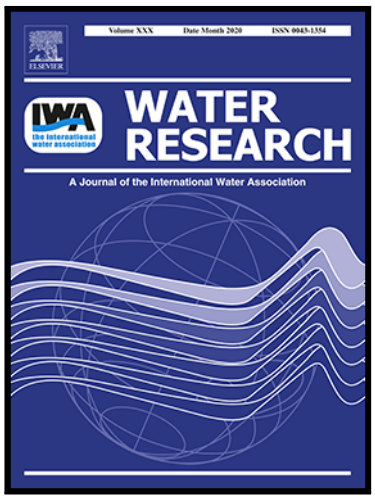

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Sequencing versus continuous granular sludge reactor for the treatment of freshwater aquaculture effluents

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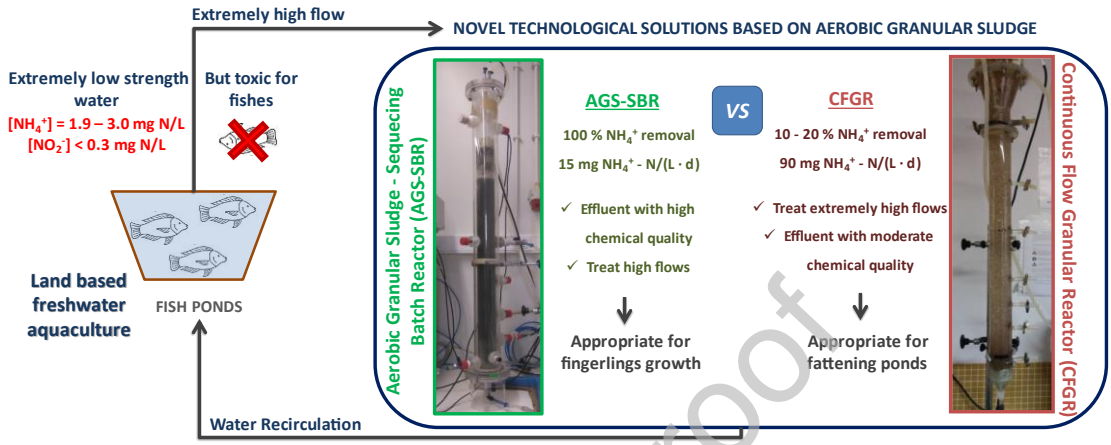
Highlights

- Granular sludge sequencing & continuous reactors cope with extremely low-strength wastewater.
- Ammonium and nitrite toxic levels for fish were avoided in both reactors effluent.
- Ammonium removal up to 100 and 20% in AGS-SBR and CFGR, respectively.
- AGS-SBR produced a high chemical quality effluent while CFGR treated 6

times higher flows.

- Biomass in CFGR granulated in 7 days at HRT of 5 min.

Graphical Abstract



Abstract

Ammonium and nitrite levels in water are crucial for fish health preservation and growth maintenance in freshwater aquaculture farms, limiting water recirculation. The aim of the present work is the evaluation and comparison of two granular sludge reactors which were operated to treat freshwater aquaculture streams at laboratory-scale: an Aerobic Granular Sludge - Sequencing Batch Reactor (AGS-SBR) and a Continuous Flow Granular Reactor (CFGR). Both units were fed with a synthetic medium mimicking an aquaculture recycling water (1.9 - 2.9 mg N/L), with low carbon content, and operational temperature varied between 17 and 25 ºC. The AGS-SBR, inoculated with mature granules from a full-scale wastewater treatment plant, achieved high carbon and ammonium removal during the 157 operational days. Even at low hydraulic retention time (HRT), varying from 474 to 237 min, ammonium removal efficiencies of approximately 87 - 100% were observed, with an ammonium removal rate of approximately 14.5 mg NH_4^+-N/(L·d). Partial biomass washout occurred due to the

extremely low carbon and nitrogen concentrations in the feeding, which could only support the growth of a small portion of bacteria, but no major changes on the reactor removal performance were observed. The CFGR was inoculated with activated sludge and operated for 98 days. Biomass granulation occurred in 7 days, improving the settling properties due to a high up-flow velocity of 11 m/h and an applied HRT of 5 min. The reactor presented mature granules after 32 days, achieving an average diameter of 1.9 mm at day 63. The CFGR ammonium removal efficiencies were of approximately 10 - 20%, with ammonium removal rates of 90.0 mg NH_4^+-N/(L·d). The main biological processes taking place in the AGS-SBR were nitrification and heterotrophic growth, while in the CFGR the ammonium removal occurred only by heterotrophic assimilation, with the reactor also presenting complete and partial denitrification, which caused nitrite production. Comparing both systems, the CFGR achieved 6 times higher ammonium removal rates than the AGS-SBR, being suitable for treating extremely high flows. On the other hand, the AGS-SBR removed almost 100% of ammonium content in the wastewater, discharging a better quality effluent, less toxic for the fish but treated lower flows.

Keywords: Freshwater aquaculture, Aerobic Granular Sludge, Sequencing batch reactor, Continuous flow reactor, Nutrient removal, Recirculation.

Abbreviations

AGS: Aerobic granular sludge

AGS-SBR: Aerobic Granular Sludge - Sequencing Batch Reactor

ALR: Ammonium Loading Rate

AOB: Ammonium Oxidizing Bacteria

ARR: Ammonium Removal Rate

CFGR: Continuous Flow Granular Reactor

CFR: Continuous Flow Reactors

COD: Chemical Oxygen Demand

DO: Dissolved Oxygen

F: Flow

GTM: Grupo Tres Mares factory

HET_{act}: Heterotrophic Activity

HPLC: High Performance Liquid Chromatography

HRT: Hydraulic Retention Time

MLSS: Mixed Liquor Suspended Solids

MLVSS: Mixed Liquor Volatile Suspended Solids

NOB: Nitrite Oxidizing Bacteria

NPR: Nitrite Production Rate

NRR: Nitrate Removal Rate

OLR: Organic Loading Rate

ORR: Organic Removal Rate

PAO: Phosphate Accumulating Organisms

RAS: Recirculating Aquaculture System(s)

RBC: Rotating Biological Contactor

SDA: Specific Denitrifying Activity

SRT: Sludge Retention Time

SVI: Sludge Volume Index

TF: Trickling Filters

TN: Total Nitrogen

TSS: Total Suspended Solids

VSS: Volatile Suspended Solids

V_{up}: Up-flow velocity

1. Introduction

Aquaculture activities have been rising in recent years due to the necessity to feed an increasing human population. From 1990 to 2012, wild fish capture increased less than 10% whereas aquaculture production increased more than five times, representing nowadays over 50% of the total fish produced (Krause et al., 2015; OECD/FAO, 2017). Also, it is predicted that in 2030 about 60% of the fish for consumption will be produced in aquaculture systems (World Bank, 2013). Inland freshwater aquaculture activities impose a high-water demand from natural nearby water streams that, in certain regions, is scarce. Competition for this resource will increase with its increasing scarcity, thus affecting the continued development of aquaculture activity. Salmonids production is one of the most important freshwater aquaculture sectors in Europe, with rainbow trout as one of the most produced species (EUROSTAT, 2018). The use of recirculating aquaculture systems (RAS) in the rainbow trout production sector reduces freshwater usage whilst maintaining fish production (Pulkkinen et al., 2019, 2018; Suhr and Pedersen, 2010). However, the application of RAS increases nutrient and organic matter concentrations in fish farms' water streams.

Nitrogen (N) compounds are major harmful water pollutants for fishes. Rainbow trout mortality increases when ammonium concentration is over 2.3 mg NH_4^+-N/L (Liao and Mayo, 1972). Nitrite is even more harmful than ammonium, with concentrations ranging from 0.14 - 0.15 mg NO_2^--N/L, causing approximately 50% of mortality on rainbow trout (Russo et al., 1974). In RAS, adequate ammonium and nitrite removal is

crucial to ensure fish health and consequently, its production. For this purpose, emerged and submerged systems are used for the development of heterotrophic and nitrifying biofilms that remove nitrogen pollutants and suspended solids (Blancheton et al., 2013; Martins et al., 2010). The most frequent water treatments used in RAS are the rotating biological contactors (RBC), trickling filters (TF) and bed filters (BF). Miller and Libey (1985) reported ammonium removal rates (ARR) between 9 - 38 and 2 - 40 mg NH_4^+-N/(L·d) in RBC and TF, respectively, that achieved ammonium removal percentages in the range 74 - 82% and 23 - 51%. The application of a fixed and a moving BF for the treatment of a rainbow trout RAS effluent achieved ARRs of 92 and 231 mg NH_4^+-N/(L d), respectively (Suhr and Pedersen, 2010). Nevertheless, when the water usage is extremely high, such as in intensive rainbow trout farms, the resulting HRT is frequently not long enough to remove the nitrogen pollutants. Thus, technologies able to achieve good removal performances at short HRT have a special interest in this sector.

Aerobic granular sludge (AGS) based technologies can be an interesting alternative to traditional biofilters used in RAS. In AGS systems, the microorganisms self-immobilize forming compact aggregates with excellent settling properties, and have proved resistance to a range of toxic compounds while keeping the main nutrient removal processes (Amorim et al., 2016; Ramos et al., 2017). AGS presents great biomass retention properties and also presents higher removal performance and lower operational costs in comparison with biological aerated filters (Di Iaconi et al., 2005). Furthermore, the AGS morphology enables that aerobic, anaerobic, and anoxic layers co-exist in granules due to different oxygen diffusion, allowing for full carbon and nutrient removal processes to take place within a single reactor (Dobbeleers et al., 2017). AGS technology requires a building area smaller than conventional activated sludge treatments, thus its application can be adequate to the needs of such farms where

the land area available is limited.

Several studies on the successful use of AGS to treat low-strength wastewater in terms of chemical oxygen demand (COD) and nitrogen (between 42 - 231 mg COD/L and 12 - 53 mg N/L) removal at laboratory scale have been reported (Awang et al., 2017; Peyong et al., 2012; Sguanci et al., 2019). Awang et al. (2017) reported that, by shortening the cycle length (reducing the HRT), the process becomes more stable due to the organic loading rate (OLR) increase. However, the nitrogen concentrations commonly found in freshwater aquaculture wastewater are much lower (0.3 - 2.0 mg NH_4^+-N/L) (Ebeling and Timmons, 2012) than those of these studies.

Up to date, the application of AGS systems for the treatment of wastewater was mostly carried out in sequencing batch reactors (SBR). Nevertheless, continuous flow reactors (CFR) have certain advantages over SBR such as an easier operation and control, and large scale high-flow treatment capacity. However, their biomass retention capacity is lower than that in SBR (Kent et al., 2018). Yang et al. (2014) cultivated AGS in an airlift CFR achieving removal performances of 30% for total nitrogen (TN) and of 75% for COD, and fast biomass granulation (25 days) using low-strength wastewater (20 - 45 mg NH_4^+-N/L and 100 - 250 mg COD/L) as inflow. Taking into account the extremely high flows of the fattening zones in freshwater aquaculture farms and the short availability of construction areas, the CFR system can be an interesting compact technology to operate at very short HRT. Moreover, We et al. (2020), when revising previous research works on the treatment of low-medium strength domestic wastewater via AGS, found that CFR are indeed an alternative to SBR, but further research on the main biological removal processes and on the granulation should be elucidated to ascertain the potential of the CFRs.

The present research work aimed to compare the performance of two different AGS based technologies (SBR and CFR) for the treatment of extremely low-strength wastewater, mimicking an aquaculture trout farm's recycling water. The challenge of the study was to accumulate enough granular sludge inside the reactors with appropriate settling properties to cope with the short hydraulic residence times imposed to treat large flows, to remove the nitrogen compounds and produce effluents suitable for recirculation in aquaculture farms. The authors hypothesized that AGS reactors will be capable to produce recycling water with enough physical-chemical quality to refill fish tanks, without affecting fish mortality and production.

2. Materials and methods

2.1 Experimental setup and seeding sludge

Two different laboratory-scale reactor configurations, namely an AGS-SBR and a CFGR were studied for the treatment of extremely low-strength freshwater aquaculture wastewater, with different operational conditions.

The AGS-SBR consisted of a column-type Plexiglas® reactor with a working volume of 2.5 L (Figure 1a). Cycles were established using automatic timers to start and stop peristaltic pumps for filling, aeration, and effluent withdrawal. Each cycle consisted of four consecutive phases: i) feeding and anaerobic reaction, during which 0.95 L of influent media was pumped into the reactor from the bottom in a plug-flow regime; ii) aerobic reaction, in which aeration was provided through the reactor bottom at 4 L/min, superficial air velocity of 84.8 m/h, controlled by a flowmeter; iii) settling and iv) effluent withdrawal. The reactor was operated at a volume exchange ratio of 40% and a corresponding HRT as shown in Table 1. The Sludge Retention Time (SRT) was not controlled over the operational period. The pH was measured online, but neither

registered nor controlled. The pH average value was 7.0 ± 0.8. Dissolved oxygen (DO) concentration was not monitored. The bioreactor was operated at room temperature (22 - 25 ºC).

[Figure 1]

The CFGR consisted of a 2 L methacrylate cylinder with a 3-phase (gas-liquid-solid) separator in the upper zone (Figure 1b). The reactor was not aerated, and the DO concentration was that of the feeding, ranging from 6.3 to 8.1 mg O_2/L. The feeding media, pumped through the reactor bottom in a continuous mode, was the mechanism to expand the biomass bed inside the reactor, favoring the mixture. The effluent was removed continuously from the reactor's top by liquid overflow. The up-flow velocity, imposed by the influent up-flow rate in the 3-phase separator, defined the settling velocity of the biomass particles that remained in the system. Temperature and pH were not controlled. Room temperature varied from 17 to 22 ºC. The pH value was imposed by the dilution water (tap water), characterized by pH ranging from 6.3 to 6.9.

The AGS-SBR was inoculated with AGS from a full-scale urban wastewater Nereda® system (Frielas, Portugal) with an initial biomass concentration of 4.8 g VSS/L. On day-98, AGS from a full-scale Nereda® system was used to re-seed the reactor, which attained a biomass concentration of 1.7 g VSS/L, and as such the adaptability of the new granules to withstand such low nutrient feeding without a prior adaptation stagewas also evaluated.

The CFGR was inoculated with activated sludge from the secondary treatment of an urban wastewater treatment plant (Silvouta; Santiago de Compostela) with an initial biomass concentration of 2.4 g VSS/L. The seeding sludge had a Sludge Volume Index (SVI) of 135 mL/g TSS. The specific denitrifying activity of the seeding sludge was 38

± 1 mg N_2-N/(g VSS·d) and the heterotrophic activity was 78 ± 10 mg COD/(g VSS·d). The biomass did not present Aerobic Oxidizing Bacteria (AOB) nor Nitrite Oxidizing Bacteria (NOB) activities.

2.2 Synthetic media

The recycling wastewater from an intensive freshwater aquaculture facility of Grupo Tres Mares (GTM) in the northwest of Spain was collected for chemical analysis. The feeding media of both reactors were prepared to mimic the composition of the water recycled in the trout farm facility (Table 1).

[Table 1]

2.2 Operational strategy

The AGS-SBR was firstly operated with 8 treatment cycles of 180 min per day, for an adaptation stageat a fixed HRT of 474 min. In this phase, the concentration of nutrients was gradually decreased to mimic the aquaculture recycling water, for adaptation of the system biomass. Afterwards, the experimental operation was split into two phases: stageI (day-0 to day-32), where the reactor was operated with 8 treatment cycles of 180 min per day, at an HRT of 474 min, and stageII (day-33 to day-157), where the reactor performed 16 treatment cycles of 90 min per day, corresponding to an HRT of 237 min, thus increasing the volume of water treated per day.

The adaptation stage was conducted with a feeding media with composition as described by De Kreuk et al. (2005). Feeding media was adjusted gradually to simulate the aquaculture station's recycling wastewater, using initial concentrations of ammonium, phosphate and COD: 3.90 mg NH_4^+-N/L, 0.18 - 1.81 mg PO_4^{3-}-P/L and

16.0 - 46.2 mg COD/L, respectively, according to equipment fluctuations. The operational cycles lasted 180 min and were distributed as: 60 min of anaerobic feeding, 112 min of aerobic reaction, 3 min of settling and 5 min of effluent withdrawal. At the end of this stage, the reactor was able to remove 61 ± 32% of COD and 99 ± 1.8%, 51 ± 37% of N as ammonium and nitrite, respectively, at the applied loads of 0.81 - 1.20 g COD/(L·d) and 0.031 g N/(L·d). During this stage, no P removal was observed, accumulation was otherwise observed throughout. Since ammonium levels are so important due to their toxicity to fish, this compound's removal percentage was taken as a reference to decide the moment of finishing the adaptation stage and starting the experimental stages. Thus, as ammonium removal was satisfactory during this period, the adaptation was ended after 58 days. Afterwards, for the experimental operation presented in the present study, on stageI, the reactor was operated with the same cycle distribution that lasted 180 min and on stageII the cycle length was shortened to 90 min (30 min of feeding, 52 min of aerobic reaction, 3 min of settling and 5 min of effluent withdrawal).

During the first 5 days of operation, the CFGR was operated in continuous mode, at an HRT of 5.2 minutes. The up-flow velocity (V_{up}) was 8.1 m/h, achieved by applying an up-flow of 285 mL/min of influent, only allowing the biomass able to aggregate in flocks and granules to remain inside the reactor. From day-5 onwards, the feeding flow was progressively increased to 385 mL/min (HRT of 8.1 min), which led to a V_{up} of 11.02 m/h. The CFGR was operated in these conditions for 98 days. The large amounts of water used to feed the CFGR made it necessary to prepare a concentrated media which was diluted with tap water in a ratio of 1:77 mL/mL. Variations in the feeding media composition were caused by the seasonal variations of the dilution tap water (Table 1). The tap water supplied contained 2.4 mg Ca^{2+}/L and 0.8 mg Mg^{2+}/L. Both

cationic species accounted for a concentration of salt of 0.19 meq/L, indicating soft water conditions. Thus, the equivalent $CaCO_3$ concentration was approximately 24.6 mg/L. Therefore, alkalinity was very low and consequently, the pH was slightly acid. Dissolved oxygen concentration depended also on the tap water composition, with concentrations close to saturation conditions throughout all the operational period (8 - 6 mg O_2/L).

2.4 Activity batch tests

To follow the CFGR biomass specific activity**,** several batch assays were performed throughout the reactor operation. The specific denitrifying activity (SDA) was determined according to the manometric method described by Buys et al. (2000), in vials of 35 mL, at 20 ºC. The concentration of substrates was 50 mg NO_3^--N/L and 225 mg COD/L (acetate) in the vials, resulting in a C/N ratio of 4.5 g/g. Liquid phase batch denitrifying assays were also conducted to follow the evolution of nitrogen compounds (nitrate and nitrite) as described by Santorio et al. (2019). The overpressure inside the vials was measured with a differential pressure transducer, 0 - 5 psi range and linearity 0.5% of full-scale, Centerpoint Electronics. Biogas composition was measured with a gas chromatograph Hewlett Packard 5890 series II.

Respirometric assays were performed to follow the specific aerobic heterotrophic activity (HET_{act}), as well as ammonium (AOB_{act}) and nitrite (NOB_{act}) oxidizing activities (López-Fiuza et al., 2002). To avoid nitrifying activity during the HET_{act} test, 0.01 mmol/L of allylthiourea was added. All batch activity tests were conducted at 20 ºC, in triplicate. The respirometric assays were carried out using a biological 152 oxygen monitor (BOM, Ysi Inc. model 5300), equipped with oxygen selective probes (YSI 5331).

2.5 Analytical methods

Throughout their operation, influent and effluent samples of the reactors were regularly withdrawn and filtered through membrane filters (0.45 mm pore-size) to remove biomass. Chemical oxygen demand (COD), total suspended solids (TSS), volatile suspended solids (VSS) and sludge volume index (SVI) were determined according to Standard Methods (APHA, 2005). The mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) concentrations were measured as TSS and VSS, respectively. The granule density was determined as the mass of the granule per granule volume, applying the blue dextran method (Beun et al., 2002).

In the AGS-SBR operation, phosphate, ammonium, nitrate and nitrite concentrations in the filtrate were determined with photometric test kits (Spectroquant®, Merck Millipore), according to the manufacturer's instructions. The AGS bed height was measured at the end of the settling period using a graduated scale, placed on the reactor's column.

In the CFGR operation, a spectrophotometric method was applied to determine ammonium concentration (Bower and Holm-Hansen, 1980). Nitrite and nitrate were determined according to the Standard Methods (APHA, 2005). Phosphate was determined by high performance liquid chromatography (HPLC) with an ion exchange column (861 Advanced Compact IC system, Metrohm, Switzerland). Total organic carbon (TOC) and inorganic carbon (IC) concentrations were determined by a Shimadzu analyzer (TOC-L, automatic sample injector Shimadzu ASI-L). The DO concentration was measured using a luminescent DO probe (LDO, Hach Lange). pH was determined with an electrode connected to a Hach Sension^{+} meter. The average diameter and size distribution of the granules were determined using a stereomicroscope

(Stemi 2000-C, Zeiss), incorporating a digital camera (Coolsnap, Roper Scientific Photometrics). The obtained images were processed using Image ProPlus® software.

2.6 Mass balances

The mass of DO consumed ($DO_{consumed}$) in the CFGR was calculated as the difference between the total COD removed ($COD_{removed}$) and the COD theoretically consumed for denitrification ($COD_{denitrification}$) (Eq. 1). The ratio of 2.85 g COD/g N-NO_3^- was chosen according to the stoichiometric expression using acetate as electron donor for denitrification (Eq. 2). The consumed nitrate was calculated as the difference between the influent and the effluent concentrations.

$$DO_{consumed} = COD_{removed} - COD_{denitrification},\ mg\ COD/L \quad \text{Eq. 1}$$

$$5\ CH_3COONa + 8\ NO_3^- + 8\ H^+ \longrightarrow 10\ CO_2 + 4\ N_2 + 9\ H_2O + 5\ NaOH \quad \text{Eq. 2}$$

The ammonium nitrogen consumed for biomass growth was estimated by a mass balance (Eq. 3). For this calculation, the theoretical biomass yield ($Y_{X/COD}$) of 0.6 g $COD_{biomass}$/ g $COD_{removed}$ was used. The ratio of 1.42 g COD/g $C_5H_7O_2N$ was used to convert COD, corresponding the biomass to the mass of the stoichiometry $C_5H_7O_2N$ that represents it (Eq. 4).

$$NH_4^+ - N = \left(COD_{removed} * Y_{X/COD}\right) \frac{g\ C_5H_7O_2N}{1.42\ g\ COD_{biomass}} \frac{14\ g\ N}{113\ g\ C_5H_7O_2N},\ g\ N \quad \text{Eq. 3}$$

$$C_5H_7O_2N + 5\ O_2 \longrightarrow 5\ CO_2 + 2\ H_2O + NH_3 \quad \text{Eq. 4}$$

3. Results and discussion

3.1 Reactors performance

Freshwater aquaculture streams are characterized by their extremely low content in carbon and nutrients and extremely high flows. In this context, a simulated freshwater aquaculture wastewater was supplied to two different granular sludge reactors, one operated in sequential mode, and the other, in continuous. The removal performance of both reactors was evaluated and is compared in the following sections.

3.1.1 Organic matter and phosphorous removal

The COD and phosphate removal during the operation of each reactor were evaluated (Figure 2).

[Figure 2]

During the phases I and II, the AGS-SBR was fed with variable influent COD concentrations between 7.3 and 70.6 mg COD/L which were mainly caused by flux variations within pumps and tubings. By the end of stageI, the reactor removed in average 52.8 ± 20.3%, achieving a maximum removal percentage of 86% of the fed carbon load. Afterwards, in stageII, which lasted for 125 days, an average removal percentage of 73.1 ± 26.3% was observed, reaching up to 100% of COD removal (Figure 2a). The organic removal rate (ORR) ranged from 44 to 334.7 mg COD/(L·d) in stageI, and 55.3 to 354.2 mg COD/(L·d) in stageII (Figure 2d). It is worth taking note that, after reinoculation, on day-98, the COD removal capacity of the reactor lowered from 88 to 62%, on average. COD is not toxic to fish but high COD concentrations in the water translate to less dissolved oxygen available for fish, which can induce hypoxia in fish, leading to several health problems and mortality.

In the CFGR, at an applied OLR of 5.3 g COD/(L·d), an overall organic carbon removal efficiency of approximately 45% was obtained, increasing up to 70% (Figure 2c). This

resulted in a competitive ORR, between 2380 - 3700 mg COD/L·d (Figure 2d), in comparison with other heterotrophic granular systems (Carrera et al., 2019), and almost ten times higher than the ORR observed for the AGS-SBR. The high heterotrophic activity of the biomass was confirmed by respirometric assays. In fact, it increased from 78 ± 10 mg COD/(g VSS·d) in the inoculum, to 999 ± 27 mg COD/(g VSS·d) after 44 days of operation. The heterotrophic activity of the biomass within the reactor continued to increase up to 1749 ± 116 mg COD/(g VSS·d) on day-78.

Despite the DO concentration being close to saturation in the feeding media (8.1 - 9.6 mg O_2/L), mass balances showed that all of it was consumed to oxidize the organic matter. The COD consumed ($COD_{removed}$) was approximately 8 - 10 mg COD/L (Figure 2c) and the COD necessary for denitrification ($COD_{denitrification}$), taking into account the nitrate consumed (Figure 4b), was 0.28 - 0.42 mg COD/L. Thus, the difference between $COD_{removed}$ and $COD_{denitrification}$ matches with the influent dissolved oxygen concentration available for the aerobic processes.

The phosphorus removal was monitored regularly in the AGS-SBR as the imposed operational conditions in the CFGR were not established to remove this compound. In the AGS-SBR, the influent phosphate concentrations varied from 0.12 - 0.89 mg PO_4^{3-}-P/L throughout the operational time (Table 1). Effluent concentrations varied from 0.26 to 0.66 mg PO_4^{3-}-P/L in stage I, demonstrating low levels of removal on some days, up to 20.5%, although P accumulation was observed over most of the operational period. During stageII, phosphorus removal efficiency seemed to improve, with higher mean removal values, from 4.1 ± 7.8% in stageI, to 46.8 ± 30.0%, and up to 100%, in stage II. Additionally, during stage II, after the reinoculation, removal efficiency lowered from 56,0 ± 21.1 to 40.5 ± 32.7%. Thus, phosphate removal efficiency in stage II varied in

the range of 40 - 100% (Figure 2 b).

In the CFGR**,** phosphate concentration in the influent was 0.8 mg PO_4^{3-}-P/L, and in the effluent it remained under the detection limit of the analytical method on several operational days analyzed. From the concentrations in the effluent it was determined that approximately 12% of the phosphorus in the feeding was removed. All phosphate consumed was probably necessary for biomass growth, taking into account the COD removal observed, and considering $C_5H_7O_2NP_{0.15}$ as the biomass elemental composition. The better phosphate removal performance in AGS-SBR can be attributed to the cycle distribution that comprised an anaerobic feeding period and a subsequent aerobic period. In this system, the phosphate can be assimilated and removed by phosphate accumulating organisms (PAO). The continuous aerobic feeding in CFGR did not promote PAO proliferation, and consequently, phosphorus was consumed only due to heterotrophic cellular growth.

Although the organic matter and phosphate concentrations measured in the trout factory (21 mg COD/L and 0.8 mg PO_4^{3-}/L) are not toxic to fish, in a recycling system these concentrations will presumably tend to increase, causing problems such as oxygen depletion and fish mortality. Thus, the COD and phosphorus removal achieved by both reactors could avoid the increase of concentration of these pollutants, allowing for water recycling.

3.1.2 Nitrogen removal and transformation

The AGS-SBR and the CFGR performed stable, in terms of nitrogen removal/transformation efficiencies during 157 and 98 days, respectively, fed with aquaculture-like synthetic media (Figure 3a and 3b).

[Figure 3]

In the AGS-SBR, ammonium removal was, on average, 98.5 ± 3.3% during stage I, slightly increasing to 98.7 ± 3.5% in stage II, achieving up to 100 % in both phases. Within stage II, before reinoculation, the removal efficiency was reduced to 97.4 ± 4.9, especially in the last 14 days. Nevertheless, the reactor recovered its ammonium removal capacity 99.8 ± 0.3% after reinoculation. In both stages, the ammonium effluent concentrations, 0.00 – 0.25 mg N/L, were below the toxicity limit for the fish. The NRR was on average of 14.5 ± 0.5 mg N/(L·d) (Figure 3c).

In the CFGR, the ammonium concentration decreased by approximately 0.3 mg NH_4^+-N/L, from the feeding media to the effluent. Overall, the ammonium removal efficiency was low, approximately 10%, achieving values up to 20% (Figure 3b). Nevertheless, taking into account the high applied ammonium loading rate (ALR) of 690 - 800 mg NH_4^+-N/(L·d), associated with the extremely short HRT, this removal percentage led to average ARR values of 90 mg NH_4^+-N/(L·d), reaching values of up to 180 mg NH_4^+-N/(L·d) (Figure 3c). Mass balances indicate that the carbon consumed was enough to assimilate all the ammonium removed. Thus, all ammonium consumption during CFGR operation seemed to serve for heterotrophic growth.

Regarding nitrate and nitrite content, the behavior observed in each reactor was different (Figure 4a and 4b). In the AGS-SBR, complete nitrification with nitrate accumulation was observed throughout both phases. During stage I, effluent concentration of nitrite was 0.016 ± 0.007 mg NO_2^--N/L, which decreased to half (0.008 ± 0.017 mg NO_2^--N/L) during stageII, indicating that shortening the cycle length, and thus the HRT, might have contributed to the nitrification improvement. On the other hand, nitrate concentration in the effluent increased to approximately 0.5 - 1.0 mg NO_3^--N/L from day-1 to day-98 (Figure 4a). It gradually decreased from day-98 onwards

(after reinoculation), indicating the possible occurrence of denitrification in the inner layers of the granules. In this period, nitrate concentrations in the effluent were negligible and nitrite concentration was always below toxic levels for rainbow trout (0.14 mg NO_2^--N/L). Probably this is a result of the addition of new granules, some of which with large diameters, allowing for denitrification.

As ammonium consumption remained constant (Figure 3a) and was over the nitrate production, this indicates that part of the ammonium was consumed for heterotrophic growth as nitrite was not produced.

In the CFGR, nitrate concentration in the effluent decreased slightly along the whole operation, with removal percentages between 3 - 25% (Figure 4b). During the first 48 days, the NRR ranged from 15 to 100 mg NO_3^--N/(L·d), decreasing thereafter to 3 - 30 mg NO_3^--N/(L·d) (Figure 4c). Thus, denitrification took place in the CFGR due to the low DO concentration achieved inside the reactor. Besides, this result matches the C/NO_3^--N ratio of 4 mg/mg that allows denitrification activity (Buys et al., 2000). Although nitrite was absent from the feeding media, it was detected in the effluent (0.03 - 0.10 mg N/L) (Figure 4b), although it always remained below the toxic level for rainbow trout. The nitrite production rate (NPR) was also higher during the first 48 operational days, producing up to 26 mg NO_2^--N/(L·d). From that moment on, the NPR decreased to 3 mg NO_2^--N/(L·d). Therefore, once the granules matured, the nitrite production decreased to below 0.05 mg NO_2^--N/L. This low concentration of nitrite which was formed could be related to a combination of low oxygen diffusion in mature granules, facilitating complete denitrification instead of partial denitrification, and low DO concentration to achieve complete ammonium oxidation.

[Figure 4]

Throughout the reactor operation, the ARR and Nitrate Removal Rate (NRR) (Figure 4c) was higher than the Nitrite Production Rate (NPR), indicating that each process separately could account for the nitrite production. To identify which of the processes was responsible for the NPR, batch activity experiments to determine AOB, NOB and denitrifying activities were carried out. The AOB and NOB activities of the CFGR biomass were measured on operational days 44 and 76 by respirometric tests. In both cases, no AOB nor NOB activities were detected, showing that the CFGR biomass was not able to oxidize neither ammonium to nitrite nor nitrite to nitrate. These results reinforce the hypothesis that ammonium consumption in the CFGR only served for biomass growth. Although the sludge used as inoculum performed the nitrification-denitrification processes in the urban wastewater treatment plant, its nitrifying activity was presumably not enough to be detected by the respirometric assays. Thus, the low AOB and NOB activities during the operational period can be related to this fact, combined with a competition for the dissolved oxygen with the heterotrophic bacteria that use it for organic matter oxidation.

Since AOB activity was not present, the nitrite production could be associated with denitratation. A denitrifying liquid phase test was conducted on day-75 showing a high specific denitrifying activity (SDA) in terms of nitrate consumption of 980 ± 53 mg NO_3^--N/(g VSS·d) (Figure 5). During the test, nitrite transient accumulation occurred, with a specific production rate of 451 ± 16 mg NO_2^--N/(g VSS·d). Therefore, the biomass was enriched preferentially in microorganisms able to reduce nitrate to nitrite. Considering this nitrite production capacity of the biomass, all the nitrate could be reduced to nitrite inside the reactor. Nevertheless, the reactor denitratation activity was lower due to the high DO concentration.

[Figure 5]

The high DO concentrations in the influent should promote the development of AOB and NOB microorganisms. However, the absence of these processes indicated that all the oxygen was consumed by the heterotrophic bacteria, avoiding nitrification. The high SDA of the biomass shows that the promotion of denitrifying activity by decreasing the DO influent concentration could improve the nitrate removal.

Influent concentrations, and consequent COD and nutrient removal variability, were observed and mainly attributed to perturbations in the plug-flow feeding system. At the high flows programmed, different pressures were applied to the tubings, forming preferential channels, inducing fluctuations in the volumes and thus concentrations of the mixture of media and water.

3.2 Biomass properties

In the AGS-SBR, mature granules collected from a full-scale reactor were used as inoculum, whereas in CFGR the formation of granules from flocculant activated sludge, using extremely low-strength wastewater and an extremely short HRT, was evaluated.

In AGS-SBR, during stageI, the biomass bed height inside the reactor progressively decreased from 17.0 to 10.5 cm, accompanied by a concomitant increase of the TSS content in the effluent (Figure 6), probably due to the biomass gradual washout attributed to biomass starvation, and caused by the low influent organic matter and nutrient concentrations. Even with the HRT reduction in stageII, by shortening the cycle length to apply 16 treatment cycles per day, the biomass bed height continued to decrease. Some authors reported that shortening the length of the operational cycles improves the performance of AGS for the treatment of low-strength wastewater (de Kreuk and van Loosdrecht, 2006; Liu et al., 2007). As in stage II the applied OLR and NLR doubled, it was expected that the biomass starvation would diminish, avoiding

biomass washout. Nevertheless, the granular biomass has difficulties adapting to the aquaculture conditions. Even after the reinoculation with mature granules on day-98, the biomass bed height started to decrease from 15 to 4 cm until day-123. After this day onwards, the biomass bed height remained stable till the end of the operation (day-157), indicating that the biomass concentration was almost constant during this period, and may have adapted to these conditions. The extremely low carbon and nitrogen content present in the feeding exerted a metabolic selective pressure, probably only supporting the viability of a small portion of biomass, leading to a reduction in the biomass concentration inside the reactor (Figure 6), along with partial disaggregation of the granules. Nevertheless, the reactor removal performance remained stable over the operation, even after changing the HRT. Moreover, with the biomass reinoculation on day-98 (stage II), ammonium removal increased up to 100%, remaining stable thereafter. Although the biomass bed height reduction again occurred, that did not affect the processes' removal efficiencies.

Since the new granules faced aquaculture conditions properly, to start-up an AGS reactor to treat aquaculture wastewater without a previous adaptation period of the inoculated granular sludge seems feasible. The maintenance of biomass properties, ammonium and organic matter removal performance, after applying low-strength wastewater to mature granules cultivated at high-strength conditions, was already reported by other authors (Liu et al., 2007; Peyong et al., 2012).

[Figure 6]

The appearance of the AGS granules was followed by visual observation throughout the reactor's operation. Since the main focus of the AGS-SBR's study was performance, granules' appearance and size were not registered. Nevertheless, at seeding, granules

appeared smooth, round, large (circa 2 - 4 mm) and dark brown/grey, and throughout operation gradually turned light brown to yellowish, small (≤2 mm), with irregular shapes, dense, and with fast settleability (less than 5 min, which was the settling time in a sequencing cycle). Similar granules have been reported in other studies on low to medium-strength wastewater, which have described smaller granules than those obtained in high-strength wastewaters (Ni et al., 2009; We et al., 2020).

In the CFGR, the granulation process took place by applying high liquid up-flow velocities (V_{up}) to the reactor, inoculated with activated sludge. The V_{up} was increased gradually from 8.1 to 11.0 m/h during the first four days of operation. Consequently, the sludge bed expanded favoring substrate mass transfer while the flocks with less density were washed out in the effluent. As a result, the biomass aggregated forming granules that started to appear on day-7, showing that this strategy promoted a fast granulation. The initial granules were almost translucid with a jelly-like appearance (Figure 7a). Afterwards, the development of new biomass in the granule body, as a dense core, was observed on day-32 (Figure 7). Since the reactor was not mechanically aerated, this result showed that the hydraulic forces (the high up-flow velocity imposed by a short HRT and the reactor's design) were crucial to promote the rapid biomass aggregation. This result proved that it is possible to obtain mature granules facing extremely low-strength concentrations with this configuration.

[Figure 7]

When mature granules were accumulated inside the CFGR, their particle diameter and density were measured. The granule average diameter was 1.9 mm, with a density of 7.12 g VSS/$L_{granule}$. Moreover, the measured SVI_{30} was 312 mL/g TSS on day-78, which is below the SVI_{30} of the seeding sludge and matches the low-density values of the granules. Additionally, SVI_1 was 321 mL/g TSS which means a ratio of 0.97

SVI_{30}/SVI_1, indicating a fast settling velocity. Only a few studies performed the treatment of low-strength wastewater with AGS in a continuous flow reactor at laboratory scale (Yang et al., 2014; Zhou et al., 2012). However, they did not treat such extremely low pollutant concentrations as the present study (approximately 20 - 70 mg N/L and 50 - 250 mg COD/L). The fast granulation achieved in the CFGR fits the granulation times reported in those studies (20 - 25 days). However, the granule diameter achieved by these authors was between 0.6 - 0.9 mm, and in the case of CFGR, particle size was larger. Besides, these studies reported low SVI_{30} of approximately 38 - 40 mL/g TSS (typical of AGS reactors) when external aeration was supplied to the system. In fact, Yang et al. (2014) reported a high SVI (225 mL/g TSS) when the oxygen availability was limited in certain operational periods. Therefore, the lack of dissolved oxygen can be the cause for the particular structure of the granules in the continuous flow reactor, which involves a high SVI. Nevertheless, despite the high SVI value, the settling properties of the granules were good enough to keep the biomass inside the CFGR even with the extremely high V_{up} applied.

3.3 AGS continuous versus sequential operation

Nitrogen species present in the water are especially harmful to fish, with ammonium and nitrite and their unionized forms the most dangerous compounds. Ammonium removal or transformation into less harmful compounds is mandatory to allow water recirculation inside a trout farm. In the present study, the operation of both granular reactors (sequential and continuous) was evaluated using, as feed, a synthetic medium with a composition mimicking the recirculating aquaculture water. The performances were different due to the different origin of the seeding sludge used in each reactor and the operational strategies imposed.

While the ammonium removal was close to 100% in the AGS-SBR, only 10 - 20% of removal was achieved in the CFGR. However, the ARR was 90.0 mg NH_4^+-N/(L·d) in the CFGR compared to the 14.5 mg NH_4^+-N/(L·d) in the AGS-SBR. These differences were mainly caused by the feeding strategy adopted and consequently the HRT. The shorter HRT of the CFGR did not allow for the development of nitrifying bacteria and the ammonium was consumed only by cellular assimilation. However, nitrification occurred in AGS-SBR. After reinoculation, denitrification also occured. Phosphorous removal followed a similar behavior as ammonium. In CFGR only 6 - 12% was consumed, mostly due to cellular growth, while in the AGS-SBR the phosphate removal was 40 - 100%, attributed to PAO activity.

Comparing the ammonium removal performances achieved in CFGR and AGS-SBR (10 - 20% in front of 100%), the air insufflation in CFGR could improve nitrification since oxygen is limited and is mostly consumed by heterotrophic bacteria. Nevertheless, it is important to highlight that the major CFGR operational cost is the feed pumping which is already necessary for aquaculture farms to maintain the water circulation of the RAS. Thus, the operational cost related to aeration would increase due to electric consumption. Besides, the aeration could reduce biomass retention performance operating at this extremely low HRT, even with the 3-phase separator.

Rotating biological contactors (RBC), trickling filters (TF), and bed filters are frequently used in RAS. Suhr and Pedersen (2010) studied a fixed bed biofilter and a moving bed biofilter, treating the effluent of a rainbow trout RAS, and reported ARRs of 92 and 231 mg NH_4^+-N/(L·d), respectively. Miller and Libey (1985) reported ARR between 9 - 38 and 2 - 40 mg NH_4^+-N/(L·d) in RBC and TF, respectively. The ammonium removal percentages in RBC were in the range of 74 - 82%, and 23 - 51% in

TF. Thus, the AGS-SBR achieved removal rates higher than those reported for TF and RBC, while CFGR achieved similar values to those of a fixed bed biofilter. Even though the ARR of the moving bed biofilter reported by Suhr and Pedersen (2010) was significantly higher than that of the CFGR, the HRT was also longer (20 - 85 min). As the moving bed biofilter treated smaller water flows, it will require a larger implantation area than the CFGR. In addition, among all the referred treatment technologies, the AGS-SBR provided the highest effluent chemical quality.

Apart from saving freshwater capture, the implementation of RAS in trout aquaculture farms is important in Mediterranean countries, which suffer from water shortage, especially in the summer period. The dry season lasts 3 - 4 months, and in this period the available volume of water in the nearby freshwater sources (rivers or lakes) diminishes, limiting the fish production of the plants. Thus, this is the period when trout farms in such conditions recycle the water. Therefore, operation lengths of 98 and 157 days, in the case of the CFGR and the AGS-SBR, respectively, could be long enough to understand if reactors performances fulfill the fish plant necessities. Both systems demonstrated a fast biomass adaptation to the imposed operational conditions. The CFGR produced granules from activated sludge in only 7 days. In AGS-SBR, after a reinoculation (day-98), the biomass concentration took approximately 3 weeks to stabilize. Some biomass loss occurred due to the extremely low nutrient concentrations, without functional performance compromise of the remaining. Nitrite concentration is a crucial issue in trout farming, and although the CFGR produced an effluent with a higher nitrite concentration than the AGS-SBR, it was mostly below the toxic limit for the fishes. Therefore, the AGS-SBR produced an effluent with good physico-chemical quality, adequate for recirculation in the trout tanks, while the CFGR was able to treat larger flows, which is also essential in freshwater aquaculture farming.

Overall, both reactors configurations could be suitable for the treatment of extremely low-strength freshwater aquaculture streams aiming at their recycling. The AGS-SBR provides a discharge stream with ammonium and nitrite concentrations close to zero. This high-quality effluent is necessary in the areas of the trout plants where the spawn and the first stages of the fish growth (fingerlings) take place (Thurston et al., 1981). It is important to highlight that the water flow demand for this part of the farms is much lower than for the fattening tanks. Moreover, the adult trouts of the fattening tanks are more resistant to nitrogen pollutants than fingerlings. Thus, the CFGR could better suit the larger water flow necessities of this area of the farm. The CFGR can recycle a water flow 35 times higher, with an ARR 6 - 12 times higher than the capacity of AGS-SBR. Besides, for most of the operational period of the CFGR, the produced effluent's nitrite concentrations were below the levels which are toxic to fish. Despite the lower removal capacity, the ammonium concentration was reduced around 0.2 - 0.3 mg N/L in the effluent. Tahar et al. (2018) monitored the ammonium concentration profile of a flow-through rainbow trout farm, showing an average increase of 0.13 mg N/L between the inlet and the outlet. Therefore, the estimated ammonium production of a trout farm could be removed by the CFGR, avoiding ammonium concentration increase in the recycled water. Thus, while the AGS-SBR could provide high quality effluent without ammonium and nitrite content to face the fingerling area requirements, the CFGR could fulfill the extremely high water flow necessities of the fattening area with suficient quality to ensure trout health. Consequently, both configurations could be suitable for different areas of freshwater aquaculture closed farms.

4. Conclusions

Adaptation of the mature granular biomass in the AGS-SBR was not required to

accomplish the removal of carbon and nitrogen compounds from the aquaculture mimicked effluent. The formation of granular biomass was feasible in 7 days by applying large liquid up-flow velocities to a CFGR.

The achieved nitrogen removal efficiencies in both granular systems depended on the HRT and NLR applied. In the AGS-SBR, the main processes taking place were nitrification and heterotrophic growth. Ammonium removal was near to 100%, resulting in an ammonium removal rate of 14.5 mg NH_4^+-N/(L·d) for AGS-SBR. In the CFGR, the processes occuring preferentially were heterotrophic growth and denitratation. In this bioreactor unit, 10 - 20% of the ammonium was consumed for biomass growth, reaching loads of 90.0 mg NH_4^+-N/(L·d).

At laboratory scale, both granular biomass systems (sequential and continuous) produced effluents with nitrogen concentrations below the toxic levels for fish. In both cases, the chemical quality of the produced water is appropriate as recycling flow in the aquaculture farm. The AGS-SBR produced an effluent with negligible nitrogen compounds concentrations, which could be suitable for recycling in the fingerling area, as fingerlings are extremely sensitive to nitrogen forms. The CFGR produced an effluent with moderate chemical quality but compatible with recycling water quality needed for the fish fattening area, and was able to treat larger flows. Besides, it achieved a fast granulation (7 days), showing a rapid adaptation to freshwater aquaculture conditions. Nevertheless, pilot-scale tests in a real freshwater aquaculture environment will help to ascertain the stability and viability of both granular reactors in treating these water streams.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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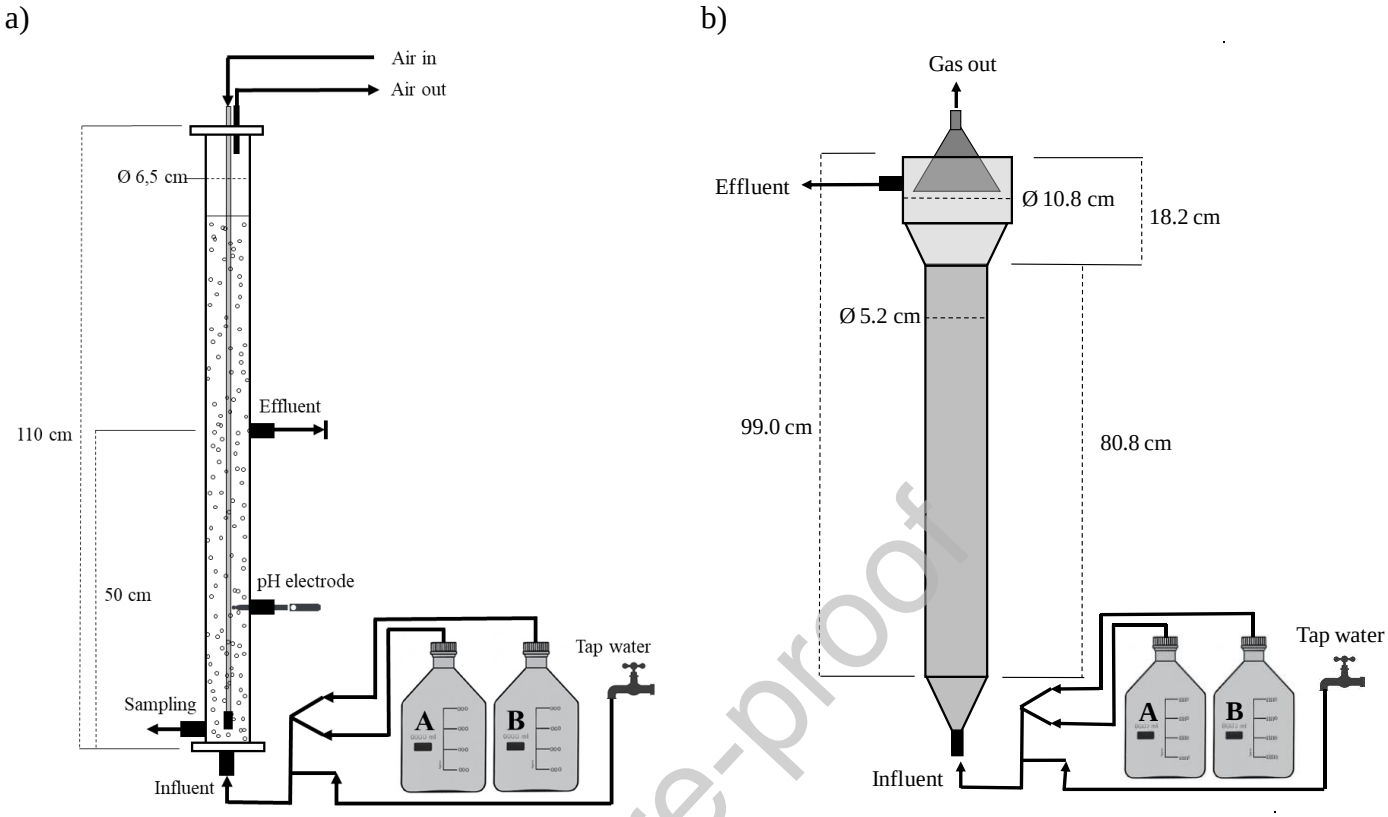


Figure 1. Diagrams of both reactors: a) AGS-SBR; b) CFGR.

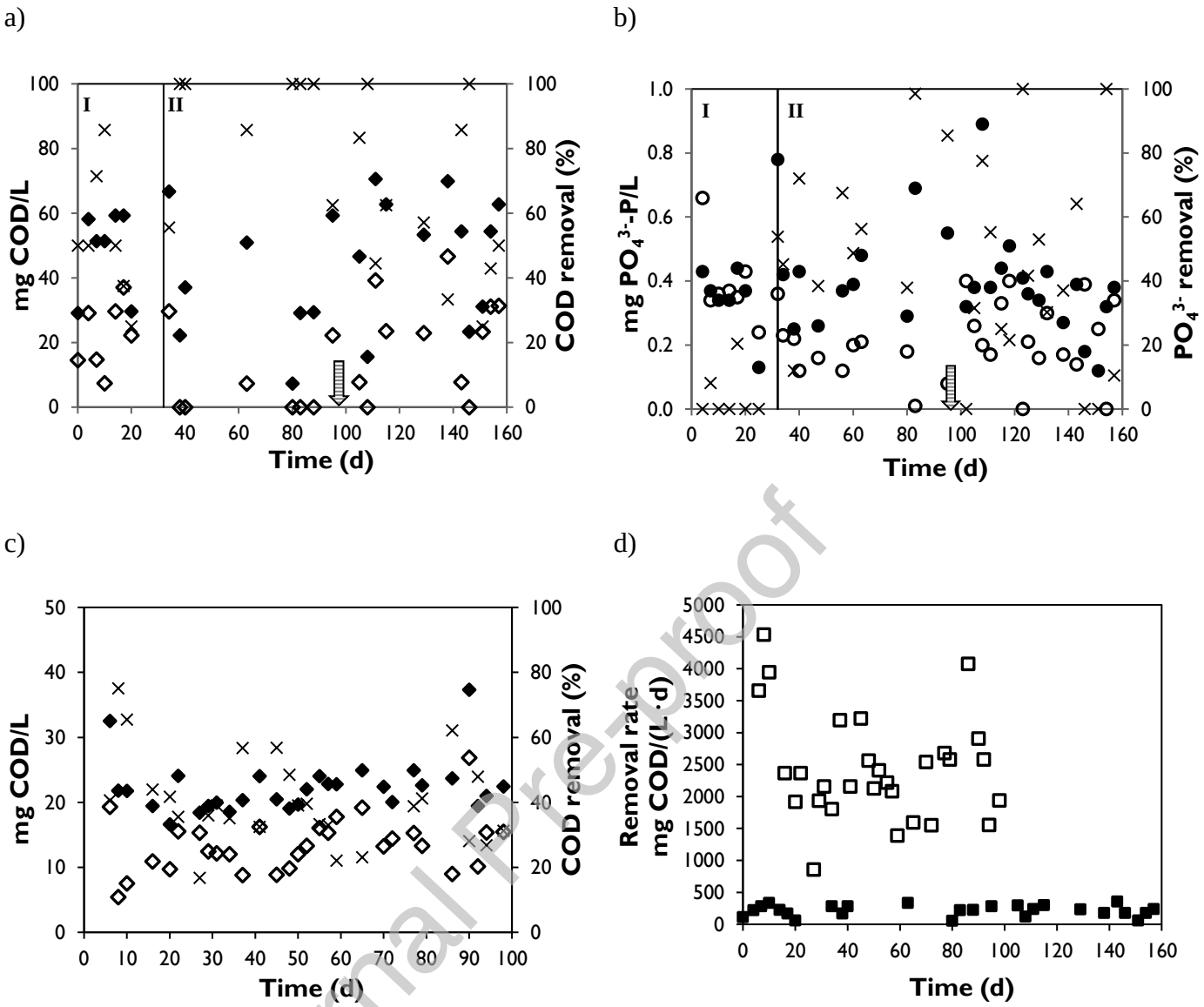
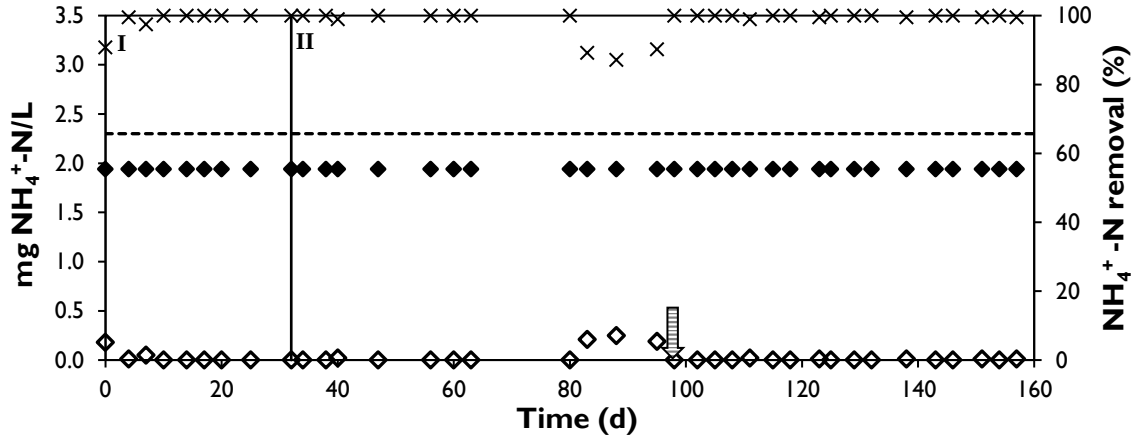


Figure 2. Organic matter and phosphorous removal profile throughout reactors' operation. COD influent (♦) and effluent (◊) concentrations, and removal percentage (x) of AGS-SBR (a) and CFGR (c); b) phosphorus removal performance of AGS-SBR: influent (●) and effluent (○) concentrations, and removal percentage (x); d) organic removal rate (ORR) of AGS-SBR (■) and CFGR (□); and. In a), b) and d), the black vertical line divided stageI and stageII, in which the HRT was reduced to half, and the vertical arrow represents the biomass reinoculation in the AGS-SBR.

a)



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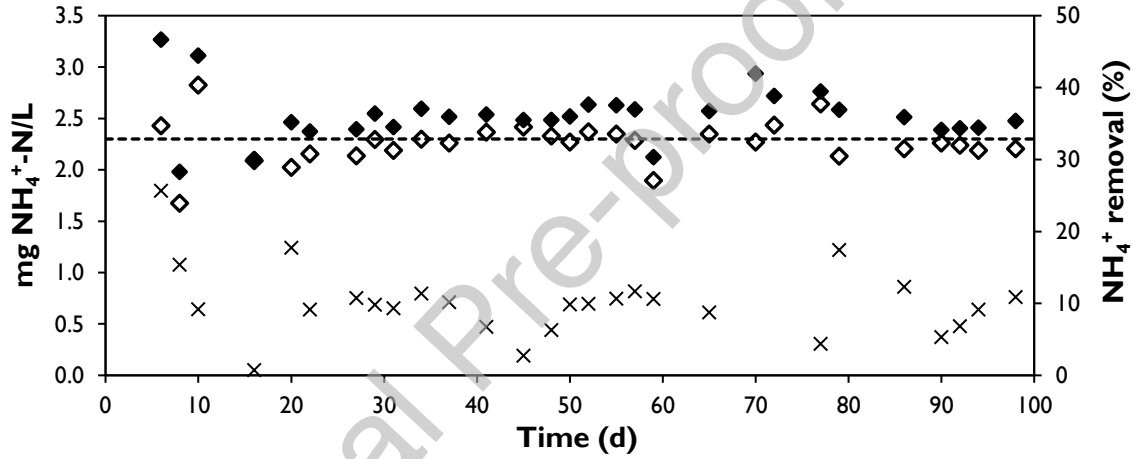

c)

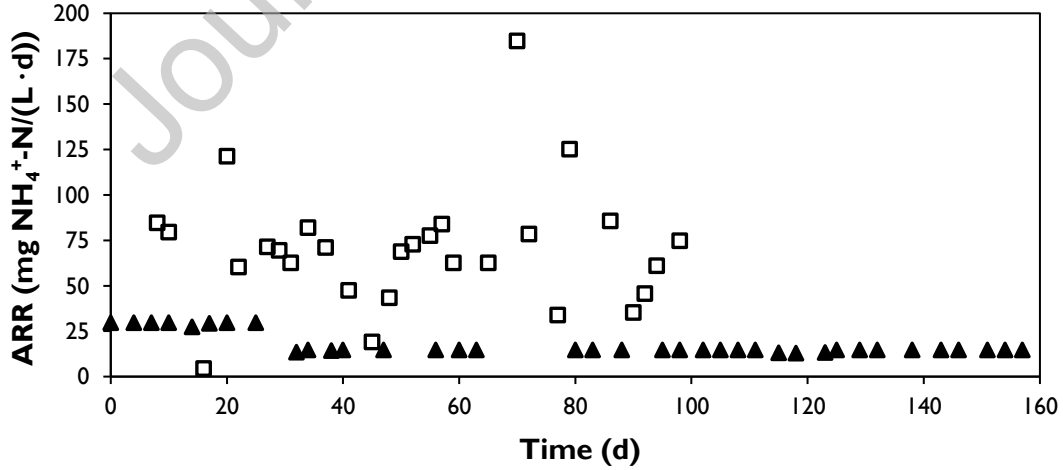

Figure 3. Ammonium removal performance of both reactors. Ammonium influent (♦) and effluent (◊) concentrations and removal percentage (x) in a) AGS-SBR and b)

CFGR (the black vertical line divides stageI and stageII, and the vertical arrow represents the moment of biomass reinoculation); and c) Ammonium Removal Rate of AGS-SBR (▲) and CFGR (□). The horizontal dashed line represents the ammonium toxic concentration for fishes.

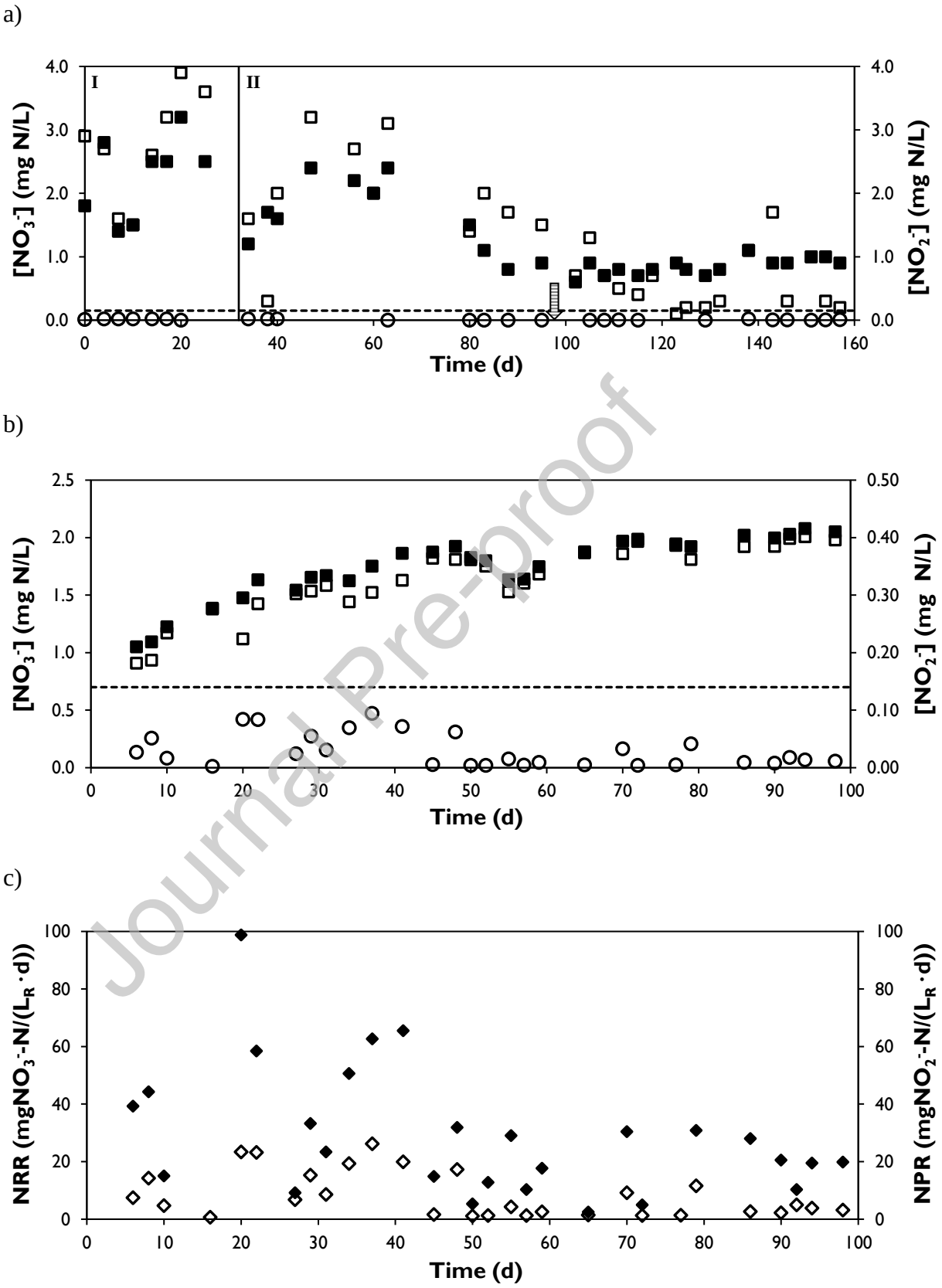

Figure 4. Evolution of nitrite and nitrate concentrations in both reactors, where the

horizontal dashed line represents the nitrite toxic concentration for fishes. Nitrate influent (■), effluent (□) and nitrite effluent (○) concentrations in a) AGS-SBR and b) CFGR (the black vertical line splits stageI from stageII, and the vertical arrow represents the moment of biomass reinoculation); and c) CFGR nitrate removal rate (♦) and nitrite production rate (◊).

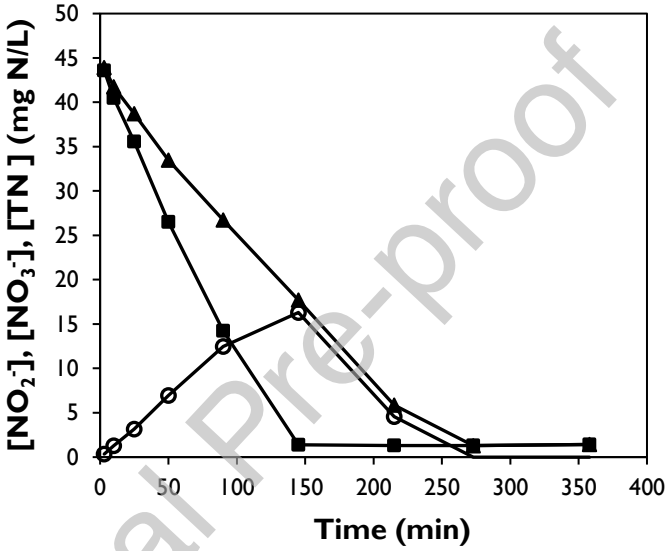

Figure 5. Evolution of nitrite (○), nitrate (■), and total nitrogen (▲) concentrations throughout the liquid phase denitrifying test performed with the CFGR reactor biomass on day-78.

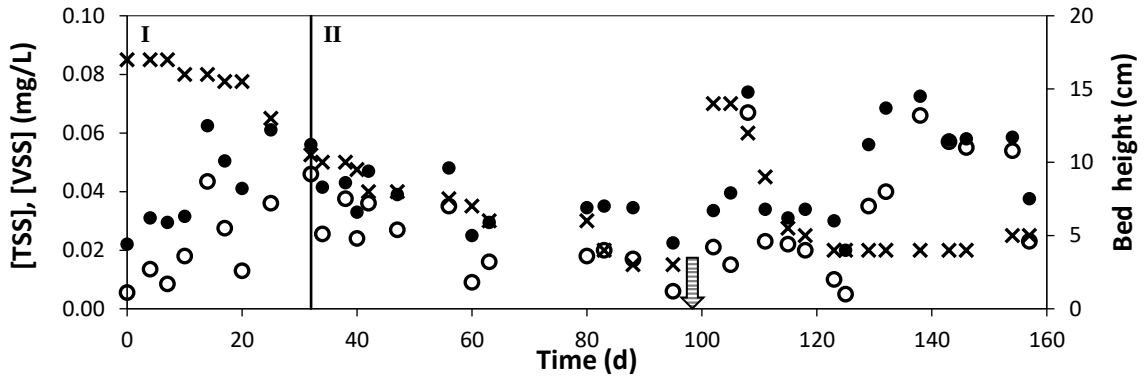

Figure 6. AGS-SBR bed height (x) and effluent suspended solids concentrations: TSS (●) and VSS (○), the black vertical line divides stage I and stageII, and the vertical arrow represents the moment of biomass reinoculation.

a) b) c)

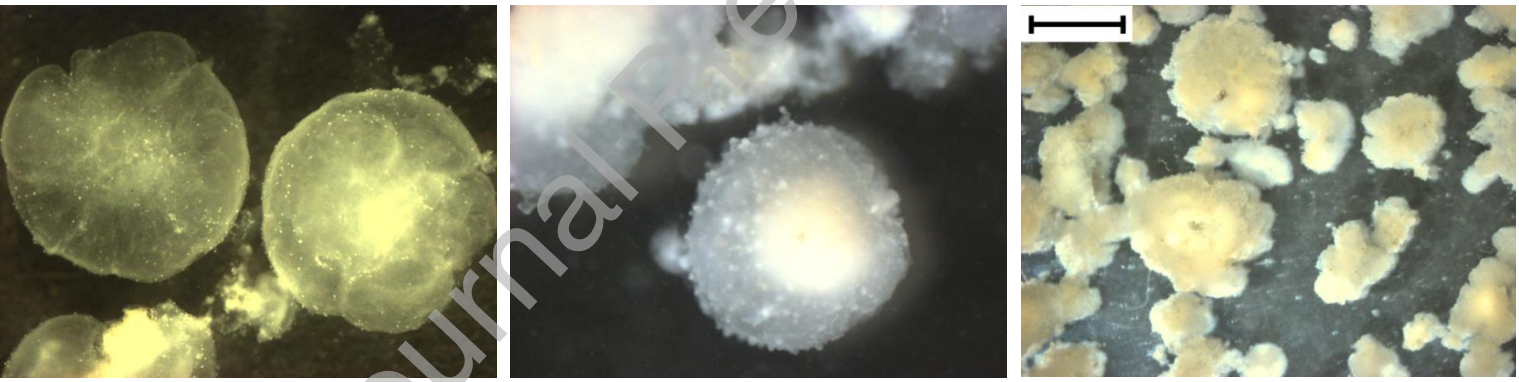

Figure 7. Images of the granules of the CFGR reactor on days 7 (a), 32 (b) and 74 (c) of operation. The size bar indicates 2 mm.

Table 1. Composition of the synthetic media used as feeding and operational conditions of the granular reactors.

Parameter	Units	AGS-SBR	CFGR
Operation time	d	157	98
pH	---	6.2 - 7.8	6.3 - 6.9
Temperature	°C	22 - 25	17 - 22
Inflow	L/d	7.6 - 15.2	355.9 - 554.4
HRT	min	237 - 474	5.2 - 8.1
V_{up}	m/h	-	8.05 - 11.02
OLR	mg COD/(L·d)	44 - 430	5380 - 6900
ALR	mg NH_4^+-N/(L·d)	15.5 - 31.0	690 - 800
NH_4^+	mg N/L	1.94	2.5 - 2.9
NO_3^-	mg N/L	-	1.5 - 2.0
COD	mg COD/L	7.3 - 70.6*	15.5 - 37.3
PO_4^{3-}	mg P/L	0.12 - 0.89*	0.42 - 0.80

* - Concentrations according to influent flow fluctuations