



## Does the feeding strategy enhance the aerobic granular sludge stability treating saline effluents?

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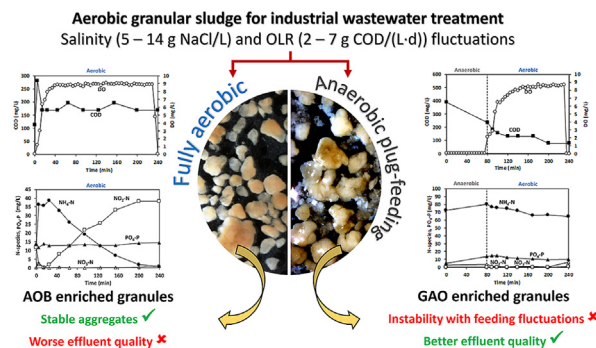
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### HIGHLIGHTS

- Fully aerobic or anaerobic-fed SBR to study aerobic granular sludge stability.
- Granulation is faster in the anaerobic-fed SBR than in the fully aerobic one.
- Granules from the fully aerobic SBR are more stable to load and salinity variations.
- Anaerobic-fed SBR has better COD removal and fully aerobic SBR has better N removal.
- The low P/COD ratio and composition variation promoted GAO enrichment.

### GRAPHICAL ABSTRACT



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### ABSTRACT

The development and stability of aerobic granular sludge (AGS) was studied in two Sequencing Batch Reactors (SBRs) treating fish canning wastewater. R1 cycle comprised a fully aerobic reaction phase, while R2 cycle included a plug-flow anaerobic feeding/reaction followed by an aerobic reaction phase. The performance of the AGS reactors was compared treating the same effluents with variable salt concentrations (4.97–13.45 g NaCl/L) and organic loading rates (OLR, 1.80–6.65 kg CODs/(m<sup>3</sup>·d)). Granulation process was faster in R2 (day 34) than in R1 (day 90), however the granular biomass formed in the fully aerobic configuration was more stable to the variable feeding composition. Thus, in R1 solid retention times (SRT), up to 15.2 days, longer than in R2, up to 5.8 days, were achieved. These long SRTs values helped the retention of nitrifying organisms and provoked the increase of the nitrogen removal efficiency to 80% in R1 while it was approximately of 40% in R2. However, the presence of an anaerobic feeding/reaction phase increased the organic matter removal efficiency in R2 (80–90%) which was higher than in R1 with a fully aerobic phase (75–85%). Furthermore, in R2 glycogen-accumulating organisms (GAOs) dominated inside the granules instead of phosphorous-accumulating organisms (PAOs), suggesting that GAOs resist better the stressful conditions of a variable and high-saline influent. In terms of AGS properties an anaerobic feeding/reaction phase is not beneficial, however it enables the production of a better quality effluent.

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## 1. Introduction

The lower footprint of aerobic granular sludge (AGS) systems in comparison with conventional activated sludge processes (Bengtsson et al., 2018), make them an attractive alternative to treat industrial wastewater. However, the instability of the granular sludge is one of the reported bottlenecks that hinder the application of AGS technologies. The growth of flocculent biomass, in addition to other factors such as organic overloads or the presence of toxic compounds like salts, is one of the causes responsible for granules integrity loss (Figuerola et al., 2015; Meunier et al., 2016). The maintenance of the aggregates stability is, even today, of major concern for AGS systems application (Bassin et al., 2019; de Kreuk and van Loosdrecht, 2004; Val del Río et al., 2013; Wagner et al., 2015a; Zhao et al., 2012). It is crucial to produce treated effluents fulfilling the discharge limits in terms of solids, organic matter and nutrient concentrations. For this reason, the assessment of different reactor configurations to avoid and/or limit the instability episodes is of interest.

The AGS is normally developed in Sequencing Batch Reactors (SBRs) operated in cycles distributed in operational phases as filling, reaction, settling and withdrawal. For this purpose two cycle configurations are preferentially used comprising: (1) a single aerobic reaction phase; or (2) an anaerobic feeding/reaction phase followed by an aerobic reaction phase. Franca et al. (2017) studied the stability of AGS in a SBR provided with an initial anaerobic feeding phase, performed in static conditions or plug-flow regime, and found that the latter was better to cope with industrial wastewater variability and to minimize the AGS instability. Thwaites et al. (2017) compared the use of long anaerobic and split anaerobic-aerobic feedings and concluded that the formation and stability of AGS does not require a conventional long anaerobic feeding. However, there are no similar studies to compare the performance of a cycle including a single aerobic phase with a cycle comprising an anaerobic feeding/reaction phase.

Salt concentration of the treated effluents, especially if they are variable, is recognized as one of the reasons for the AGS instability. Although many research studies were conducted to evaluate the effects of high salinity on granule formation and biological activity inhibition (Bassin et al., 2011; Ou et al., 2018; Pronk et al., 2014; Ramos et al., 2015; van den Akker et al., 2015; Wang et al., 2017), few studies investigated the influence of the variable salinity on biological processes and metabolic activities (Sun et al., 2010; Wang et al., 2009). Moreover, most of these studies have been performed with synthetic media and under a gradual increase of salinity, while studies that address the issue of granule stability under fluctuations (increase and decrease) of salinity and organic loading rate (OLR) have not been found yet in case of industrial wastewater treatment.

Taking this into consideration, the present study aims to evaluate the best SBR cycle configuration, to maintain the stability of AGS, between two options: one with short feeding and a single aerobic reaction phase and another with a plug-flow anaerobic feeding/reaction phase followed by aerobic reaction. Both reactors treated fish canning wastewater with variable composition in terms of salt and organic matter concentrations.

## 2. Materials and methods

### 2.1. Reactors set-up

Two laboratory SBRs, R1 and R2, were utilized with a useful volume of 1.75 L. Both units were operated at  $25 \pm 1$  °C in a thermostatic room and without pH control. The air was supplied with fine bubble diffusers located at the bottom of the reactors, to provide an air flow of 7 L/min, so that the superficial air velocity (SAV)

was of approximately 2 cm/s. A programmable logic controller (PLC) Siemens model S7–224CPU was used to control the activation of the different devices like pumps, valves, aeration system, and the length of the cycles. Both SBRs were operated with a volume exchange ratio of 50%.

Two cycle lengths were tested in both reactors, 3 h (0–92 days) and 4 h (93–226 days), distributed in feeding, aeration, settling and withdrawal (see details of each phase length in Supplementary Material, Table S1).

R1 was fed from the top in a short period of time (5 min) followed by the aerobic reaction phase. R2 was continuously fed from the bottom with plug-flow regime throughout the whole anaerobic phase (60 and 80 min) (De Kreuk et al., 2005), and afterwards the aerobic reaction phase took place. The two feeding methods selected were because according to Pronk et al. (2014) they influence the morphology and stability of aerobic granular sludge.

The first 15 days of operation the settling time of both reactors was set at 7 min, which implied an imposed minimum settling velocity ( $v_s$ ) inside the reactors of 1.33 m/h. Then, it was reduced to 4 min corresponding to a  $v_s$  of 2.33 m/h. From day 23 onwards the settling time imposed was only 1 min in R1 ( $v_s = 9.30$  m/h) and 2 min in R2 ( $v_s = 4.65$  m/h).

### 2.2. Operational conditions

Both reactors were operated for 226 days fed with wastewater from a fish canning company located on the coast of Galicia (NW region of Spain). The industrial effluent was collected after its pre-treatment in a dissolved air flotation (DAF) unit, where fats, oils and greases were removed. Gathered wastewater presented significantly different compositions due to the seasonal variations of processed fish products, so that each collected batch was fully characterised in composition. The experimental period was divided into four stages, depending on the salt concentration of the collected batches of wastewater (Table 1).

In Stages II and III, two sub-stages were defined on days 93 and 152, respectively. In Stage II the cycle length was extended from 3 h (Sub-Stage II. a) to 4 h (Sub-Stage II. b), resulting in an increase of the hydraulic retention time (HRT) from 6.0 h to 8.0 h. During Stage III, the applied OLR was diminished from  $4.28 \pm 0.70$  kg CODs/( $m^3 \cdot d$ ) (Sub-Stage III. a) to  $1.80 \pm 0.38$  kg CODs/( $m^3 \cdot d$ ) (Sub-Stage III. b) associated to a decrease of the chemical oxygen demand (COD) concentration in the fed wastewater.

Both reactors were inoculated with 875 mL of flocculent sludge from the biological SBR in operation in the fish cannery which provided the wastewater. The inoculum contained 3.23 g TSS/L and 2.27 g VSS/L and was characterised by a sludge volume index at 30 min ( $SVI_{30}$ ) of approximately 300 mL/g TSS.

### 2.3. Analytical and microbiological procedures

The pH and the concentrations of ammonium ( $NH_4^+$ ), nitrite ( $NO_2^-$ ), nitrate ( $NO_3^-$ ), total nitrogen (TN), phosphate ( $PO_4^{3-}$ ), total suspended solids (TSS), volatile suspended solids (VSS) and total organic carbon (TOC), and the sludge volume index after 30 ( $SVI_{30}$ ) and 5 ( $SVI_5$ ) minutes, were measured according to the Standard Methods (APHA/AWWA/WEF, 2012). The concentrations of chloride ( $Cl^-$ ) and sulphate ( $SO_4^{2-}$ ) ions were determined by means of Ion Chromatography (Metrohm 816 Advanced Compact IC). The COD concentration was determined according to Soto et al. (1989) taking into account the different salt concentrations of the sample. Total COD ( $COD_T$ ) concentration was determined in the sample without filtering, whereas soluble COD ( $COD_S$ ) concentration was measured in samples filtered through 0.45  $\mu m$  pore size filters. Dissolved oxygen (DO) concentration was measured with an on-line probe

(Hach® HQ40D). The morphology and size distribution of the granules was measured with a stereomicroscope (Stemi 2000-C, Zeiss) and using an image analysis procedure (Tijhuis et al., 1994).

Main microbial populations were identified in AGS samples by fluorescent *in situ* hybridization (FISH) analysis. Nitrifying bacteria, phosphate accumulating organisms (PAO) and glycogen accumulating organisms (GAO) were identified using the set of fluorescent labelled 16S rRNA-targeted DNA probes indicated in Table S2 (Supplementary Material). All probes were 5' labelled by fluorochromes FITC (Fluorescein-5-isocyanate) or Cy3 (Carbocyanine 3). Inoculum and fresh samples collected from the reactors on days 34, 92, 145, 163 and 222, were analysed. DAPI (4, 6-diamidino-2-phenylindole) was used as universal dye to label all DNA in the sample. Fluorescence signals were observed under an epifluorescence microscope (Axioskop 2, Zeiss, Germany) and registered with an acquisition system (Coolsnap, Roper Scientific Photometrics). To determine the relative abundance of each population, referred to the total bacteria domain, at least 15 images from each sample were taken and processed using DAIME software (Daims et al., 2006).

### 3. Results and discussion

#### 3.1. Granulation process with variable feeding

The biomass used as inoculum (from the fish canning treatment plant) was supposed to be acclimated to the wastewater conditions and for this reason it was chosen despite its bad settling properties (SVI<sub>30</sub> of 300 mL/g TSS). In Fig. 1 the evolution of the SVI values in both AGS reactors as well as the solids concentration are represented. Furthermore, for a better interpretation of the solids concentration inside the reactors and in the effluents the Table S3 in Supplementary Material can be consulted. To enhance the granulation process, by removing the biomass which settle worse, the settling time of both reactors was gradually decreased during the first 22 days of operation from 7 to 1–2 min. The fixed SAV of 2 cm/s was high enough to promote the granulation process (Liu and Tay, 2004). Since in R2 AGS comprise slow-growing organisms (GAOs and PAOs) the imposed settling time was 1 min higher than that of

R1 to favour the biomass retention and compensate for its low growth rate.

The first aggregates were observed in R1 on day 22 (Figure S1a in Supplementary Material) while in R2 the first notice of aggregates occurred before (day 6). On days 15–25, a sudden increase of OLR from  $8.17 \pm 0.66$  kgCODs/(m<sup>3</sup>·d) to  $9.40 \pm 0.30$  kgCODs/(m<sup>3</sup>·d) provoked a growth of flocculent biomass in both the reactors that caused high values of SVI<sub>5</sub> (300 mL/g TSS) and SVI<sub>30</sub> (150 mL/g TSS) (Fig. 1). Then, in R2 approximately on day 34 the SVI<sub>5</sub> and SVI<sub>30</sub> values were equal and as low as 27 mL/g TSS (Fig. 1b), meaning that the granulation process was completed in that moment, based on the physical characteristics of the granules (Figure S1b) (see details at Campo et al., 2017).

After only one month, the composition of the industrial wastewater fed changed (Stage II), mainly characterised by a decrease of the salt concentration to  $5.48 \pm 0.59$  g NaCl/L (Table 1). This change provoked a significant increase of the aggregates size in both reactors, from 1.51 to 2.52 mm in R1, and from 0.89 to 1.63 mm in R2 (Table 2). However, the most significant difference was an improvement in the biomass settleability of R1 (SVI<sub>30</sub> of 45 mL/g TSS) and a worsening in R2 (SVI<sub>30</sub> maximum achieved values as high as 155 mL/g TSS) (Fig. 1). Therefore, the salinity change did not affect the evolution of the granulation process in R1 and on day 90 it was considered completed, since the SVI<sub>5</sub> was similar to the SVI<sub>30</sub> (Fig. 1). However, in R2 this change was detrimental for the stability of the AGS due to the growth of flocculent biomass. To recover the stability in R2 the cycle length was increased from 3 to 4 h (Sub-Stage II. b), to give it more time to uptake the COD during the anaerobic feeding/reaction phase. To maintain the same loading rate in both reactors, the cycle length was also extended to 4 h in R1. However, this change did not have significant effects in both reactors in the initial 10 days (Fig. 1).

In Stage III, the salinity of the wastewater increased suddenly to  $13.45 \pm 0.51$  g NaCl/L. This change implied a higher buoyancy force in the mixed liquor and, consequently, occasional events of biomass washout took place (Sub-Stage III. a) in both reactors. This effect was faster in R1 while in R2 a progressive increase of the solids concentration in the effluent was observed. Therefore, the maximum solids concentration discharged was higher in R1 (3.96 g

**Table 1**

Characteristics of the fish canning wastewater and operational conditions in the different stages. Provided data are average values plus standard deviation corresponding to the analytical measurements performed in each stage.

Stage	I	II.a	II.b	III.a	III.b	IV
Days	0–35	36–92	93–102	103–151	152–182	183–226
Wastewater composition						
Salinity (g NaCl/L)	10.19 ± 0.63	5.48 ± 0.59		13.33 ± 0.57		4.97 ± 0.52
SO <sub>4</sub> <sup>2-</sup> (mg/L)	386.97 ± 108.58	240.87 ± 58.50		208.29 ± 134.77	263.47 ± 158.26	137.27 ± 75.55
COD <sub>T</sub> (g/L)	2.17 ± 0.24	1.21 ± 0.23		1.81 ± 0.32	0.78 ± 0.15	0.91 ± 0.08
COD <sub>S</sub> (g/L)	1.71 ± 0.22	0.96 ± 0.07		1.47 ± 0.24	0.61 ± 0.12	0.84 ± 0.07
TOC (mg/L)	537.60 ± 75.07	236.15 ± 36.27		506.72 ± 83.46	156.10 ± 54.64	300.06 ± 36.42
TN (mg/L)	99.13 ± 13.81	129.86 ± 12.43		106.78 ± 18.09	77.36 ± 6.95	107.64 ± 53.23
NH <sub>4</sub> <sup>+</sup> -N (mg/L)	90.55 ± 7.77	102.13 ± 27.03		86.08 ± 14.32	72.56 ± 7.15	105.80 ± 5.30
PO <sub>4</sub> <sup>3-</sup> -P (mg/L)	22.34 ± 9.10	15.78 ± 4.89		20.17 ± 9.55	10.55 ± 2.23	13.65 ± 3.45
TSS (mg/L)	237.51 ± 49.07	108.13 ± 35.79		194.18 ± 98.47	160.71 ± 60.52	58.66 ± 26.45
VSS (mg/L)	167.77 ± 27.30	81.69 ± 33.98		120.92 ± 83.75	78.59 ± 39.50	41.50 ± 25.19
pH (-)	5.89 ± 0.35	7.37 ± 0.26		6.64 ± 0.30	7.34 ± 0.31	7.46 ± 0.16
Operational conditions						
HRT (h)	6.0	6.0 <sup>a</sup>	8.00 <sup>a</sup>		8.00	8.00
OLR (kg CODs/(m <sup>3</sup> ·d))	6.65 ± 0.83	3.22 ± 0.32		4.28 ± 0.70 <sup>b</sup>	1.80 ± 0.38 <sup>b</sup>	2.37 ± 0.16
NLR (kg NH <sub>4</sub> <sup>+</sup> -N/(m <sup>3</sup> ·d))	0.37 ± 0.03	0.45 ± 0.07		0.25 ± 0.22	0.22 ± 0.02	0.31 ± 0.02
PLR (g PO <sub>4</sub> <sup>3-</sup> -P/(m <sup>3</sup> ·d))	88.7 ± 36.5	53.15 ± 42.45		59.7 ± 29.8	31.6 ± 7.0	38.9 ± 9.9
COD/N (g/g)	19.28 ± 3.52	7.62 ± 1.60		17.66 ± 4.05	8.39 ± 2.40	7.93 ± 0.55

COD<sub>T</sub>: total chemical oxygen demand; COD<sub>S</sub>: soluble chemical oxygen demand; TOC: total organic carbon; TN: total nitrogen; TSS: total suspended solids; VSS: volatile suspended solids; HRT: hydraulic retention time; OLR: organic loading rate; NLR: nitrogen loading rate; PLR: phosphorus loading rate.

<sup>a</sup> Stage II has two sub-stages corresponding to the modification of the HRT due to the change in the operational cycle length from 3 to 4 h.

<sup>b</sup> Stage III has two sub-stages corresponding to the shift of OLR provoked by the change in the industrial wastewater composition mainly of COD concentration.

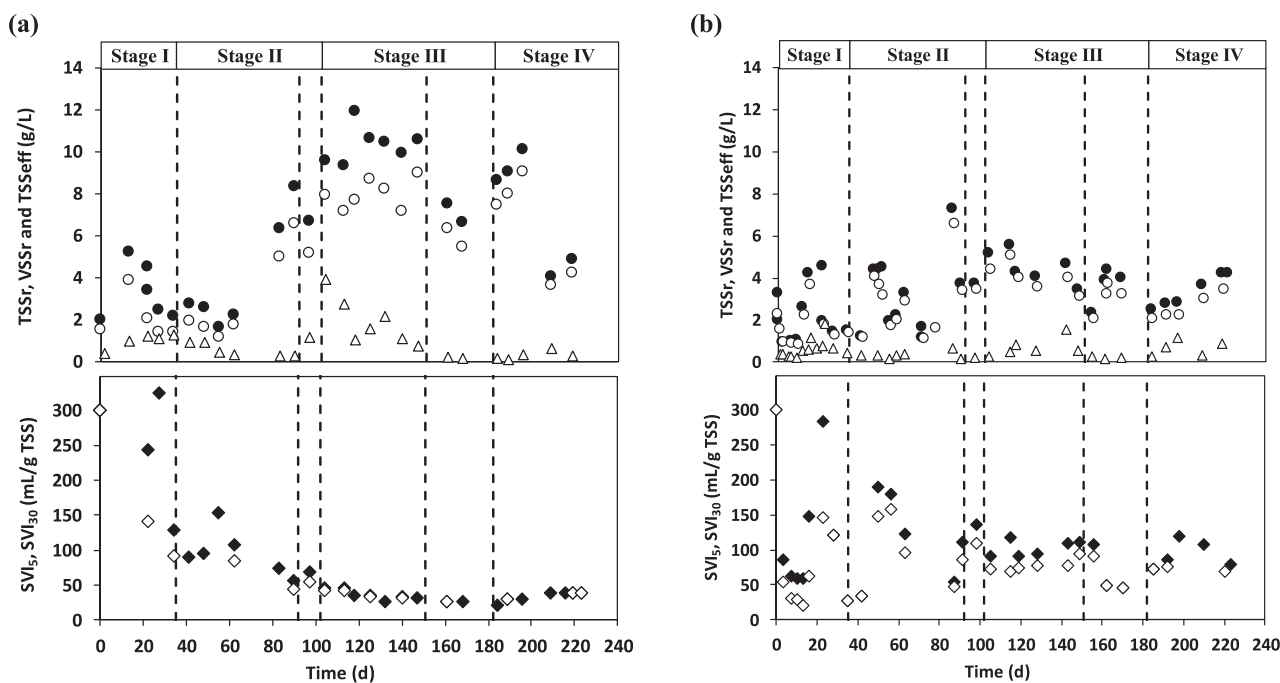


Fig. 1. Concentrations of solids in the reactor TSSr (●), VSSr (○) and the effluent TSSeff (Δ) (g/L); and SVI<sub>5</sub>, SVI<sub>30</sub> (mL/g TSS) of the granules (mL/g TSS) in R1 (a) and R2 (b).

TSS/L, day 104) than in R2 (1.54 g TSS/L, day 142) (Fig. 1). The average diameter of the granules remained in both reactors slightly lower than in the previous stage (Table 2), while the settling ability as SVI<sub>30</sub> value was better in R1 (38 mL/g TSS) than in R2 (77 mL/g TSS) (Fig. 1). Thus, in R1 well-formed granules were observed (Figure S1c), while in R2 the granules were more irregular with the presence of some flocculent biomass (Figure S1d).

Then, the reduction of the applied OLR (Sub-stage III. b) provoked the decrease of solid concentrations in both reactors. In R1 from 9.0 to 5.5 g VSS/L and in R2 from 4.0 to 2.1 g VSS/L (Fig. 1). Although in R1 no significant effect was observed in the AGS characteristics, in R2 the AGS settleability improved and the SVI<sub>30</sub> went down from previous values of 90 to 45 mL/g TSS (Fig. 1b).

In Stage IV the salt concentration in the feeding suddenly decreased again to  $4.97 \pm 0.52$  g NaCl/L (as in Stage II). However, due to the fact that the granular biomass was at that moment more “mature” than in Stage II, in R1 this change did not significantly influence the AGS properties and its SVI<sub>30</sub> and average diameter values were similar to those previous to the change, while the biomass concentration increased a little (Fig. 1a and Table 2). In R2 partial degranulation and a loss of AGS stability took place after day 192, with an increase of solids concentration in the effluent up to

1.16 g TSS/L (day 197, Fig. 1b), and a new event of flocculent biomass growth. Since the OLR remained almost equal to that of Stage III, it could be asserted that the new growth of flocculent biomass was related to the salinity decrease. Nevertheless, it is noteworthy to mention that in this case the instability episode was less significant than in the previous event of salt decrease (from Stage II to Stage III).

In summary, comparing both SBR cycle configurations, granulation process was faster when the anaerobic feeding/reaction was applied and the formed AGS exhibited better settling properties. Nevertheless, the AGS from the fully aerobic reactor responded better to the changes in composition of the wastewater, and higher biomass concentration inside the reactor was achieved while its SVI followed a decreasing trend until achieving low and stable values.

### 3.2. Reactors performance

#### 3.2.1. Organic matter removal

During the first days of Stage I the CODs removal gradually increased up to an average value of  $79 \pm 8$  and  $82 \pm 6\%$  in R1 and R2, respectively (Fig. 2). These percentages remained almost constant during the whole operational time (Stages I to IV) and the trends

Table 2  
Summary of AGS properties and reactor performance of R1 and R2 in the different operational stages.

Stage	I	II.a	II.b	III.a	III.b	IV
Change from previous stage	Start-up	Decrease salt	Increase HRT	Increase salt	Decrease OLR	Decrease salt
Main features	R1 – R2 granulation	granulation instability (flocculent growth)	– –	biomass loss (quick) biomass loss (slow)	nitrification better settle ability	biomass loss instability (flocculent growth)
Diameter (mm)	R1 $1.51 \pm 0.53$ R2 $0.89 \pm 0.51$	$2.52 \pm 1.13$ $1.63 \pm 0.18$	$1.72 \pm 0.57$ $1.38 \pm 0.05$	$1.23 \pm 0.31$ $1.18 \pm 0.19$	$1.25 \pm 0.39$ $0.94 \pm 0.11$	$1.10 \pm 0.34$ $1.12 \pm 0.25$
SRT (d)	R1 $0.78 \pm 0.41$ R2 $1.00 \pm 0.38$	$2.88 \pm 3.05$ $3.06 \pm 1.64$	$1.98 \pm 0.03$ $5.93 \pm 0.05$	$2.56 \pm 1.46$ $3.07 \pm 1.94$	$15.18 \pm 4.50$ $4.04 \pm 2.49$	$11.60 \pm 10.54$ $2.26 \pm 1.20$

SRT: solids retention time.

HRT: hydraulic retention time.

OLR: organic loading rate.

were very similar to the  $COD_T$  (Figure S2 in Supplementary Material). Only in Stage III, when the OLR decreased from  $4.28 \pm 0.70$  to  $1.80 \pm 0.38$  kg  $COD_s/(m^3 \cdot d)$  resulted in a slight decrease of the removal efficiencies in both reactors, probably due to the transient conditions during this period and the decrease of biomass concentration in the reactors.

R2 (anaerobic feeding/reaction phase) showed a slightly more stable performance in terms of  $COD_s$  removal (80–90%) than R1 (fully aerobic) (75–85%) (Fig. 2), while the fluctuations of salinity and OLR resulted in a physical stress over the AGS more than in biological inhibition, as proved by Bassin et al. (2011) by means of batch tests.

### 3.2.2. Nitrogen removal

Nitrogen removal took place basically due to heterotrophic biomass growth during the whole operation of R2 and most part of the operation of R1 (Stages I to Sub-Stage III. a) (Fig. 2). The removal percentages ranged between 20 and 40% depending on the load of  $COD$  removed and the corresponding ammonium concentration. The short solid retention times (SRTs) in both reactors (Table 2) were responsible for the absence of detectable nitrifying bacteria activities, as found by Wagner et al. (2015b). They observed that when SRT values were lower than 3 days only 15% of ammonium was removed, whereas with SRTs of 7 days the ammonium removal increased to 70%.

In R1, when the SRT was lower than 5 days, the growth of ammonium oxidising bacteria (AOB) did not occur and neither nitrite nor nitrate were measured in the effluent. Then, when the SRT increased over 9 days (from day 153 onwards, Stage III), nitrite production considerably increased to values between 3.72 mg  $NO_2^-N/g$  VSS and 7.05 mg  $NO_2^-N/g$  VSS, highlighting the AOB development.

The absence and presence of AOB activity inside R1 at different SRTs is proven by the comparison of nitrogen species concentration profiles during different cycle measurements (Figs. 3a and 4a). As an example, on day 91 when the SRT was 3 days the ammonium

oxidation was negligible (Fig. 3a). However, on day 162, when the SRT increased to 15 days, complete ammonium oxidation to nitrite took place, and the nitrite formed was removed by denitrification during the feast period of the next cycle (Fig. 4a). This removed nitrogen, in addition to the ammonium removal due to biomass growth, allowed the improvement of the nitrogen removal percentages to  $49 \pm 7\%$  and  $77 \pm 8\%$  in Stages III and IV, respectively (Fig. 2a). Furthermore, no nitrite oxidation to nitrate was detected during the whole experiment, probably because of the salt concentration in the reactor, which might have provoked the inhibition of nitrite oxidising bacteria (NOB) (Giustinianovich et al., 2018).

The proliferation of AOB in the fully aerobic configuration (R1) was confirmed by the results of the FISH analysis. In AGS samples from days 0 (inoculum), 34 (Stage I) and 92 (Stage II. b) no positive signal for AOB (probe NSO 190) was detected. However, on samples from days 145 (Stage III. a), 163 (Stage III. b) and 222 (Stage IV) the results for AOB (probe NSO 190) were positive and their relative abundance had an increasing trend with values of 1.3%, 3.3% and 5%, respectively (Figure S3a in Supplementary Material).

With respect to R2, the SRT values remained shorter than 6 days (except for some days) during the experimental period (Table 2), which meant that AOB retention was not favoured. Results from the FISH analysis of AGS samples from days 0 (inoculum), 34 (Stage I), 92 (Stage II), 145 (Stage III. a), 163 (Stage III. b) and 222 (Stage IV) showed no positive signal for AOB (probe NSO190). The low values of SRT in R2 can be associated to instability episodes and the higher proliferation of flocculent biomass in comparison with R1, which hindered the enrichment of aggregates inside the reactor.

### 3.2.3. Phosphorus removal

The SBR configuration with a fully aerobic reaction phase was not designed for phosphorus removal. Therefore, in R1 phosphorus was mainly removed to meet the metabolic demand of heterotrophic organisms.

In case of R2 configuration, comprising an anaerobic feeding/reaction phase, the metabolic selection of slow-growing organisms,

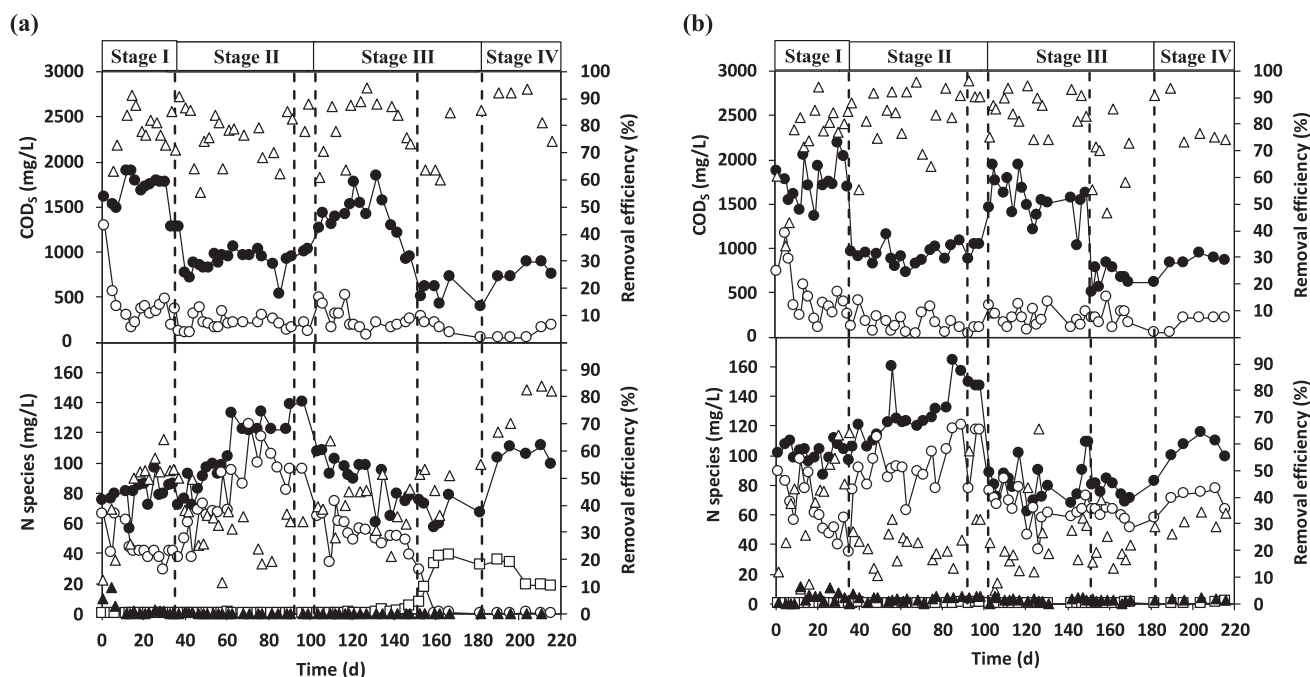


Fig. 2. Evolution of concentrations of  $COD_s$  and  $NH_4^+-N$  in the influent (●) and effluent (○), removal efficiencies (Δ), and concentrations of  $NO_2^-N$  (□),  $NO_3^-N$  (▲) in the effluent, throughout the different operational stages in R1 (a) and R2 (b).

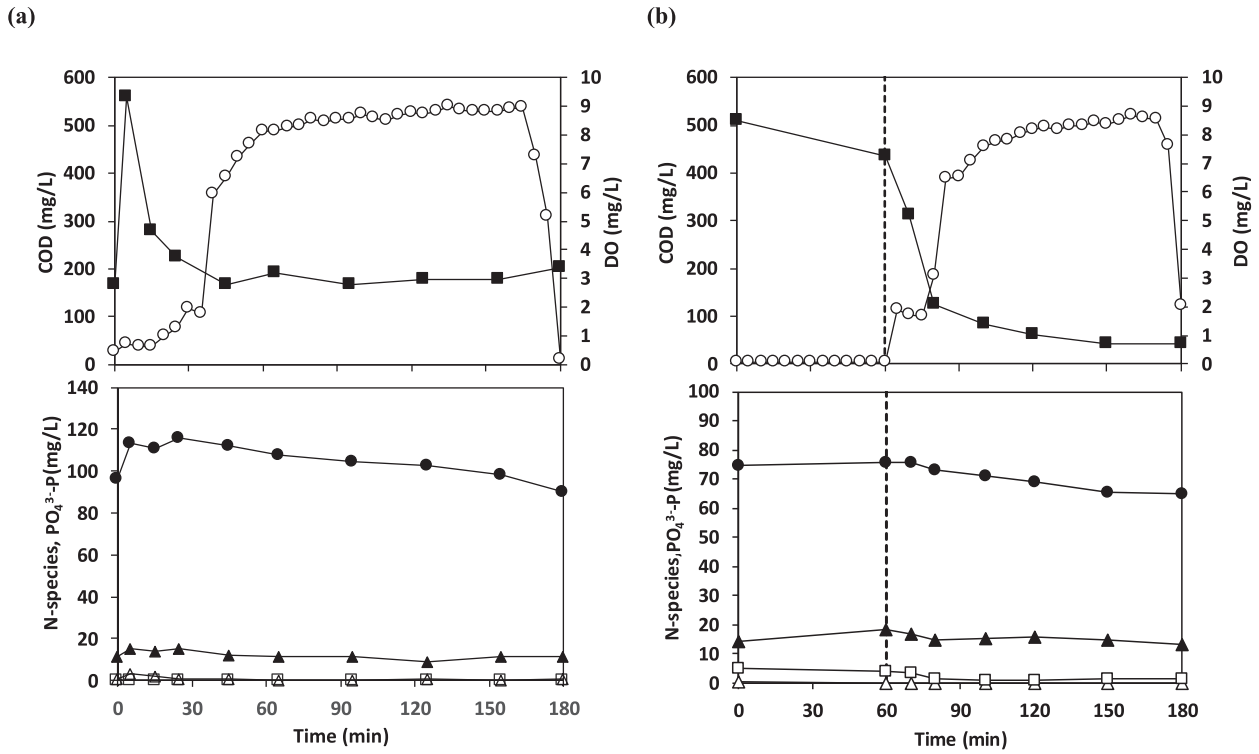


Fig. 3. Concentration profiles measured throughout an operational 3-h cycle on day 91 in R1 (a) and R2 (b). Concentrations of soluble COD (■), DO (○), NH<sub>4</sub><sup>+</sup>-N (●), NO<sub>2</sub><sup>-</sup>-N (□), NO<sub>3</sub><sup>-</sup>-N (Δ), PO<sub>4</sub><sup>3-</sup>-P (▲).

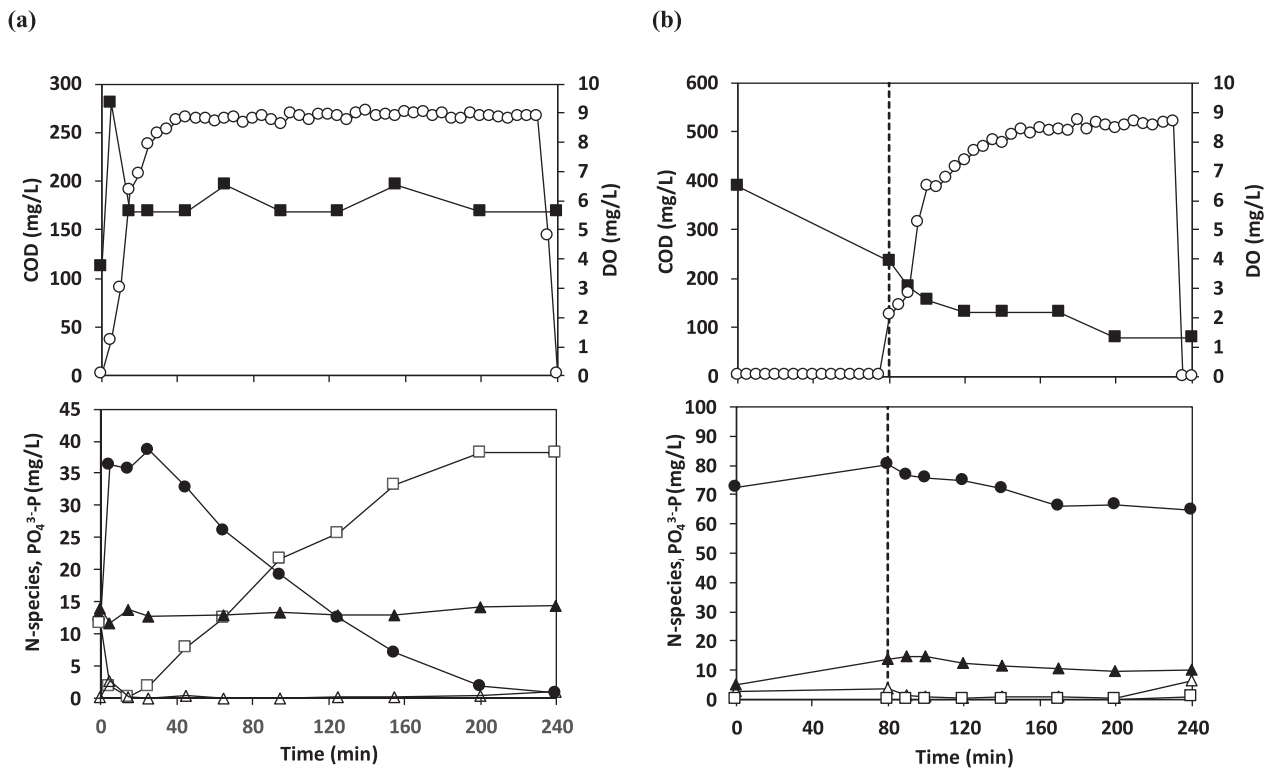


Fig. 4. Concentration profiles measured throughout an operational 4-h cycle on day 162 in R1 (a) and R2 (b). Concentrations of soluble COD (■), DO (○), NH<sub>4</sub><sup>+</sup>-N (●), NO<sub>2</sub><sup>-</sup>-N (□), NO<sub>3</sub><sup>-</sup>-N (Δ), PO<sub>4</sub><sup>3-</sup>-P (▲).

such as PAOs or GAOs is promoted. PAOs are preferentially selected under high P/COD ratio and GAOs develop better under low P/COD ratio (Weissbrodt et al., 2013). Both, PAOs and GAOs, uptake COD under anaerobic conditions, which represents an advantage in terms of energy-saving associated to the absence of air requirements, compared with a fully aerobic granular sludge reactor.

Although R2 cycle distribution was conceived to biologically remove phosphorus, the low P/COD ratio in the industrial wastewater (0.016 g P/g COD) was approximately near the minimum value required for P removal (0.010 g P/g COD), as found by Weissbrodt et al. (2013). Consequently, the accumulation of PAOs was limited, and the growth of GAOs occurred.

From mass balance calculations, it was estimated that PAO activity contributed to phosphorus removal only in Stages I and II, being the main route of phosphorus removal in these stages the heterotrophic growth. Nevertheless, PAOs were not detected in the inoculum and they were not observed by FISH analysis (probes PAO462, PAO651, PAO846) during these stages. This fact could be due to the contribution of other organisms responsible for phosphorus removal, different from *Accumulibacter*, such as *Tetrasphaera* (Muszyński and Mitobędzka, 2015; Marques et al., 2017), which grows when the wastewater presents complex substrates. From the Stage III onwards the phosphorus removal due to PAO activity was negligible by mass balances. The obtained results suggest that PAOs are highly sensitive to operational conditions, as recently found from other authors (Wang et al., 2017). Not only the low P/COD ratio, but also the fluctuations of the OLR and salinity of the feeding led to the inhibition of PAOs.

The presence of GAOs, which were the main responsible organisms of the anaerobic COD uptake, was confirmed by the positive signals of probes GAOQ989 and GAOQ431 applied to AGS samples collected during the entire operation of the reactor (Figure S3b). The trend of GAOs relative abundance fluctuated very likely due to the high variability of the influent composition, maintaining a value always above 30%, with peaks next to 70% and 60% during Stages I and IV, respectively. The highest abundances of GAOs coincided with the operational periods with lower fraction of flocculent biomass and better physical properties of the biomass.

### 3.3. Instability episodes

The dynamic behaviour, due to the industrial wastewater variability, of the aggregation process of the biomass was observed in both SBRs. The worst instability episode occurred in the reactor with anaerobic feeding/reaction phase (R2), when the salinity decreased from 10.2 to 5.5 g NaCl/L (Stage II). Although it happened again when it decreased from 13.3 to 5.5 g NaCl/L, (Stage IV) it was less severe, probably because of the fact that the AGS was more mature.

In both episodes of R2 the growth of flocculent biomass was observed. However, specific measures were taken only in the first instability event when the fraction of fluffy biomass corresponded to approximately 30% of the biomass weight inside the reactor. In this case, most of the granules remained entrapped in this flocculent biomass (see Figure S4 in Supplementary Material), which worsened their settling properties and increased the values of the SVI<sub>30</sub> from 34 mL/g TSS (day 42) to 149 mL/g TSS (day 50). As a consequence, the entrapped granules were washed-out with the effluent. To cope with this drawback, the granules were manually recovered, by filtering the effluent with a 200 µm sieve, and put back inside the reactor. Although, this strategy is not feasible at full scale, it was used in the present research work at laboratory scale to continue the operation of R2, in order to compare its performance in the following stages with R1. This procedure was more complicated than that applied by Liu and Tay (2004) who operated an AGS

reactor fed with industrial wastewater and observed a stratification between granular biomass, settled at the bottom, and flocculent biomass, suspended on top of the sludge bed. In this case, the flocculent sludge can be relatively easily removed with the effluent withdrawal. Nevertheless, in the present study these two separate phases did not occur in R2, probably due to the different kind of wastewater used, for this reason the strategy of increasing the settling time to retain the granules was not applied, as it could have implied also the retention of undesirable flocculent biomass.

The growth of flocculent biomass, only observed in R2, was related to the COD storage capacity of the biomass during the anaerobic feeding and can be explained by the COD profile consumption in the two applied cycle configurations (Figs. 3b and 4b). In R2 the length of the anaerobic feeding/reaction phase was not long enough to guarantee the complete organic matter uptake, and the remaining COD (as hydrolysis products) was consumed during the initial minutes of the aerobic phase (Fig. 3b). Consequently, when aerobic conditions were imposed in the cycle, the hydrolysis products were directly consumed for growth by the organisms at the surface of the granules with steep substrate diffusion limitation gradients (Mosquera-Corral et al., 2003), promoting the filamentous growth, as stated by Pronk et al. (2015). This fact agrees with van den Akker et al. (2015), who observed the growth of filamentous bacteria when the applied COD exceeded the anaerobic storage capacity of the PAOs. Conversely, in R1 the fully aerobic strategy allowed a more efficient consumption of COD during the feast phase (Fig. 3a), which favoured the selection of microorganisms forming aggregates and obtaining a more stable granular system.

According to Meunier et al. (2016), the increase of the anaerobic feeding length enhances the anaerobic COD depletion and improves the selection of PAOs and GAOs. Consequently, the COD removal during the subsequent aerobic phase decreases, hindering the formation of flocculent sludge and filamentous microorganisms. For this reason the cycle length was increased from 3 to 4 h in Stage II. b, which implied an increase in the anaerobic feeding period from 60 to 80 min (Table S1).

Furthermore, the decrease of the OLR in Stage III. b allowed an almost complete anaerobic substrate hydrolysis (Fig. 4b), so the selection of slow-growing microorganisms and the biomass granulation improved.

### 3.4. Quality of the effluent

Comparing the effluent composition with the threshold limits suggested in “Reference Document on Best Available Techniques in the Food, Drink and Milk Industries (Prevention, I. P. (2006))”, the configuration with a previous anaerobic feeding/reaction phase (R2) presented lower concentrations of TSS during the whole experiment, whereas in the fully aerobic reactor (R1) there were important fluctuations due to the quick washout of the biomass with the change of the wastewater conditions (Table 2). In this sense, the slower washout of flocculent biomass in R2 when fluctuations occurred, avoided the peaks of solids in the effluent but provoked the instability of the aggregates which remained entrapped in it. However, none of the configurations respected the discharge limit for TSS (50 mg/L) (Fig. 1), which was probably related to the difficulty to achieve stable AGS when treating a wastewater characterised by high fluctuating values of salinity and OLR. Therefore, for industrial application, both configurations will require a posterior solid separation system.

Regarding the CODs removal, R1 did not fulfil the discharge requirements (125 mg COD/L), except for Stage IV, reaching values below 90 mg COD/L (Fig. 2a). The COD<sub>5</sub> concentration in the effluent was lower in R2 and with less fluctuations during the whole operational period (Fig. 2b). It fulfilled the discharge

requirements in each stage, very often in Stage II, characterised by low salinity and low OLR.

In terms of TN, the concentration in the effluent was similar in both configurations, with the exception of the operational periods when the SRT was higher than 10 days in R1 (Sub-Stage III. b and Stage IV, Fig. 2a). At that moment the TN concentration in the effluent from R1 was lower than in R2 due to the ammonium oxidation to nitrite and its further denitration, but it was still above the discharge requirements for TN (10 mg N/L).

In R1, the phosphorus concentration in the effluent was always higher than the discharge limit of 5 mg P/L. In R2, the phosphorus concentration in the effluent frequently fulfilled the discharge limit during Stage I and Stage II, suggesting a small PAOs contribution from mass balance calculations. Then, during the remaining days of operation, when PAOs activity decreased, the concentration was above the discharge limit.

#### 4. Conclusions

In the fully aerobic configuration (R1) the granulation process was slower (completed on day 90) than in the anaerobic feeding/reaction configuration (R2) (completed on day 34) when fish canning wastewater was used as feeding. However, the granules obtained in R1 were more stable against alternating OLRs and salinity concentrations in the industrial wastewater treated. In general, the granular biomass in R1 presented lower SVI<sub>5</sub>/SVI<sub>30</sub> ratios and larger average diameter than that from R2.

With respect to the reactor performance, R2 responded slightly better in terms of COD<sub>5</sub> removal (80–90%) than R1 (75–85%). Nitrogen removal was significantly larger in R1 (80%) when the values of SRT were high enough to promote nitrification, while in R2 nitrification did not occur, probably due to the instability episodes of the AGS which led to low values of the SRTs (<6 days). Although the biological phosphorus removal took place only in R2 due to the alternating anaerobic/aerobic reaction conditions, the predominant microorganisms observed were GAO instead of PAO. In the treated wastewater the P/COD ratio was not enough to support PAOs development growth.

The correct management of the anaerobic feeding/reaction phase, assuring a complete anaerobic storage of the COD, is crucial to assure the development of stable granular biomass and the absence of filamentous bacteria.

#### CRedit authorship contribution statement

**P. Carrera:** Data curation, Formal analysis, Investigation, Methodology, Writing - original draft. **R. Campo:** Data curation, Formal analysis, Investigation, Methodology, Writing - original draft. **R. Méndez:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision. **G. Di Bella:** Funding acquisition, Validation, Visualization, Writing - review & editing. **J.L. Campos:** Conceptualization, Formal analysis, Supervision, Validation, Writing - review & editing. **A. Mosquera-Corral:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing - review & editing. **A. Val del Rio:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing - review & editing.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.chemosphere.2019.03.127>.

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