR principles (Li et al., 2019). These complexities undermine the practicality and scalability of such systems for widespread application in wastewater treatment plants.

Facing these challenges, the main objective of this thesis was to develop and assess a new CFR model that enables the full-scale implementation of this technology. The proposed model aims to offer a simplified design and operation framework while ensuring the long-term retention of granular biomass, achieving high removal performance.

To solve this engineering issue, we proposed four novel single-chamber CFR designs aimed at reducing costs, simplifying construction and maintenance, and preserving granular stability while avoiding the negative impacts associated with sludge return systems. In contrast to existing designs, which are often complex and difficult to scale while maintaining granular stability (Rosa-Masegosa et al., 2021; Yan et al., 2021), our proposed designs offer a simpler construction, operation and maintenance compared to existing bioreactors.

The developed bioreactors R1, R2 and R3 featured eccentric cones, and bioreactor R4 had a concentric cone (Rosa-Masegosa et al., 2023). R1′s baffle was a truncated, conical settler positioned in the water outlet zone and connected to an elbow tube. R2′s baffle was a vertical plate placed above the air diffusor. R3′s baffle was an internal tube in an eccentric position, running over the air diffuser. Finally, R4’s baffle was an internal concentric tube located in the centre of the reactor. After characterising the granular biomass and the organic matter removal performance of the four novel configurations, the selected design was R1 (Fig. 11). This configuration demonstrated superior biomass concentration with excellent settling ability, which indicated the highest compaction and dense structure of granules formed, in comparison to granules from rest of bioreactor configurations (Rosa-Masegosa et al., 2023).

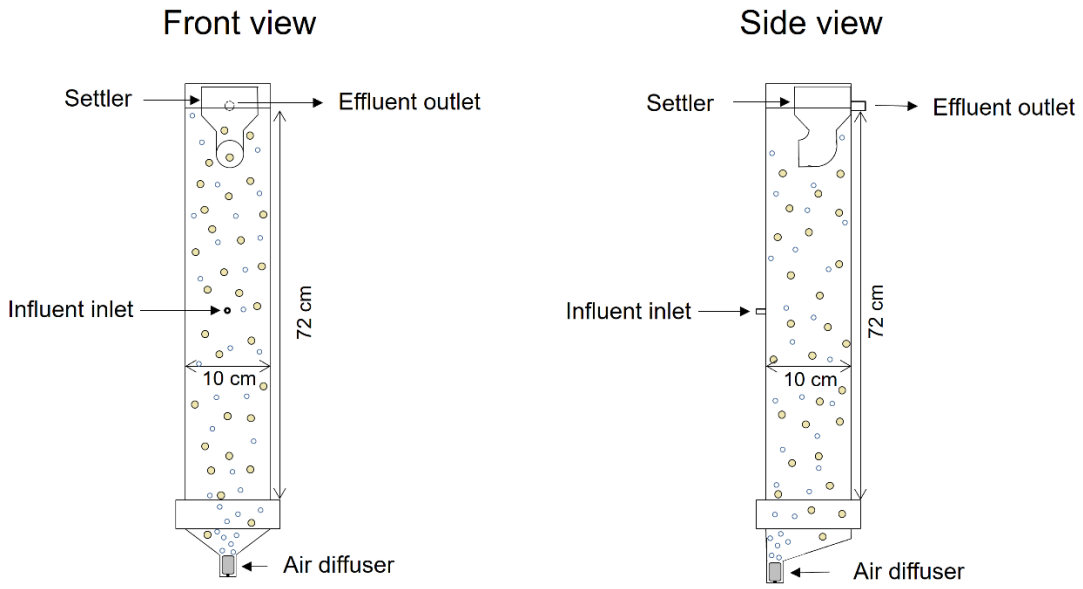

Figure 11. Schematic diagram of the single-chamber AGS-CFR configuration developed in this doctoral thesis.

In terms of biomass concentration, R4 did not retain more than 2.2 $g \cdot L^{-1}$. Contrarily, R1, R2 and R3, allowed a higher biomass retention, being biomass present within R1 bioreactor more compact than that of R2 and R3 bioreactors. These results suggested that the concentric cone in R4 contributed to greater biomass loss for the hydraulic stress. In addition, R1 configuration allowed a faster start-up period (11 days) compared to the rest of bioreactor models studied in this research, and also in comparison with configurations designed by other authors, even employed several strategies (Xu et al., 2022, 2021). In this way, the granules conformed from R1 bioreactor reached an average settleability of 106.5 $m \cdot h^{-1}$, even higher than other bioreactors operated in SBR, such as reported Muñoz-Palazon et al. (2020a), van Dijk et al. (2020) and Kocaturk and Erguder et al, (2015), which reported average values ranging from 90 to 74 $m \cdot h^{-1}$. Furthermore, the settleability reached from R1 design was significantly superior to the values reported by other CFRs configurations (43.2 and 39.6 $m \cdot h^{-1}$ reported by Xu et al. (2020) and Liu et al. (2012b), respectively). This improvement in settling velocity suggested that the R1 design produces granular with enhanced physical properties, likely resulting from more efficient biomass aggregation and compactness during the long-term operation in comparison with the abovementioned studies. On the other hand, the granules described by Cofré et al. (2018) achieved a similar settling velocity (113 $m \cdot h^{-1}$), however

the CFR model used consisted of two tanks and an external settler, requiring extra efforts for construction and operation comparing with the single-chamber design. These results are indicative of the potential benefits of R1 system as selected design in enhancing biomass aggregation and exploitation of the technology, making it a more effective configuration for applicating to pre-existing installation. The rest of reactor designs with eccentric cone (R2 and R3) showed better settleability than reactor with concentric cone In this sense, the R4 design did not promote the washout of fluffy flocs, increasing the presence of filamentous microorganisms, affecting the compaction of granules, which only achieved 40 $m \cdot h^{-1}$ of settling velocity, whereas granules from the R2 and R3 bioreactors had settleability values ranging from 50 to 65 $m \cdot h^{-1}$. Zhou et al., (2013), with a similar concentric design to R4 bioreactor, reported granules with filamentous structure and a poor settling ability. These findings are consistent with the results observed in the reactor with the concentric cone (R4), which exhibited poorer settleability performance. In spite of the lower settling ability showed by granules from R2, R3, and R4 bioreactors in comparison with R1, mean granular velocity was higher than settleability presented by other studies performed with CFRs (Liu et al., 2012b; Xu et al., 2020).

The R1 bioreactor design exhibited a superior ability for promoting the formation of compact granules within a short start-up period and for washing out filamentous microorganisms compared to R2, R3, and R4 bioreactors. This enhanced performance was not only due to its superior settleability, but also attributed to better physicochemical interactions, making the R1 configuration a more advantageous and profitable choice for wastewater treatment in comparison to the bioreactor models assessed in this doctoral thesis and in previous studies (Kent et al., 2018; Rosa-Masegosa et al., 2023, 2021; Yan et al., 2021).

Regarding the organic matter, mean removal performance was higher for R1 in comparison to R2, R3, and especially, to R4 bioreactor. These results were also corroborated by the carbon mass balance, which indicated that R1 bioreactor removed a higher organic load (9.84 and 7.32 g $O_2 \cdot d^{-1}$ of COD and BOD_5, respectively) than R2, R3, and R4, and achieving greater carbon load remotion than in comparison to another CFR bioreactor (Santorio et al., 2021).

These results contributed to select the R1 bioreactor model for performing further studies, due to its successful design which allows the dense biomass retention and achieves high organic matter removal under a simple single-chamber configuration.

The proliferation of *Acinetobacter* genus (OTU09 and OTU21) within R1, R2 and R3 bioreactors may be associated to the role that this taxon plays in the granule formation and stability, attributed to its capacity to secrete EPS, as reported Liébana et al. (2019) and Iorhemen et al. (2020), due to these bioreactors presented more stabilised granules than R4 design. The extra-compaction observed in granules from R1 bioreactor could be reinforced by the proliferation of the *Pseudomonas* genus (OTU16 and OTU20), a genus with strong self-aggregation capacity, which also promotes the granular stability (Han et al., 2022). Both *Acinetobacter* and *Pseudomonas* are fast-growing microorganisms which affect granule structure Franchi et al., (2024), therefore the absence or low abundance of both may induce granular instability, as occurred in R4. *Pseudomonas* is a genus widely described in granules cultivated in CFR regardless of configuration design of reactor (Franchi et al., 2024; Li et al., 2020a), which is described as denitrifying phosphate-accumulating organism. Contrarily, *Acinetobacter* is a genus more described in SBR operation and recently all its interesting functions for biological treatment have been recognized (Jiang et al., 2020; Liu et al., 2023a; Othman et al., 2020; Zhao et al., 2024). Consequently, biotechnological approaches for the elimination of various contaminants are focusing on the identification and enrichment of this bacteria (Chen et al., 2022; Liang et al., 2024b; Sun et al., 2020). The EPS production and granular stability abilities of *Devosia* in AGS systems have been reported by different authors (Alves et al., 2022; Rosa-Masegosa et al., 2024b). However, the presence of this genus in R4 bioreactor did not enhance the granular stability, indicating that the granular stability is not only promoted by *Devosia*, but rather by a microbial consortium that includes *Devosia* alongside other microorganisms, as it was reported in other biotechnological applications to completion metabolic pathways (Wang et al., 2023b; Wang et al., 2023c; Zhang et al., 2022b). Furthermore, the disintegration of R4 granules may not be directly linked to the presence of dominant phylotypes in granules but rather to the low hydrodynamic pressure (Zhou et al., 2021).

On the contrary, the hydrodynamic shear force of R1 design induced to that the prokaryotic community had higher stability and a faster selection in response to operational conditions, corroborating the shorter start-up period needed by this configuration. β-diversity results also corroborate the highlighted ability of R1 design to quickly select the granule-forming microorganisms. On the contrary, R2, R3 and R4 bioreactors had progressive changes in comparison to the inoculum, which indicated that

these bioreactor models did not exert a strong selection pressure in the biofilm, suggesting a long start-up process as reported Liu and Tay (2015).

Dynamics of eukaryotic community pointed out the importance of a taxon belonging to the *Ascomycota* phylum, whose lower relative abundance in R4 could be related to the granular disintegration, due to these granules were the most unstable. However, further in-depth analyses and the development of more comprehensive databases are essential for advancing our understanding of eukaryotic communities, particularly within fungal communities. The fungal kingdom, with its vast and largely unexplored taxonomic and functional diversity, represents an area of significant research potential (Nilsson et al., 2019).

In summary, data resulting from this research pointed out the ability of R1 design for achieving high remotion of fluffy flocs, rapid selection of microorganisms that promote dense and stable granules, fast start-up, greater organic matter removal performance and higher microbial diversity. These reasons and the simplicity of this design made this configuration a suitable option for treating wastewater at full scale.

To assess the capability and functioning of the novel and promising design, we studied the response of granular biomass and removal performance operating at different OLRs of domestic wastewater maintaining 6 h of HRT. This design maintained granular stability even withstanding 1.85 kg COD·m^{-3}·d^{-1}, in contrast with previous investigations under SBR operation, which reported granular disintegration at OLRs close to 2 kg COD·m^{-3}·d^{-1} (Silva et al., 2022).

The results showed a direct relation between the OLR and the granular size, consistent with findings from some authors that reported that higher OLRs favour the formation of larger granules (Cui et al., 2015; Muñoz-Palazon et al., 2023c; Tang et al., 2022). In spite of the effect of OLRs on the granular size and density, our results highlighted the ability of this novel CFR configuration to maintain granular stability when this bioreactor is treating domestic wastewater with an OLR from 0.45 to 1.85 kg COD·m^{-3}·d^{-1}. The preservation of granular stability under different OLRs was described operating SBRs, due to the facility to remove fluffy flocs and the feast-famine conditions (Iorhemen and Liu, 2021; Muñoz-Palazon et al., 2023c). However, strategies to achieve long-term granule stability in CFRs are more complicated, often requiring granule recirculation or

multiple chambers, impairing the ease of bioreactor scaling (Kent et al., 2018; Xu et al., 2022). This is the reason why this single-chamber bioreactor could be a promising alternative in real wastewater treatment, due to the success for treating a wide range of OLRs, retaining structured granules under a simple design and operation.

Moreover, this bioreactor configuration operated under several OLRs adjusted its biomass concentration (5.0, 2.6, 1.6, 0.4 $g \cdot L^{-1}$ for R1, R2, R3 and R4, respectively) to obtain high organic matter performance regardless the influent characteristics. These biomass concentrations were able to achieve more than 80% of COD removal performance for all OLRs tested, without finding statistically significant differences between bioreactors.

Nonetheless, the ammonium removal performance was not optimal for any OLR tested. Some authors have reported the reduction of nutrients removal (including ammonium) at short HRTs (Li et al., 2021; Samaei et al., 2023; Wan et al., 2013a). Therefore, a longer HRT may enhance the ammonium removal ability. Kosar et al. (2022) reported the influence of seed sludge on nutrient removal, hence this parameter could be also responsible for the lower ammonium removal performance.

From microbiological point of view, OLR modulated the microbial community present in each bioreactor. In line with other studies, the lower OLR allowed the increase of diversity of prokaryotic and eukaryotic communities, whereas at higher OLRs, microbial diversity was reduced due to the competitive advantage of only few taxa in consuming organic matter effectively, as it has been reported treating high OLR or toxics wastewaters (Hua et al., 2020; Muñoz-Palazon et al., 2023c; Szabó et al., 2017; Tang et al., 2022). In addition, faster community changes were reported at higher OLRs, possibly due by the competence of dominant phylotypes for substrate. In this study, it was observed that OLRs of 1.40 and 1.85 kg $COD \cdot m^{-3} \cdot d^{-1}$ promoted the proliferation of *Hypocreales*, *Pseudomonas*, *Spirosomaceae*, *Dysgonomonadaceae* and *Rhodocyclaceae*. The increment of the relative abundance of some of these phylotypes have been reported at higher OLRs in AGS systems (Chen et al., 2019b; Min et al., 2024). On the other hand, OLRs of 0.45 and 0.90 kg $COD \cdot m^{-3} \cdot d^{-1}$ favoured the selection of *SM1A02*, *Rhodobacteraceae*, *Sphingobacteriaceae*, *Rhizobiaceae*, the order *OPB56*, *Sericytochromatia*, and NS9 marine group. NS9 marine group has been reported in oligotrophic environments (Muñoz-Palazon et al., 2023c). The *Rhodobacteraceae* phylotype is highly related to the organic matter degradation and EPS formation,

contributing to granular stability (Hamza et al., 2018; Rajeev and Cho, 2024). In addition, it has been described in high-strength wastewater, indicating its high versatility (Hamza et al., 2018). Although, *Pseudomonas* was described ubiquitous in most of AGS as dominant phylotype, in scenarios with low organic matter this genus showed low competitiveness for the substrate in comparison with others bacterial taxa.

One of the most intriguing aspects revealed by this research is the resilience and robustness of the granules, independent of the influent characteristics. In this context, the scalability of the continuous-flow design highlights its ability to withstand daily and/or seasonal fluctuations, as the bioreactor could adapt to changes in organic matter concentration. Furthermore, these fluctuations, particularly on a daily basis, could potentially promote feast-famine cycles. Our study demonstrated the ability of the new single-chamber CFR to treat a wide range of OLRs of domestic wastewater maintaining granular stability and achieving great organic matter removal performance. This finding is particularly significant, as it emphasizes the strength and adaptability of the system, which is crucial for real-world applications where influent characteristics are subject to variation. The ability to maintain high performance despite fluctuations in OLR not only enhances the operational flexibility of the system but also underscores its potential for long-term stability and efficiency in diverse environmental conditions. This research provides valuable insights into the design and optimization of wastewater treatment systems, with implications for both full-scale operations and decentralized treatment solutions, where variability in influent quality is often a challenge.

The demonstrated resilience of the CFR design highlighted under several OLR has to be also supported by the evaluation of other key parameters for the operation of a WWTP in order to offer a sustainable and reliable solution, paving the way for more resilient and adaptive environmental technologies. For that, we assessed the response of granular biomass characteristics and macro-nutrients removal performance when the bioreactor is operated at 8, 6, 4 and 2 h of HRT, in order to evaluate the effect of selection pressure and organic load.

Our results suggested that 2 h of HRT was not optimum for the operation of the novel CFR design, due to the excess sludge produced, which impeded the appropriate functioning of the bioreactor and required continuous withdrawal. In spite of this, the four conditions tested maintained high biomass concentrations, with average values of 3.9, 4.2, 4.8 and 3.5 $g \cdot L^{-1}$ from day 70 onwards for R1, R2, R3 and R4 bioreactors. This CFR

design was able to retain even more biomass than SBRs and other CFRs configuration, disagreeing with some authors that affirm the incapacity of CFRs for biomass retaining (Kent et al., 2018; Samaei et al., 2023; Wan et al., 2014a). Furthermore, some researchers affirmed that CFR configurations operating at HRTs shorter than 3h suffered the washout of biomass (Samaei et al., 2023; Wan et al., 2014a), however, this design was able to effectively retain granular biomass even at 2 h of HRT.

Bioreactors operated at longer HRT (8 and 6 h) punctually suffered a proliferation of filamentous microorganisms, such as *Chaetothyriales* order, possibly due by the lower selection pressure exerted at those HRTs (Hou et al., 2021; Samaei et al., 2023; Wang et al., 2023a). But no overgrowth of filamentous microorganisms was recorded for the bioreactor operated at 4 hours of HRT and the biomass did not need to be withdrawn. These results pointed out that these conditions exerted satisfactory selection pressure for selecting dense granules and remove filamentous-forming microorganisms, refuting hypothesis that CFRs do not provide enough selective pressure to avoid the proliferation of filamentous microorganisms (Wang et al., 2023a).

As highlighted in this study, the statement "higher organic loads promote larger granules" was not strictly fulfilled, suggesting that other parameters such as aeration intensity and the COD/N ratio could also influence the granular size, as reported Galant et al., (2023). This is consistent with the finding in Chapter 3, where the COD/N ratio was 3.7, whereas in chapter 2 the COD/N ratio varied across the different reactors (7.0, 5.4, 3.4 and 1.7 for R1, R2, R3 and R4, respectively).

For organic matter removal, excluding the bioreactor R4 operated at 2h of HRT, the rest of bioreactors achieved values below the discharge limit stipulated by the Urban Wastewater Treatment Directive (Directive 91/271/EEC). In addition, the removal performance achieved by the novel design operated at 4 h of HRT was comparable to the removal rate achieved by more complex CFR configurations even when using longer HRTs (Berzio et al., 2023; Sun et al., 2019). This result demonstrates that the system providing an effective solution with reduced cost and space requirement, and together with the resilience observed under varying OLRs, the system is positioned as an optimal choice for upgrading existing WWTP. Bioreactors operated at 6 and 8 h of HRT reached also high removal performance in spite of the microbial succession which induced changes in structure of granules, resulting in deterioration of water quality at some intervals. The high OLR tolerated by the bioreactor operated at 2 h of HRT (2.93 kg

$COD \cdot m^{-3} \cdot d^{-1}$) resulted in continuous withdrawal of biomass, leading fluctuations to MLSS and removal performance. These conditions thus deviated to be the optimal for the operation of this bioreactor. Results derived from organic matter removal were corroborated with the carbon mass balance. At longer HRT, the organic load removed was lower, whereas at shorter HRT, the carbon load remotion was higher, even the bioreactor operated at 2 h of HRT, which suffered biomass purging. Despite elevated organic load remotion, R4 bioreactor conditions were not the most favourable for treating this influent.

In terms of nitrogen removal, the scientific consensus suggest that it would be enhanced at longer HRT, however, although the nitrogen removal performances of bioreactors operated with the longest HRTs were higher than those of the rest of bioreactors, and their average effluent concentrations were the lowest, differences with the other bioreactors were not statistically significant. Li et al., (2019), indicated that HRT of 9 h could enhance the nutrients removal, but their research need to combine SBR and CFR to be successful. Other authors pointed out excellent nutrients removal simultaneously with the need to employ four chambers and a total retention time of 12 hours (Chen et al., 2024).Following our findings, other AGS-CFR operated in a single chamber reached maximum value of 70% for total nitrogen removal (Li et al., 2020a). The obtained results corroborated the research that highlights that the feeding strategies and enrichment of group of microbial taxa are essential to enhance the nitrogen removal (da Silva et al., 2023; Wan et al., 2021), but the main studies related were carried out in SBR and fed the bioreactor in the anaerobic period (da Silva et al., 2021). For phosphorus removal, in spite of the low removal rate (averages values from 20 % to 33 %), the bioreactors operated at 8 and 6 h of HRT reached statistically significant higher phosphorus removal that the rest of bioreactors. These results showed that longer HRTs promote nutrient removal, however, that is not the unique reason, due to a more adapted inoculum may be necessary (Kosar et al., 2022; Wan et al., 2014a). In this sense, qPCR results suggested that PAO activity was not only influenced by its presence in granules, but also by other operational factors such as aerobic/anaerobic conditions and the feeding strategy (da Silva et al., 2021). The obtained phosphate removal is in the line with other research that shows the reduction of nutrients removal in CFRs (Li et al., 2016c). Likewise, denitrification and phosphate removal are not common processes using an aerobic single-chamber CFRs (Tchobanoglous et al., 2003), therefore, achieving higher

nutrient removal performance under these conditions will be a future challenge to investigate on.

From a microbiological point of view, the presence of *Brevundimonas* and *Comamonas* genera, described for its implication in P removal, suggested that they were present but not active, due to the low P removal achieved. With the objective to obtain information not only by the presence, but the metabolically active microbial community, transcriptomic analyses should be carried out (Rogers et al., 2024).

Some taxa were enhanced by conditions of R1, R2 and R3 bioreactors, such as *Proteiniphilum*, *Saccharimonadales* and *Chitinophagaceae*, whereas *Hypocreales*, *Spirosomaceae* and *Brevundimonas* were present independently of the bioreactor's conditions. The *Chitinophagaceae* family has a relevant role in granular core, contributing to granular stability (Suhonen et al., 2023). *Brevundimonas* is related to N and P removal and EPS secretion, maintaining granular structure (Pastore and Sforza, 2018; Song et al., 2022a). The bioreactor operated at 2 h of HRT experienced the greatest changes in microbial community. In terms of diversity, for both eukaryotic and prokaryotic communities, diversity suffered a reduction at shorter HRTs, possibly due by the greater selection pressure and the increase of organic load (Liu et al., 2016; Szabó et al., 2017; Tang et al., 2022).

Briefly, this single-chamber CFR was able to operate at different HRTs, being 4 HRT the most advantageous conditions, due to its to selection pressure, biomass retention and short HRT, which allow the treatment of higher volumes of wastewater in a more cost-effective mode. In terms of nutrients removal, setting up longer HRTs or selecting an optimal seed sludge may enhance it.

In summary, this novel single-chamber CFR for treating urban wastewater with AGS technology faces the current issues described for AGS-CFR systems (Samaei et al., 2023; Yan et al., 2021): need to employ an effective selection pressure; need to maintain granular stability, need to operational flexibility and automatic control system and need to avoid the granule return. This novel configuration represents an advantageable design for its implementation at full scale due to its effective selection pressure for keeping dense granules and high biomass retention within the bioreactor, even at very short HRTs and without the need of feast-famine regime. It achieves a fast start-up and is able to treat a

wide range of OLRs, maintaining long-term granular stability and achieving more than 80% of organic matter removal. Additionally, it can be operated at different HRT, being 4 h the most cost-effective in terms of removal performance and granular stability. All this in a simple design of a single chamber, without need for biomass recirculation and complex control.

This novel CFR design was patented with the objective of advancing societal progress while protecting its intellectual property. The patent validated the accomplishment of the main objective of this doctoral thesis (to develop and evaluate a functional simple CFR for the treatment of urban wastewater), which reinforce the idea of its novelty and possibilities in the area of wastewater treatment. In addition, a spin-off company from the University of Granada is exploiting the patent. It was established to further develop and research this technology, with the focus in providing water treatment by mean of this single-chamber CFR. Following the successful assessment of this novel design at lab scale and using synthetic wastewater, further research is required to better understand the performance of this CFR configuration when treating real wastewater at pilot-scale and then at full scale. Both the scaling up and the use of real wastewater will give rise to new challenges. In addition, deeper microbiological analysis should be done, due to only by knowing microorganisms, their activities and optimal conditions in depth we will be able to achieve the maximum system performance with the lowest cost. Microbial community studies are essential, such as metagenomics and metatranscriptomics analysis, considering the role played by eukaryotic community. Further investigations should be done also elucidating the enhancement of nutrient removal in a single-chamber CFR.

VI - GENERAL CONCLUSIONS

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1. This Ph.D. thesis has succeeded in building and developing a novel single-chamber continuous-flow aerobic granular sludge reactor for treating urban wastewater, which allowed the retention of biomass, long-term stability of granules, and fast start-up (11 days).
2. The innovative design developed was able to operate in a wide range of organic loading rates (0.45 to 1.85 kg $COD \cdot m^{-3} \cdot d^{-1}$) of urban wastewater and at different hydraulic retention times (2 to 8 hours), achieving an efficient organic matter, nitrogen and phosphorus removal performance, reaching values superior to 97 %, 65 % and 50 %, respectively.
3. The granular biomass generated in this continuous-flow bioreactor was mainly composed by microbial groups belonging to *Brevundimonas*, *Pseudomonas*, *Spirosomaceae*, *Chitinophagaceae* and *Hypocreales* taxa. Although prokaryotic and eukaryotic communities were complex and dependent on operational conditions.
4. The organic loading rate modulated the microbial community of granules, favouring *Pseudomonas* genus and the *Spirosomaceae* family at organic loading rates from 1.40 to 1.85 kg $COD \cdot m^{-3} \cdot d^{-1}$, whereas the *OPB56* order, the *Rhodobacteraceae* and *Rhizobiaceae* families were promoted from 0.45 to 0.90 kg $COD \cdot m^{-3} \cdot d^{-1}$.
5. Hydraulic retention times from 4 to 8 hours encouraged *Proteiniphilum*, *Chitinophagaceae* and *Saccharimonadales* taxa. At 2 hours of hydraulic retention time, the eukaryotic and prokaryotic communities suffered a depletion in terms of diversity and evenness.
6. This Ph.D. thesis stablished that the novel single-chamber continuous-flow reactor design possesses feasible characteristics to be scaled-up and therefore, implemented at full-scale for the treatment of real wastewater.

VII - CONCLUSIONES GENERALES

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1. Esta tesis doctoral ha conseguido construir y desarrollar un novedoso biorreactor de flujo continuo de una sola cámara para tratar agua residual urbana mediante la tecnología de fango aeróbico granular, que permitió la retención de biomasa, la estabilidad de los gránulos a largo plazo y una rápida puesta en marcha (11 días).
2. El novedoso diseño desarrollado fue capaz de operar en un amplio rango de carga orgánica (de 0,45 a 1,85 kg $DQO \cdot m^{-3} \cdot d^{-1}$) de agua residual urbana, y a diferentes tiempos de retención hidráulico (de 2 a 8 horas), consiguiendo un eficiente rendimiento de eliminación de materia orgánica, nitrógeno y fósforo, alcanzando valores superiores al 97 %, 65 % y 50 %, respectivamente.
3. La biomasa granular generada en este biorreactor de flujo continuo estuvo principalmente compuesta por grupos microbianos pertenecientes a los taxones *Brevundimonas*, *Pseudomonas*, *Spirosomaceae*, *Chitinophagaceae* e *Hypocreales*. Aunque las comunidades de procariotas y eucariotas fueron complejas y dependientes de las condiciones operacionales.
4. La carga orgánica moduló la comunidad microbiana de los gránulos, favoreciendo al género *Pseudomonas* y a la familia *Spirosomaceae* a cargas orgánicas desde 1,40 a 1,85 kg $DQO \cdot m^{-3} \cdot d^{-1}$, mientras el orden *OPB56*, y las familias *Rhodobacteraceae* y *Rhizobiaceae* fueron potenciadas de 0,45 a 0,90 kg $DQO \cdot m^{-3} \cdot d^{-1}$.
5. Los tiempos de retención hidráulicos de 4 a 8 horas fomentaron los taxones *Proteiniphilum*, *Chitinophagaceae* y *Saccharimonadales*. A 2 horas de tiempo de retención hidráulico, las comunidades de procariotas y eucariotas sufrieron una reducción en términos de diversidad y equidad.
6. Esta tesis doctoral estableció que el novedoso diseño de biorreactor de flujo continuo de una sola cámara posee suficientes características para ser escalado y, por lo tanto, implementado a escala real para el tratamiento de agua residual real.

VIII - REFERENCES

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IX - ANNEX

OFICINA ESPAÑOLA DE PATENTES Y MARCAS

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Título: **Biorreactor granular aeróbico**

Resumen: La presente invención describe un biorreactor y procedimiento de flujo continuo para el tratamiento de aguas y obtención de biomasa donde el biorreactor comprende un tanque (2) con una entrada (5) y una salida (6), un medio de aireación (7) y, además, un medio deflector (13) configurado para extraer agua clarificada y evitar la salida de materia granulada (11) con al menos una velocidad de decantación de 10 m/h que comprende un cuerpo principal (14) hueco con una primera apertura (15), situada en su parte inferior, configurada para permitir la entrada del fluido acuoso (9) a su interior y devolver la biomasa granular (11) al tanque (2), una segunda apertura (16), situada en su parte lateral, conectada a la salida (6) del tanque (2), y una tercera apertura (17), situada en su parte superior, configurada para permitir la salida de burbujas de aire (10).

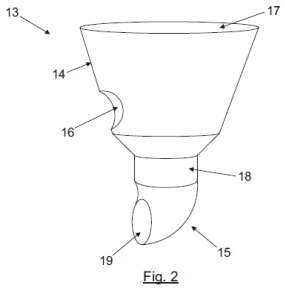


Fig. 2

DESCRIPCIÓN

Biorreactor granular aeróbico

Campo de invención

La presente invención se encuadra dentro del campo de tratamiento de agua, específicamente en los equipos de tratamiento empleados para el tratamiento de aguas residuales, tratamiento de aguas potables y de aquellos efluentes industriales biodegradables.

Estado de la técnica

La contaminación causada por la descarga de las aguas residuales al medio ambiente es uno de los principales problemas medioambientales. El agua residual contiene una variedad amplísima de compuestos químicos y biológicos que pueden afectar a los ecosistemas naturales y/o a la salud de las personas, siendo por tanto una prioridad el tratamiento de las mismas antes de su vertido. De manera general, se puede definir una composición de un agua residual urbana según la Tabla 1.

Tabla 1: Composición de un agua residual urbana estándar

Residuos	Concentración (mg/L)
Sólidos totales	700-800
- Disueltos totales / en suspensión / sedimentables	450-550 / 150-250 / 5-15
DBO_5	200-300
COT	150-170
DQO	300-600
Nitrógeno total	40-80
- Orgánico / amoniaco libre	15-30 / 25-50
Fósforo total	8-16
- Orgánico / inorgánico	3-6 / 5-10

Cloruros	40-60
Sulfato	25-35

Las tecnologías más extendidas de tratamiento de aguas residuales urbanas a nivel mundial son tecnologías basadas en reactores biológicos de fangos activos.

A lo largo de sus más de 100 años de historia estas tecnologías han sufrido no obstante numerosas modificaciones, intentando mejorar su eficiencia a nivel de eliminación de materia orgánica, fosforo y nitrógeno, al mismo tiempo que una reducción en el espacio requerido para su instalación y en los costes económicos derivados del proceso.

El sistema de depuración biológica por fangos activos, también denominado lodos activos, consiste en el desarrollo de un cultivo bacteriano en un depósito agitado, aireado y alimentado con el agua residual, que es capaz de metabolizar como nutrientes los contaminantes biológicos presentes en esa agua.

Permite la eliminación de diferentes sustancias contaminantes mediante un proceso biológico natural, realizado por diferentes grupos microbianos responsables de la depuración, para posteriormente eliminar la biomasa generada del agua mediante sedimentación.

Sin embargo, la gran generación de biomasa, en forma de lodo, y el gran gasto energético en bombeo del agua de recirculación, lo que supone cerca del 50% del total del coste del tratamiento, provoca un gran incremento en los costes de tratamiento, así como la generación de importantes cantidades de lodos que necesitan de un tratamiento adecuado y de alto coste.

Además, las mayores restricciones establecidas en cuanto a la calidad de las aguas residuales urbanas tratadas, como por ejemplo la Directiva 271/91 UE; 98/15/CE), hace necesario modificar los sistemas convencionales de fangos activos para solucionar los problemas relacionados con la eutrofización que el nitrógeno causa en áreas especialmente

Durante la última década, se ha mostrado un gran interés en el tratamiento de aguas residuales domésticas e industriales mediante la utilización de tecnologías basadas en sistemas granulares aeróbicos.

La biomasa granular aeróbica opera en condiciones con aireación, es decir, en presencia de oxígeno. Es especialmente útil para tratar altas cargas orgánicas, así como para realizar la eliminación de nitrógeno y fósforo. En la actualidad la biomasa granular aeróbica se obtiene en sistemas secuenciales aeróbicos, alimentados con agua residual, los cuales se basan en 4 etapas operacionales: aireación, decantación de la biomasa, vaciado del agua tratada y llenado con agua contaminada. Estas etapas permiten la obtención de una película de biomasa efectiva para el tratamiento del agua.

La granulación aeróbica permite una mayor retención de biomasa gracias a que existe una etapa de decantación previa al vaciado del agua tratada. Todo ello, permite realizar una selección de la biomasa con mayor capacidad de decantación, lo que impide la perdida de esta por el efluente en el proceso de vaciado. Esto provoca, una mejor capacidad de sedimentación, menores requerimientos de recirculación de fangos y mayor resistencia a cambios bruscos en el influente y a compuestos tóxicos, en comparación con el fango activo.

La biomasa granular está compuesta por una gran diversidad microbiana que es la encargada de eliminar los contaminantes existentes en las aguas residuales. Sin embargo, entre todos los microorganismos existentes (heterótrofos, desnitrificantes, nitrificantes…) hay algunos que presentan una mayor capacidad de crecimiento dentro de los sistemas de biopelículas granulares, tales como las familias *Comamonadaceae*, *Zooglea*, *Pseudomonas*, entre otras. Estos microorganismos permiten obtener una mayor compactación y estabilidad mediante la producción de Exopolisacáridos (EPS).

La estructura estratificada de la biomasa granular permite conseguir zonas aeróbicas y anaeróbicas dentro de dicha biomasa. Esto permite realizar múltiples procesos biológicos al mismo tiempo, como por ejemplo los procesos de nitrificación, desnitrificación, eliminación de fosforo y materia orgánica, los cuales se pueden realizar de manera simultánea en un solo biorreactor bajo condiciones de aireación.

En la actualidad, la granulación aeróbica es una tecnología alternativa para el tratamiento de agua, y son ya numerosas las plantas de tratamiento en Europa (fundamentalmente de aguas residuales industriales) que incorporan este sistema, en biorreactores granulares secuenciales (BGS). Los BGS son por tanto una tecnología la cual permite obtener las condiciones operacionales requeridas para la formación de los gránulos (Isanta et al., 2015; Gonzalez-Martinez et al., 2017; Liu et al., 2017; Muñoz-Palazón., 2018).

Los reactores BGS utilizan una secuenciación seriada de alimentación, aireación, sedimentación y vaciado. Dicho proceso es complicado y requiere un control separado de cada paso. La alimentación y el vaciado son especialmente problemáticos ya que deben realizarse en un corto período de tiempo, necesitando sistemas de bombeo y cambios radicales en las condiciones operacionales.

El proceso de granulación aeróbica se encuentra favorecido en los BGS, gracias al proceso de decantación previo al vaciado y al movimiento circular dentro del biorreactor, sin que ello determine pérdidas de biomasa. Si bien los últimos sistemas BGS desarrollados han tratado de simplificar la operación del sistema realizando simultáneamente las fases de rellenado y vaciado, la dificultad sigue siendo un obstáculo técnico. En cualquier caso, el tiempo de llenado y vaciado en los reactores BGS es siempre una desventaja operativa intrínseca en estos sistemas (Pronk et al., 2015).

Por ello, la utilización de reactores secuenciales estaría limitado al tratamiento de localidades que generen bajos caudales, ya que operar con múltiples BGSs en paralelo o instalar tanques de ecualización para poder manejar mayores caudales de entrada se vislumbra como una alternativa irreal.

Sin embargo, en los últimos años, ha crecido el interés en desarrollar biorreactores granulares de flujo continuo (BRFC) (Chen et al., 2017; Xin et al., 2017; Corsino et al., 2016; Qian et al, 2017).

La aplicación de esta tecnología se estima que permitiría llevar a cabo el tratamiento de efluentes con altos caudales, tales como, los que se tratan en los sistemas de depuración de aguas urbanas en grandes ciudades superiores a 20.000 habitantes equivalentes.

Al contrario de los reactores BGS, los reactores BRFCs brindan ventajas en términos de mayor control y mayor simplicidad de manejo en el proceso de depuración (Corsino et al., 2016; Qian et al., 2017). A su vez, es posible su utilización en infraestructuras previamente existentes en las Estaciones Depuradoras Convencionales de fangos activos actualmente operativas, las cuales funcionan con flujo continuo debido al gran volumen del influente tratado.

Para ello, se ha descrito el empleo de reactores con láminas de separación que definían una primera zona aireada de una segunda sin airear, el empleo de otros diseños tales como tubos concéntricos con una zona de aireación central y una zona externa sin aireación.

Sin embargo, la eliminación del periodo de decantación y la posibilidad de pérdida de biomasa por la salida constante de agua tratada por el efluente en los sistemas BRFCs actuales se traduce como un efecto negativo sobre la compactación y estabilidad de los gránulos, haciendo imposible el empleo de este tipo de sistemas para tratar una corriente de agua de manera continua a largo plazo.

De forma resumida, estos equipos presentan importantes limitaciones operacionales:

- Reactores BGS requieren trabajar con bajos caudales de operación

- Los reactores BGS requieren de una automatización elevada por su operación en modo secuencial.

- Reactores BRFCs existentes presentan elevada pérdida de biomasa

- Imposibilidad de tratamiento en continuo en un tiempo prolongado

- Falta estabilidad y compactación de los gránulos aérobicos en reactores BRFCs,

- Elevada pérdida de eficiencia de los sistemas BRFCs

Con el objeto de superar las limitaciones reportadas en el estado del arte en cuanto a biorreactores para el tratamiento del agua, se propone un nuevo equipo para dicho fin.

Descripción de la invención

La presente invención describe un biorreactor de flujo continuo para la formación de biomasa granular aeróbica y el tratamiento de aguas potables y aguas residuales, tanto urbanas como industriales.

La presente invención logra solventar los problemas de estado de la técnica en relación con el tratamiento de agua residuales en modo continuo, logrando la formación de gránulos estables a largo plazo. De este modo, a diferencia con las soluciones actuales de biorreactores en flujo continuo, la formación de estos gránulos permite el empleo de estos sistemas para el tratamiento de aguas de manera continua y estable en el tiempo.

Para conseguir dicha granulación estable, la presente invención describe una novedosa configuración de biorreactor, operable bajo unas condiciones de operación que permiten la optimización en el desarrollo de la biomasa. Dichas condiciones operacionales están recogidas en la siguiente Tabla 2.

De este modo, una aireación en el intervalo 2–15 mg O_2 / L logra crear un movimiento de la biomasa dentro de biorreactor que permite la compactación de la biomasa hasta la creación de una estructura granular, donde dicha compactación permite la realización de procesos anaeróbicos dentro del granulo. Por otro lado, los intervalos de pH (6–9) y temperatura (15–30 ºC) utilizados, son los óptimos para el crecimiento de los microorganismos que favorecen la formación granular. Finalmente, el tiempo de retención hidráulicos establecido, es el tiempo necesario para poder obtener una concentración de biomasa suficiente para poder eliminar los contaminantes requeridos en las aguas residuales, como, por ejemplo, según lo establecido en la Directiva Europea 91/271/CEE.

Tabla 2: Condiciones operacionales del reactor BRFC.

Condición operacional	Cantidad
pH	6-9
Aireación (mg O_2/L)	2-15
Tiempo de retención hidráulico	2-24
Temperatura (ºC)	5-45

A diferencia con reactores granulares secuenciales, donde la formación de la biomasa y el tratamiento del agua se lleva a cabo por medio de periodos de decantación, vaciado y llenado, la presente invención presenta una alimentación de una corriente acuosa en flujo continuo. De este modo, se obtiene una importante mejora en el sistema al no requerir la existencia de algunas de estas etapas.

En este sentido, el presente biorreactor fomenta la estabilización de contacto multicelular resultado de las fuerzas de atracción inicial, tales como fuerzas físicas (fuerzas de Van der Waals, tensión superficial, etc.), químicas y bioquímicas.

Para ello, el presente biorreactor comprende un tanque donde se acumula la corriente acuosa. Dicho tanque presenta una base con una pared en torno a la base, con una entrada de un influente preferentemente agua residual y una salida para un efluente clarificado.

Adicionalmente, el biorreactor comprende un medio aireador configurado para introducir aire en el tanque del biorreactor. De este modo, la entrada de aire presenta dos funciones. Por una parte, aporta el oxígeno necesario para el desarrollo de los microorganismos y, por otro lado, genera un movimiento físico de carácter convectivo que favorece el contacto entre las bacterias existentes en el fluido, mediante procesos hidrodinámicos de difusión por transferencia de materia y de movilidad celular.

Como consecuencia de la existencia del medio aireador, el biorreactor presenta un flujo ascendente de aire, preferentemente, en forma de burbujeo en un intervalo 0,5 y 8,5 mg O_2/L. Esta configuración es la más eficiente para poder obtener la biomasa granular por dos principales razones:

1.- Debido a que las burbujas de aire siempre tienden a subir en el agua, se logra una mayor difusión de oxígeno de la burbuja de gas al agua para poder realizar los procesos aeróbicos. De este modo, se reducen las necesidades bombeo del aire y, por tanto, el coste.

2.- La aireación ascendente, permite que la biomasa no decante en el fondo impidiendo la formación de la biomasa granular permitiendo mantener en movimiento constante la biomasa para llevar a cabo la formación de la biomasa granular.

Por tanto, sin la presencia de esta corriente ascendente, la biomasa granulada del fango tiende a descender al fondo del tanque, mientras que las partículas en suspensión se mantendrán en el seno del fluido. Al introducir una corriente fluida ascendente, dará lugar a un lecho fluidificado, donde la materia particulada presenta una fuerza ascendente que tiende a llevarlas hacia la parte superior.

Ahora bien, las partículas granuladas presentan un tamaño, preferentemente en el intervalo 1 – 40 mm de diámetro, y una configuración tal que no se ven suficientemente alteradas por la corriente de aireación y descenderán por su propio peso en contra del flujo de aireación, mientras que los flóculos, partículas suspendidas en el fluido, son desplazadas por el propio efecto de la aireación y separadas por la parte superior del tanque.

Por tanto, la presente invención permite mantener la biomasa particulada en el tanque, dando lugar al proceso de granulación, mientras que se elimina aquella biomasa sobrante que no es requerida para el proceso de granulación.

En función del flujo de aire se puede controlar el tiempo de las partículas en el interior del tanque, dando lugar a un proceso de estabilización de los gránulos de forma correcta.

Sin embargo, el proceso de granulación es un proceso progresivo, por lo que la biomasa puede presentar inicialmente un tamaño menor, donde el efecto de la aireación del tanque sí fuese relevante y la empujase a la parte superior del tanque dando lugar a la pérdida de biomasa necesaria en el tanque.

Para evitar este efecto y la consecuente pérdida de biomasa como ocurre en otras soluciones del estado de la técnica, el biorreactor comprende un medio deflector conectado a la salida del tanque del biorreactor.

Este medio deflector está configurado para limitar la salida de la biomasa granular, es decir, su mecanismo de funcionamiento es equivalente a una trampa para gránulos, donde en caso de introducirse algún gránulo en el interior del medio deflactor, éste tiende a regresar al tanque del biorreactor. Para ello, el medio deflector comprende un cuerpo principal hueco con tres aperturas.

El medio deflector comprende una primera apertura, o entrada principal, situada en su parte inferior. La situación de esta entrada desfavorece la entrada de la biomasa granular, debido a la tendencia a sedimentarse de este tipo de partículas. Es decir, la primera apertura está configurada para permitir la entrada al cuerpo principal de un flujo de agua tratada con una concentración reducida de biomasa granular. Así mismo está configurada para devolver la biomasa granular decantable al tanque del biorreactor en caso de que ésta entrase en el interior del medio deflector.

Por otro lado, la segunda apertura, o salida del medio deflector, se encuentra conectada con la salida del tanque del biorreactor. Dicha segunda apertura se encuentra situada preferentemente de manera lateral. El efluente que sale del medio deflector es, por tanto, una corriente de agua tratada, que comprende los flóculos presentes en el fluido.

Por último, la tercera apertura si encuentra en la parte superior del medio deflector. El medio deflector se sitúa sumergido en el fluido dentro del tanque del biorreactor. De manera preferente, la parte superior se encuentra a la misma altura o por encima del nivel del agua, lo que permite evitar que los gránulos puedan pasar por encima, es decir, entren en el interior del medio deflector a partir de esta tercera apertura. La función de la apertura de la parte superior es permitir la salida de aire en el caso de que alguna burbuja de aire

entre dentro del medio deflector, evitando así su acumulación, lo que dificultaría la salida del flujo de agua tratado del biorreactor. De manera preferente, esta tercera apertura ocupa la parte superior del cuerpo principal hueco de manera completa. De este modo, se evita la existencia de una superficie sobre la que se formaría una biopelícula que podría llegar a taponar la salida del medio deflector.

Por tanto, el biorreactor descrito en la presente invención comprende un medio deflector con un cuerpo principal hueco, donde dicho cuerpo principal hueco comprende

· una primera apertura, situada en la parte inferior del cuerpo principal, configurada para permitir la entrada del fluido al interior del cuerpo principal y devolver la biomasa granular decantable al tanque

· una segunda apertura, situada en la parte lateral del cuerpo principal, conectada a la salida del biorreactor aeróbico

· una tercera apertura situada en la parte superior del cuerpo principal configurada para permitir la salida de burbujas.

Así, a diferencia con otros biorreactores actuales, este medio deflector puede actuar como separador del fluido tratado de la biomasa granular. La presencia de un medio deflector como el descrito en la presente invención se traduce en que el biorreactor que lo comprende puede operar con un caudal de alimentación y salida continuo, al reducir la pérdida de biomasa en el interior del tanque, mejorando la estabilidad del biorreactor en el tiempo.

En definitiva, el deflector está configurado para la salida del biorreactor de aquella biomasa que no alcanza una decantación superior de 10 m/h, velocidad necesaria para poder considerarla como óptima para la formación de la biomasa granular.

De esta manera, bajo un caudal de alimentación continuo, se consigue mantener la biomasa granular dentro del biorreactor BRFC, al mismo tiempo que permite la salida de la biomasa sobrante. Al incrementar el tiempo de retención de la biomasa en el interior del tanque, se fomenta la maduración de la agregación celular a través de la producción de polímeros extracelulares dando lugar a una biomasa granular, con una velocidad de decantación óptima.

Todo ello, se traduce en que el biorreactor BRFC de la presente invención permite obtener las ventajas de un sistema granular como la de obtener unos rendimientos y una concentración de biomasa muy superior a los sistemas convencionales de fangos activos, pero, además, trabajando bajo una configuración de caudal continuo, en lugar de hacerlo en modo secuencial como ocurre en otros reactores granulares que se utilizan en el tratamiento de aguas residuales. Por tanto, al eliminar los flóculos, los cuales compiten por los mismos nutrientes, se favorece el crecimiento de la biomasa granular dentro del reactor BRFC, dando un enriquecimiento progresivo del fango aérobico granular en un régimen continuo.

El resultado de esta configuración es la formación de biomasa granular en la parte inferior del tanque y un efluente tratado de manera continua.

En un segundo aspecto de la invención, se puede definir un procedimiento desarrollado en el biorreactor de la presente invención que comprende las siguientes etapas:

- Entrada y acumulación de manera continua de un influente en el tanque del biorreactor, donde el tanque del biorreactor presenta un caudal de aire en el tanque del biorreactor en el intervalo 0,5 - 8,5 mg O_2/L y un tiempo de retención hidráulico durante 2 – 24 h, a un pH entre 6 y 9, y una temperatura entre 5 – 45 ºC

- Inoculación de microorganismos y formación de biomasa granular en el tanque del biorreactor

- Separación de la biomasa granular y el agua tratada en el interior del medio deflector

- Salida de un efluente clarificado de manera continua.

Así, gracias al biorreactor de la presente invención, la biomasa granulada formada presenta, preferentemente, una velocidad de decantación superior a 10 m/h y una concentración de biomasa de hasta 30 g/L, preferentemente en el intervalo 1 – 20 g/L, de manera estable en el tiempo.

Por un lado, a diferencia con las soluciones actuales, la velocidad de decantación de la biomasa formada en el biorreactor de la presente invención permite evitar la necesidad de una elevada recirculación de fangos en un decantador secundario para mantener una concentración de bacterias óptima, como sí ocurre en los sistemas convencionales de depuración. Adicionalmente, se logra una mayor compactación de la biomasa formada,

lo que se traduce en la capacidad de tener mayor número de biomasa por litro permitiendo realizar procesos aeróbicos y anaeróbicos en un mismo biorreactor.

Por otro lado, el biorreactor según la presente invención presenta la salida en la parte superior. De este modo, existe la posibilidad de poder tener biomasa granular en todo el volumen del biorreactor, lo que provoca un incremento importante de biomasa por litro. En otras palabras, gracias a la presente solución, se logra una mayor cantidad de bacterias dentro del sistema, por lo que el rendimiento en la eliminación de contaminantes es mucho mayor, superior al 95%.

Debido a la baja pérdida de biomasa que hay en el biorreactor en comparación con otros biorreactores, no se requiere decantar la biomasa en un segundo biorreactor y volver a introducirla en el biorreactor inicial, y capacita al reactor a funcionar en modo continuo durante un tiempo prolongado. Por tanto, el biorreactor de la presente invención permite reducir el volumen, con una reducción de hasta un 50% del volumen de un biorreactor equivalente, necesario mejorando las tasas de depuración obtenidas en sistemas convencionales de fangos activos. A su vez, la configuración en continuo del presente biorreactor logra incrementar en un 50% el volumen de agua tratada por unidad de tiempo en comparación con los reactores BGS. Además, la presente invención logra evitar la pérdida de biomasa de los biorreactores en continuo, lo que capacita a la presente invención a una operación estable a lo largo del tiempo.

Por tanto, desde un punto de vista económico, la presente invención mejora la sostenibilidad y competitividad del sector de tratamiento de las aguas, ya que la calidad de los efluentes se incrementa por medio de un tratamiento más eficaz y rentable logrando una disminución del gasto energético en recirculación superior al 50%. La presente invención alcanza unos rendimientos aceptables para el tratamiento de agua, logrando reducir hasta un 80% la cantidad de nitrógeno y un 90% la cantidad de materia orgánica.

Entre las ventajas de la invención respecto a otros sistemas ya existentes cabe señalar las siguientes:

- Capacidad de tratamiento de caudales grandes

- Carencia de medios de decantación previa al vaciado del biorreactor.

- Menor tamaño del biorreactor

- Mejora de la eficiencia del proceso

- Incremento de la cantidad de biomasa por volumen de biorreactor.

- Crecimiento de la biomasa granular en todo el volumen del biorreactor

- Operación estable a lo largo del tiempo

Estos resultados muestran como este sistema es una alternativa viable a las soluciones actuales de los sistemas de tratamiento de aguas.

Esto podría traducirse en un beneficio económico para las empresas pudiendo incrementar los beneficios y la liberación de recursos económicos para el fomento de la empleabilidad con la creación de nuevos puestos de trabajo con una mayor cualificación.

En las figuras, se muestran los siguientes elementos:

1. Biorreactor

2. Tanque

3. Base del tanque

4. Pared del tanque

5. Entrada del tanque

6. Salida del tanque

7. Medio de aireación

8. Difusor

9. Fluido acuoso

10. Burbujas de aire

11. Biomasa granular

12. Flóculos

13. Medio deflector

14. Cuerpo principal

15. Primera apertura

16. Segunda apertura

17. Tercera apertura

18. Saliente

19. Orificio lateral

A lo largo de la descripción y las reivindicaciones la palabra "comprende" y sus variantes no pretenden excluir otras características técnicas, componentes o pasos. Además, la palabra "comprende" incluye el caso "consiste en". Para los expertos en la materia, otros objetos, ventajas y características de la invención se desprenderán en parte de la descripción y en parte de la práctica de la invención. Los siguientes ejemplos y dibujos se proporcionan a modo de ilustración, y no se pretende que sean limitativos de la presente invención.

Además, la presente invención cubre todas las posibles combinaciones de realizaciones particulares y preferidas aquí indicadas.

Breve descripción de las figuras

La Figura 1 muestra una realización del biorreactor de flujo continuo

La Figura 2 muestra un alzado de una realización del medio deflector del biorreactor.

La Figura 3 muestra una planta de una realización del medio deflector del biorreactor.

La Figura 4 muestra una gráfica comparativa de los resultados de concentración de biomasa granular (CBG), en g/L, de un biorreactor de flujo continuo (BRFC1), de acuerdo con la presente invención, frente a otras soluciones existentes (BRFC2 y BRFC3) a lo largo del tiempo (t), en días.

Descripción detallada de la invención

La presente invención describe un biorreactor (1) de flujo continuo para el tratamiento de agua mediante la formación de biomasa granular aeróbica. El agua a tratar puede consistir en un influente de aguas potables o aguas residuales, tanto urbanas como industriales. Por tanto, para poder desarrollar el tratamiento de agua y formación de la biomasa granular en el interior del tanque del biorreactor, funcionando en flujo continuo, el biorreactor requiere una configuración específica.

De este modo, la Figura 1 muestra una realización del biorreactor (1) de flujo continuo.

El biorreactor (1) de flujo continuo según la presente invención comprende un tanque (2) configurado para tratar un fluido acuoso (9) en su interior. Dicho tanque (2) presenta una base (3) con una pared (4) dispuesta en torno a dicha base (3), con una entrada (5) de un influente, preferiblemente agua residual a tratar, y una salida (6) para un efluente clarificado, con presencia de flóculos (12) y una cantidad de biomasa granular (11) reducida.

Preferentemente, la entrada se encuentra situada en la parte más alejada del tanque (2) de la salida de modo que el influente cargado de contaminantes se homogeniza en el interior del tanque, siendo tratado antes de su salida. En una realización preferente, la entrada (5) se encuentra situada en una parte inferior, próxima a la base (3) del tanque (2), mientras que la salida (6) se encuentra dispuesta en la parte superior, lo más alejada posible de dicha entrada (5).

Adicionalmente, el biorreactor (1) comprende un medio de aireación (7) configurado para introducir burbujas de aire (10) en el interior del tanque (2) del biorreactor (1). En una realización preferente, el biorreactor (1) presenta un flujo ascendente de aire, en forma de burbujeo. Para ello, el medio de aireación (7) puede comprender un difusor (8), por ejemplo, en forma de placa porosa, que distribuya de manera homogénea las burbujas de aire (10).

Sin embargo, el proceso de granulación requiere un tiempo de retención de la biomasa en el tanque (2). Para evitar la pérdida de dicha biomasa en el tanque (2), el biorreactor (1) comprende un medio deflector (13) conectado a la salida del tanque (2) del biorreactor (1) configurado para extraer agua clarificada y evitar la salida de biomasa granulada (11) con al menos una velocidad de decantación de 10 m/h.

Como se aprecia en la Figura 1, el medio deflector (13) se dispone sumergido en la parte superior del tanque (1), preferentemente a la misma altura o por encima del nivel del agua, lo que permite evitar que la biomasa granular (11) pueda situarse por encima del medio deflector (13).

Adicionalmente, en otra realización preferente, el medio deflector se sitúa de manera alejada a la corriente de burbujas de aire (10) producido en el medio de aireación (7). De este modo, se reduce la entrada de biomasa granular (11) al interior del medio deflector

(13), logrando mantener a este tipo de biomasa en el tanque (2) por el tiempo suficiente para la formación de los gránulos.

La capacidad de mantener la biomasa en el interior del tanque (1) del biorreactor descrito en la presente solución hasta la formación de biomasa granular (11) logra evitar la pérdida de biomasa, de modo que puede ser empleado como biorreactor de flujo continuo de manera estable con el paso del tiempo, a diferencia de las soluciones conocidas en el estado de la técnica.

En este sentido, Figura 2 muestra un alzado de una realización preferente del medio deflector (13) del biorreactor (1).

Como se aprecia en esta Figura 2, el cuerpo principal (14) es hueco y comprende tres aperturas (15, 16 y 17), que pueden ser atravesadas por el fluido acuoso:

- una primera apertura (15) o entrada, situada en la parte inferior del cuerpo principal (14), configurada para permitir la entrada del fluido acuoso (9) al interior del cuerpo principal (14). Adicionalmente, está configurada para devolver la materia granulada (11) al interior del tanque (2) en caso de que ésta acceda al interior del medio deflector

- una segunda apertura (16) o salida, situada en la parte lateral del cuerpo principal (14). Esta segunda apertura (16) se encuentra conectada a la salida del tanque (2) del biorreactor (1).

- una tercera apertura, situada en la parte superior del cuerpo principal (14) configurada para permitir la salida de burbujas de aire (10).

La primera apertura (15), permite la entrada al interior del cuerpo principal (14) del medio deflector (13) por parte del fluido acuoso (9). Este fluido (9) presenta diferente material particulado incluyendo tanto biomasa granulada (11) como flóculos (12), que pueden desplazarse en el seno del fluido por efecto de la aireación. La diferencia entre este material particulado es la capacidad de decantación de la biomasa granular (11). Específicamente, se pueden señalar como biomasa granular (11) como aquellas partículas compuestas por microorganismos que presentan una velocidad de decantación de al menos 10 m/h. La situación de esta entrada en la parte inferior del medio deflector (13) se opone a la entrada de la biomasa granular (11), debido a la tendencia a sedimentarse de dicha biomasa granular (11).

Por su parte, la segunda apertura (16), se encuentra conectada con la salida (6) del tanque (2), y se sitúa preferentemente de manera lateral, y aún más preferente en una zona elevada del cuerpo principal (14) al favorecer la separación de la biomasa granular (11). De este modo, por un lado, se reduce el impacto de la aireación en la zona donde se dispone el medio deflector (13) y, adicionalmente, podría ser posible emplear infraestructuras como el depósito existente en las Estaciones Depuradoras Convencionales, los cuales proporcionan una instalación aceptable para la presente invención al funcionar con flujo continuo debido al elevado volumen del influente tratado. La estructura de estos depósitos sería modificada para la adaptación de los medios de aireación (7) o la incorporación de los medios deflectores (13), si bien el coste podría verse reducido al reutilizar las estructuras ya existentes.

Por último, la tercera apertura (17) se encuentra situada en la parte superior del cuerpo principal (14). Su principal función es permitir la salida de burbujas de aire (10) que hayan entrado en el medio deflector (13). La posibilidad de la salida de estas burbujas de aire (10), existente en el interior del medio deflector (13) supone la creación de una zona en el interior del tanque (2) donde el efecto de la aireación ascendente se ve reducido. De este modo, el volumen hueco existente en el cuerpo principal supone un espacio idóneo para la decantación de la biomasa granular (11).

En una realización preferente, se presenta un medio deflector (13) con un cuerpo principal (14) en forma troncocónica invertida. Como se mencionaba anteriormente, esta forma contribuye a que la biomasa granular (11), arrastrada por la aireación e introducida dentro del medio deflector (13), pueda tener una zona donde no haya burbujas de aire (10) y regresen al interior del tanque (2). El cuerpo principal (14) presenta un volumen tal que permite la separación de la biomasa granular (11). Adicionalmente, es preferible la devolución de dicha biomasa granular (11) al tanque (2), sin la formación de una biopelícula en el interior del medio deflector (13). De este modo, la forma troncocónica aporta un espacio central aceptable para llevar a cabo la separación de la biomasa y paredes por las que la biomasa granular (11) puede rodar hasta la primera apertura (15), cuyo diámetro es preferiblemente reducido en comparación con el cuerpo principal (14).

Por su parte, la primera apertura (15) del medio deflector (13), situada en la parte inferior, provoca que, por las propias características de decantación de la biomasa granular (11), no pueda subir hacia zonas más altas del medio deflector (13) donde se encuentra la

segunda apertura (16), y la salida (6) del tanque (2). Para mejorar esa limitación en la entrada de biomasa granular (11), la primera apertura (15) puede presentar un saliente (18) adicional, que reorienta la primera apertura (15) al comprender, el saliente (18), un orificio lateral (19) de manera paralela al flujo ascendente de las burbujas de aire (10). En una realización aún más preferente, este orificio lateral (19) se encuentra orientado hacia la pared (2) del tanque (2), reduciendo el efecto del flujo ascendente de la aireación.

Ahora bien, a pesar de la mejora que supone la configuración presente por el medio deflector (13), en el caso de que algún granulo de la biomasa granular (11) entrara dentro del medio deflector (13), el propio cuerpo principal (14) del medio deflector está configurado para permitir el retorno hacia el tanque del biorreactor de los gránulos (por gravedad), mientras que los flóculos (12), al tener una menor velocidad de decantación, no serían capaz de retornar al tanque (2) y atravesarían la segunda apertura (16) hacia la salida (6) del tanque (2).

La Figura 3 muestra una planta de una realización del medio deflector (13) del biorreactor (1). En esta Figura 3, se muestra de manera detallada como la tercera apertura (17) abarca la superficie completa de la parte superior del cuerpo principal (14). Esta configuración, además de permitir la salida de las burbujas de aire (10), facilita la capacidad de limpieza en el interior del medio deflector (13). La alternativa de presentar una cubierta parcial que permite la salida de las burbujas de aire (10) sería viable operacionalmente, e impediría la entrada de agentes externos por la parte superior del medio deflector (13). Sin embargo, la existencia de una superficie como la descrita generaría la posibilidad de acumulación de una biopelícula, que podría reducir el rendimiento del biorreactor (1).

La corriente saliente del biorreactor (1) es una corriente acuosa clarificada, que comprende los flóculos (12) presentes en el fluido, pero cuya concentración de biomasa granular (11) se ha visto minimizada. Desde una perspectiva superior de esta realización, se puede comprobar como la primera apertura (15), situada en la parte inferior, presenta un diámetro inferior al volumen presente en el interior del cuerpo principal (14) del medio deflector (13).

Esta primera apertura (15) es por donde sale la biomasa granular (11) una vez que el efecto de la aireación es suprimido.

En definitiva, el medio deflector (13) está configurado para la salida del biorreactor (1) de aquella biomasa que no alcanza una decantación superior de 10m/h que es la necesaria para poder considerarla como óptima para la formación de la biomasa granular (11).

De esta manera, bajo un caudal de alimentación continuo, se consigue mantener la biomasa granular (11) dentro del biorreactor BRFC, al mismo tiempo que permite la salida de la biomasa sobrante, como los flóculos (12), capacitando la formación de biomasa granulada a la vez que se trata una corriente de agua.

En un segundo aspecto de la invención, se descrite un procedimiento para el tratamiento de aguas. El procedimiento para el tratamiento de aguas empleado presenta las siguientes etapas

- Entrada y acumulación de manera continua de un influente en el tanque (2) del biorreactor (1),

- Inoculación de microorganismos y formación de biomasa granular (11) en el tanque (1) del biorreactor (2)

- Salida de un efluente de agua clarificada de manera continua

De este modo, de acuerdo con el procedimiento de tratamiento de agua mediante la formación de biomasa granular según la presente invención, el resultado es un efluente depurado obtenido gracias al metabolismo producido por las poblaciones microbianas existentes en la biomasa granular (11) formada en el biorreactor (1).

La alimentación del influente al tanque (2) se puede realizar mediante bombeo o por gravedad, de este modo, es posible realizar una alimentación constante en base al caudal del influente de entrada que sea necesario tratar.

La aireación en el biorreactor (1) se produce mediante el uso de medio de aireación (7) que comprende, preferentemente un difusor (8) como, por ejemplo, una placa porosa, en la base. Dicho medio de aireación (7), al localizarse en la zona inferior del biorreactor (1), permite mantener a la biomasa granular (11) en un movimiento continuo que da lugar a la formación granular. Específicamente, la cantidad de aire existente en el tanque se encuentra en un intervalo de 0,5 – 8,5 mg O_2/L. De manera preferente, el intervalo de operación es 2 – 4 mg O_2/L. Esta corriente de aire aporta el oxígeno suficiente para todos

aquellos procesos aeróbicos que se requieren para la eliminación de los contaminantes en el agua residual.

En adicción al aporte de aire, otros factores deben ser controlados para el correcto desarrollo del procedimiento de formación de la biomasa. En una realización preferente, la acumulación de la corriente acuosa se lleva a un pH entre 6 y 9, aún más preferente 6,5 – 8.

Adicionalmente, la temperatura durante el proceso de granulación se encuentra entre 5 y 45 ºC, preferentemente 15 – 25 ºC. El empleo de estas condiciones afecta al comportamiento de los microorganismos inoculados, lo que reduce el rendimiento del procedimiento de tratamiento de aguas.

Para un correcto tratamiento del agua, se requieren, además, los procesos anaerobios. La formación de la biomasa granular (11) es completamente necesaria para poder realizar dichos procesos anaeróbicos, dado que se realizan en las zonas internas de los gránulos.

Adicionalmente, la formación de biomasa granular (11) es imprescindible para poder incrementar la concentración de microorganismos dentro del sistema.

Por otro lado, gracias a la presente solución, toda aquella biomasa que se encuentre dentro del biorreactor (1) pero no se encuentre en forma de biomasa granular (11), no tendrá capacidad de multiplicarse al irse junto con el efluente por la salida, reduciendo o incluso impidiendo la proliferación de microorganismos que puedan alterar el rendimiento del sistema. En otras palabras, los microorganismos existentes en los flóculos (12), competidores con la biomasa granular (11) por los nutrientes existentes, son eliminados en la corriente saliente del biorreactor (1).

Por tanto, para desarrollarse el tratamiento de agua, debe formarse una biomasa granular (11) con las poblaciones microbianas adecuadas para poder eliminar todos los contaminantes. Entre las poblaciones microbianas empleadas para el tratamiento de aguas se pueden destacar: Bacterias Oxidadoras de Amonio (AOB), Bacterias Oxidadoras de Nitrito (NOB), Arqueas Oxidadoras de Amonio (AOA), y Bacterias Acumuladoras de Fosfato (PAO).

La retención de la biomasa es una etapa fundamental en el proceso. Esta etapa define el tratamiento del agua, así como la formación de un fango activo que comprende la biomasa

granular (11), al permitir tiempo necesario para desarrollarse la actuación de los microorganismos.

Por tanto, a diferencia con otras soluciones existente en el sector, el resultado de esta etapa es la posibilidad de acumulación de biomasa granular (11) con una velocidad de decantación superior a 10 m/h en un fango activo lo que permite el empleo de la presente solución de manera estable para la depuración del caudal continuo de agua contaminada, a lo largo del tiempo.

Ejemplo 1 - Variación de tiempos de retención

En el ejemplo 1 se muestra un ejemplo de una realización del procedimiento empleado para el tratamiento de aguas mediante la formación de biomasa granular en un biorreactor según la presente invención. Dicho biorreactor presenta un volumen de 6 L de capacidad y se alimentó con una corriente acuosa de caudal continuo. El fluido es una corriente acuosa con una composición como la mostrada en la Tabla 3.

Tabla 3. Composición del agua residual empleada para la obtención de gránulos

Compuesto	Concentración (g/L)
$CH_3COONa \cdot 3H_2O$	0,79
NH_4Cl	0,25
KCl	0,04
$MgSO_4 \cdot 7H_2O$	0,10
K_2HPO_4	0,085
KH_2PO_4	0,03

La inoculación de los microorganismos se llevó a cabo mediante la utilización de un fango activo proveniente de una Estación Depuradora de Aguas Residuales. Se empleó un pH igual a 7, una aireación de 6 mg O_2/L y una temperatura de 22ºC, dando lugar a unas condiciones óptimas para el desarrollo de los microorganismos empleados en este ejemplo.

En este sentido, en la siguiente Tabla 4 se muestran los resultados de concentración de biomasa granular (CBG), velocidad de decantación (VD), rendimiento de eliminación de materia orgánica (RMO) y rendimiento de eliminación de nitrógeno total (RNT) obtenidos en 6 muestras de utilización del sistema de la invención variando el tiempo de retención Hidráulico (TRH).

Tabla 4. Resultados según la variación de tiempos de retención

Muestra	TRH (h)	CBG (g/L)	VD (m/h)	RMO (%)	RNT (%)
Muestra 1	24	4,7	33	99	99
Muestra 2	16	4,9	33	99	96
Muestra 3	8	8,2	35	99	87
Muestra 4	6	8,1	39	97	77
Muestra 5	4	6,2	28	96	70
Muestra 6	2	5,8	20	92	61

Ejemplo 2 – Variación de temperatura

En el mismo biorreactor del ejemplo 1, con un TRH de 8 horas, se llevó a cabo la inoculación del biorreactor y se llevó a cabo la operación a una temperatura variable. De acuerdo con la Tabla 5, se aprecia como el rendimiento disminuye al descender la temperatura a niveles inferiores a 10ºC.

Tabla 5. Resultados según la variación de temperatura

	Temperatura (ºC)	RMO (%)	RNT (%)
Muestra 7	30	99	89
Muestra 8	20	93	82
Muestra 9	10	80	47
Muestra 10	5	60	26

Ejemplo 3 – Evolución con el tiempo

Tres biorreactores granulares de flujo continuo con distintos diseños (BRFC1, BRFC2 y BRFC3) se inocularon con el mismo fango activo y fueron operados bajo las mismas condiciones. En este sentido las condiciones operacionales expuestas fueron una aireación de 6 mg O_2/L, una temperatura de 22ºC, un pH de 7 y un TRH de 8h.

Los diferentes modelos de biorreactor de flujo continuo (BRFC) utilizados fueron:

- BRFC1: reactor, según la presente invención, que comprende un medio deflector (13) acoplado a la salida del biorreactor, que permitía la sedimentación de los gránulos en la zona de salida de agua;

- BRFC2: reactor que comprende una lámina que separaba en dos el biorreactor, estableciendo una primera zona de aireación y subida de los gránulos y una segunda zona de no aireación y bajada de los mismos, donde se encontraba la salida del agua; y

- BRFC 3: reactor que comprende dos zonas, interior y exterior, definidas por dos tubos concéntricos. La zona interior, está definida por el interior del tubo concéntrico de menor radio, donde se introducía la aireación haciendo subir los gránulos, que volvían a bajar en ausencia de aireación por la zona exterior, definida por el espacio anular existente entre los tubos, donde se encontraba la salida del agua.

Como se aprecia en la Figura 4, el rendimiento en el tratamiento de agua se ha mantenido estable a lo largo del tiempo (t - en días), confirmándose la mejora frente a otras soluciones donde la pérdida de la concentración de biomasa granular (CBG – g/L) es tal que no pueden ser empleados en flujo continuo y requieren la continua incorporación de nueva biomasa. En este sentido, se puede observar cómo los diseños alternativos de un biorreactor de flujo continuo, BRFC2 y BRFC3, ya desarrollados previamente, alcanzan rendimientos de biomasa muy inferiores a los alcanzados por el biorreactor BRFC1, reactor de acuerdo a la presente invención.

REIVINDICACIONES

1. Biorreactor de flujo continuo configurado para el tratamiento de aguas que comprende:

- Un tanque (2) que comprende una base (3) y una pared (4) en torno a dicha base (3), configurado para tratar un fluido acuoso (9) en su interior,

- Una entrada (5) para un influente y una salida (6) para un efluente, y

- Un medio de aireación (7) configurado para introducir burbujas de aire (10) en el tanque (2),

caracterizado por que el biorreactor comprende, además

- Un medio deflector (13) configurado para extraer agua clarificada y evitar la salida de materia granulada (11) con al menos una velocidad de decantación de 10 m/h que comprende un cuerpo principal (14) hueco, donde dicho cuerpo principal (14) hueco comprende

· una primera apertura (15), situada en la parte inferior del cuerpo principal (14), configurada para permitir la entrada del fluido acuoso (9) al interior del cuerpo principal (14) y devolver la biomasa granular (11) al tanque (2),

· una segunda apertura (16), situada en la parte lateral del cuerpo principal (14), conectada a la salida (6) del tanque (2), y

· una tercera apertura (17), situada en la parte superior del cuerpo principal (14), configurada para permitir la salida de burbujas de aire (10).

2. Biorreactor aeróbico de acuerdo con la reivindicación 1, donde el medio deflector (13) comprende una forma troncocónica.

3. Biorreactor aeróbico de acuerdo con cualquiera de las reivindicaciones 1-2, donde la tercera apertura (17) del medio deflector (13) abarca la superficie completa de la parte superior del cuerpo principal (14).

4. Biorreactor aeróbico de acuerdo con cualquiera de las reivindicaciones 1-3, donde el cuerpo principal (14) del medio deflector (13) comprende un saliente (18) conectado a través de la primera apertura (15) que comprende un orificio lateral (19) paralelo a la pared (4) del tanque (2), configurado para permitir la entrada del fluido acuoso (9) al interior del cuerpo principal (14) y devolver la biomasa granular (11) al tanque (2).

5. Biorreactor aeróbico de acuerdo con la reivindicación 4, donde el saliente (18) del cuerpo principal (14) está orientado hacia la pared (4) del tanque (2).

6. Biorreactor aeróbico de acuerdo con cualquiera de las reivindicaciones 1-5, donde el medio de aireación (7) se sitúa en la base (3) del tanque (2).

7. Biorreactor aeróbico de acuerdo con cualquiera de las reivindicaciones 1-6, donde el medio de aireación (7) comprende un difusor (8) configurado para formar burbujas de aire (10).

8. Procedimiento de tratamiento de agua mediante la formación de biomasa granular en un biorreactor (1) de acuerdo con cualquiera de las reivindicaciones 1-7, caracterizado por que comprende las siguientes etapas:

- Entrada y acumulación de manera continua de un influente en el tanque (2) del biorreactor (1), donde el tanque (2) presenta un caudal de aire en el intervalo de 2 - 15 mg O_2 / L y un tiempo de retención hidráulico durante 2 – 24 h, a un pH entre 6 y 9, y una temperatura entre 5 – 45 ºC;

- Inoculación de microorganismos y formación de biomasa granular (11) en el tanque (2) del biorreactor (1)

- Separación de la biomasa granular (11) y el agua tratada en el interior del medio deflector (13)

- Salida de un efluente de agua clarificada de manera continua.

9. Procedimiento de acuerdo con la reivindicación 8, donde la entrada de aire forma una corriente de burbujas de aire.

10. Procedimiento de acuerdo con cualquiera de las reivindicaciones 8 - 9, donde el pH se encuentra entre 6,5 y 8.

11. Procedimiento de acuerdo con cualquiera de las reivindicaciones 8 - 10, donde la entrada de aire presenta un caudal entre 2 – 4 mg O_2 / L.

12. Procedimiento de acuerdo con cualquiera de las reivindicaciones 8 - 11, donde la temperatura se encuentra entre 15 – 35 ºC.

13. Procedimiento de acuerdo con cualquiera de las reivindicaciones 8 - 12, donde la separación de la biomasa granular (11) es una etapa de decantación.

14. Biomasa granular (11) obtenida según el procedimiento de acuerdo con cualquiera de las reivindicaciones 9 – 13.

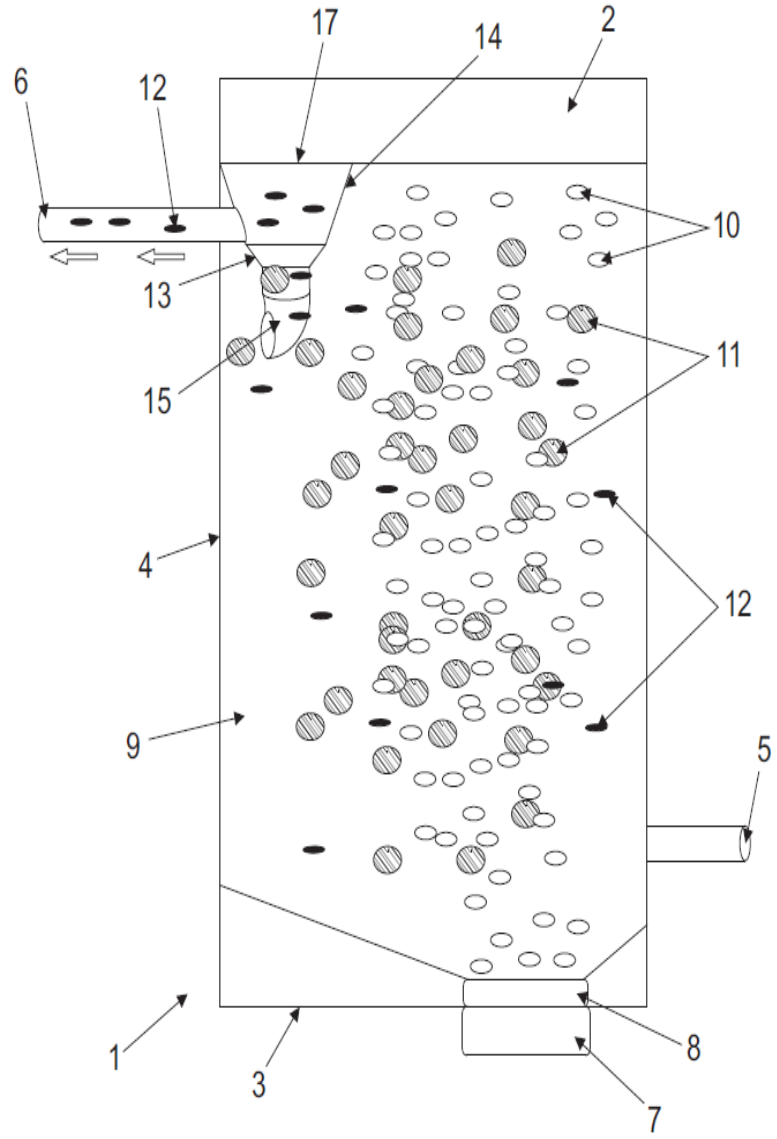


Fig. 1

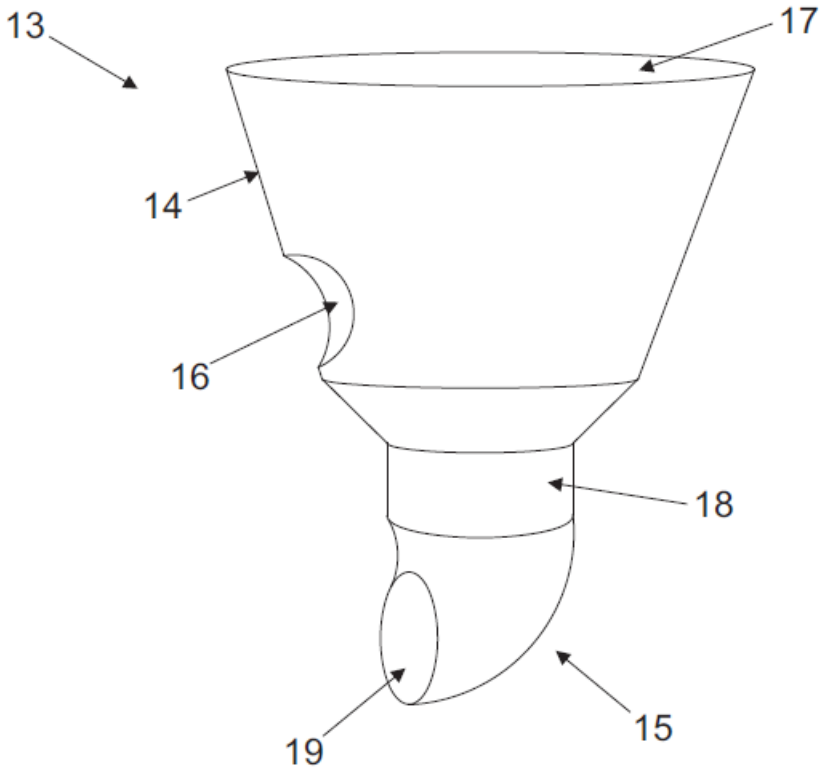


Fig. 2

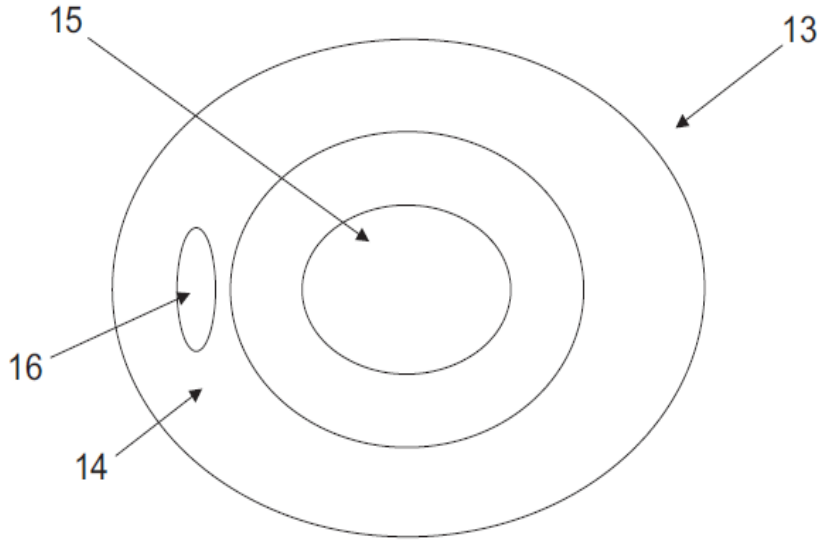
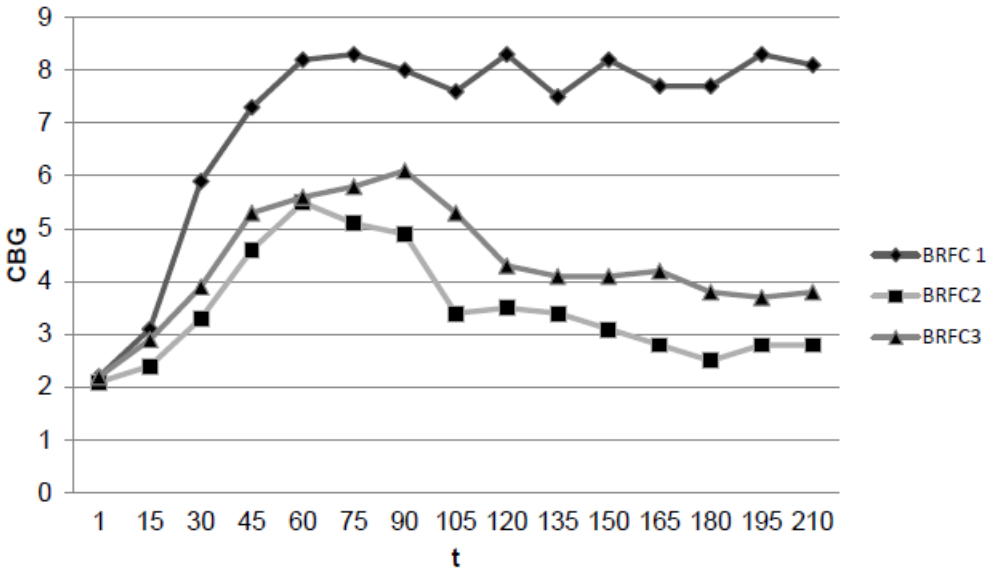


Fig. 3


9
8
7
6
5
4
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2
1
0
CBG
1 15 30 45 60 75 90 105 120 135 150 165 180 195 210
t
BRFC 1
BRFC2
BRFC3

Fig. 4