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**EFFECT OF EXTENDED FAMINE CONDITIONS ON AEROBIC  
GRANULAR SLUDGE STABILITY IN THE TREATMENT OF BREWERY  
WASTEWATER**

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**Abstract**

Results obtained from three aerobic granular sludge reactors treating brewery wastewater are presented. Reactors were operated for 60 d days in each of the two periods under different cycle duration: (Period I) short 6 h cycle, and (Period II) long 12 h cycle. Organic loading rates (OLR) varying from  $0.7 \text{ kg COD m}^{-3}\text{d}^{-1}$  to  $4.1 \text{ kg COD m}^{-3}\text{d}^{-1}$  were tested. During Period I, granules successfully developed in all reactors, however, results revealed that the feast and famine periods were not balanced and the granular structure deteriorated and became irregular. During Period II at decreased 12 h cycle time, granules were observed to develop again with superior structural stability compared to the short 6 h cycle time, suggesting that a longer starvation phase enhanced production of proteinaceous EPS. Overall, the extended famine conditions encouraged granule stability, likely because long starvation period favors bacteria capable of storage of energy compounds.

**Keywords:** Extracellular polymeric substances, Feast/Famine, Aerobic granular sludge, Industrial wastewater, Stability.

## 1. Introduction

Brewery industry consumes large volumes of water for both brewing and cleaning processes. It was estimated that 3-10 L of wastewater is generated for the production of 1 L of beer (Chen et al., 2015). Brewery wastewater typically has a high chemical oxygen demand (COD) which is derived from all the organic components (e.g., yeast, sugars, soluble starch, etc), while nitrogen and phosphorus concentrations are mainly dependent on the amount of yeast present in the effluent (Fillaudeau et al., 2006). Typically, biological treatment of brewery wastewater involves a combination of anaerobic and aerobic processes. The anaerobic process, as a pre-treatment, is capable of removing more than 75% of the BOD, with generation of biogas, with residual BOD, COD, nitrogen and phosphorous are removed to comply with discharge limits in the subsequent aerobic process (Simate et al., 2011).

During the last decade, aerobic granular sludge (AGS) technology has been widely proven to be a suitable solution for treatment of several industrial wastewaters containing xenobiotic, recalcitrant and hardly biodegradable organic matters (Corsino et al., 2016a; Maszenan et al., 2011; Moreira et al., 2015; Zhang and Tay, 2016). Most of these studies reported a gradual deterioration in the granular sludge structure leading in some cases to irregular granules with fluffy appearance and poor settling capability. In some cases complete degranulation was observed. No clear explanation for these phenomena was reported by the authors. De Kreuk et al. (2010) stated that the presence of suspended organic matter or slowly biodegradable substrates in the influent wastewater favoured the growth of filamentous and pin-type structures on the granular surface that caused structural deterioration. Wagner et al. (2015) observed that an anaerobic feeding phase extended to as much as 90 min, was not sufficient to hydrolyze all of the particulate substrate, and filamentous structures were still present in the bulk liquid. These contributed to enlarged granule diameter with structural instability and poor settleability.

The structural stability is the main issue of the AGS technology. The key advantages over conventional processes are enhanced settling properties, coexistence of different microorganisms in a discrete environmental distribution and compact structure. All of these characteristics are strictly related to granules integrity. A majority of the AGS related studies reported that EPS has a crucial role for both granulation and stability properties (Adav et al., 2008; Xiong and Liu, 2013; Rene et al., 2008; Zhu et al., 2012b). The exact role of EPS in maintaining the granular structure stability has not been investigated in detail on real industrial wastewater and remains to be elucidated. Since EPS is produced from bacterial metabolism, its generation is strictly related to the substrate availability and microorganism activity. Previous works reported that the EPS content of granular sludge consistently varied during sequencing batch reactor (SBR) reaction cycle (Zhu et al., 2012a). Most research on synthetic wastewater demonstrated that EPS is mainly produced during the feast phase of the sequencing batch reactor (SBR) cycle, following the feeding period in which the readily biodegradable COD is mainly degraded. A significant consumption, however, was observed during the famine phase in which substrate is present in limiting concentrations and bacteria utilize EPS as carbon and energy source for endogenous respiration (Zhu et al., 2012a; Corsino et al., 2015). However, no information was available on how the EPS characterization under different lengths of the famine phase affects granular stability in treating actual high-strength wastewaters. The main objective of this study was to develop a better understanding of the EPS distribution and characterization under different operating conditions. In particular, the impact of organic loading rates (OLRs) on the EPS structure and composition was evaluated.

## 2. Materials and methods

### 2.1 Experimental and reactors set-up

Experiments were carried out in three geometrically identical SBRs. Each reactor was a column-type (100 cm height) with a working volume of 4 L. Air was supplied at a flow rate of  $2.5 \text{ L min}^{-1}$  via three fine bubble aerators placed on the reactor bottom with  $120^\circ$  angle spacing between each other. The filling height was 80 cm and therefore the height/diameter ratio corresponded to 8. The volumetric exchange ratio (VER) applied was different for each reactor. The reactors called R1, R2 and R3 were operated with a VER equal to 75%, 50% and 25%, respectively. Therefore, 3 L, 2 L and 1 L of effluent were withdrawn by a peristaltic pump at the end of the reaction cycle.

Sequencing batch reactors were operated for 120 days with two different periods of 60 days each in which the studied conditions were maintained. During Period I, the reactors were operated with a 6 h cycle. Because of the different VER applied, settling time differed in order to maintain the same hydraulic selection pressure. The reaction cycle consisted of 40 min of upflow anaerobic feeding, 310-312 min of aeration, and 4.5, 3 and 1.5 min of settling in R1, R2 and R3, respectively, before 2 min of effluent discharge. During Period II, the cycle length was extended to 12 h maintaining the same phase duration excepted for the aeration time, which was increased accordingly. Five automatic timers ensured the SBR cycle operations. The main operational parameters are reported in Table1.

[INSERT TAB. 1]

The SBRs were seeded with conventional activated sludge from West End Water Pollution Control Centre (WEWPCC; Winnipeg, CA) and fed with brewery wastewater collected from a local brewery (Fort Garry Brewery). The raw wastewater was pre-treated in an anaerobic moving bed

biofilm reactor (AMBBR; di Biase et al., 2016), and its effluent was used as influent for the AGS reactors.

In Period I, because the raw brewery wastewater lacked nutrients, nitrogen ( $\text{NH}_4\text{Cl}$ ) and phosphorous ( $\text{KH}_2\text{PO}_4$ ) were added to maintain a nutrient ratio of 100 COD: 10 N: 1 P by weight, to avoid heterotrophic growth limitation and filamentous bacteria appearance (Wang et al., 2007). In Period II the composition of the wastewater from the plant changed to the point that the ratio was maintained without external nitrogen and phosphorus additions..

The influent characteristics of anaerobically pre-treated wastewater are reported in Table 2.

### [INSERT TAB. 2]

Sludge retention time (SRT) was not directly controlled. The geometrical features of the reactors, in particular the VER applied, allowed R1 to have the smallest volume (1 L) available for biomass retention compared to R2 and R3. At the end of the settling phase, R1 was discharging the solids above the valve located at 1 liter level. Above this point, the excess of solids were wasted. The sludge retention time in reactor R2 and R3 was manually maintained as close as possible to R1. Thus, SRT was estimated ~ 12 d and ~ 30 d during Period I and Period II, respectively.

## 2.2 Analytical methods

All of the chemical-physical analyses (COD, BOD, TN, TP, TSS, and VSS) were performed according to standard methods (APHA, 2012). Samples were tested for total COD (TCOD). Soluble COD (SCOD) was determined after filtration through a 0.45  $\mu\text{m}$  membrane. By the difference between TCOD and SCOD it was possible to quantify the particulate COD fraction (PCOD). Extracellular polymeric substances determination were carried out according with the heating two-steps extraction method described by Le-Clech et al. (2006). A sample of mixed liquor (50 mL) was first centrifuged at 5000 rpm for 5 min at room temperature ( $20 \pm 0.1$  °C) and the supernatant was

immediately filtered through a 0.22  $\mu\text{m}$  pore-size membrane. In this way, the not-bound EPS (NB-EPS) was extracted. The precipitate, was resuspended with deionized water to its original volume and then placed in a thermal bath at  $80 \pm 1$   $^{\circ}\text{C}$  for 10 min. Afterwards, the sample was centrifuged at 7000 rpm for 10 min at  $4 \pm 1$   $^{\circ}\text{C}$  and the supernatant was filtered through a 0.22  $\mu\text{m}$  membrane. During this step, the tightly-bound EPS (TB-EPS) were extracted. For both NB-EPS and TB-EPS, carbohydrate and protein concentrations were determined according to the phenol–sulphuric acid method with glucose as standard (DuBois et al., 1956) and by the Folin method with bovine albumin serum as standard (Lowry et al., 1951), respectively. Morphological features of the granules were evaluated through stereomicroscope observations (Zeiss, Toronto, ON, CA), while their size distribution were determined by measuring approximately 100 granules through an image analyser software (IM50-Leica).

### 3. Results and Discussions

#### 3.1 Formation and morphology of granular sludge

In Period I, aerobic granulation was successfully achieved in all three reactors. The granulation process rapidly occurred and only one week after the reactors were seeded the first aggregates were distinctly visible in the mixed liquor. At steady state, the mixed liquor solids concentration was close to 3.6 g TSS  $\text{L}^{-1}$  in R1 and R2, and 1.9 gTSS  $\text{L}^{-1}$  in R3. The VSS/TSS ratio was close to 90% in all reactors. Rapid aerobic granules formation was also observed in other studies treating real industrial wastewater (Abdullah et al., 2013; Rosman et al., 2013). Other authors indicated that to achieve granulation on actual industrial wastewater a longer time is required (Wagner et al., 2015). The granules formation in the present study may have been enhanced by the biofilm detachment present in the effluent of the anaerobic pre-treatment system (Yu et al., 2013). The inactive cells and particulate material in suspension could have acted as aggregation nuclei for granular structure development.



Aerobic granules were characterized by a yellow-brownish appearance with a regular and smooth surface. Aerobic granules were dominant after 4 weeks, when their average sizes reaching 1.2 mm, 1.9 mm and 1.6 mm in R1, R2 and R3 respectively. The granular sludge structure remained stable in the first month of operation in which the granulometric distribution was quite homogeneous and no apparent signs of degranulation were observed. After 35 days, irregular shaped granules were visible within all reactors. These granules were characterized by uneven structure and filamentous structures were clearly visible on their surface. Afterwards, the biomass was a mixture of flocculent and granular sludge. Gradually, a consistent shift to flocculent sludge was observed. Similar results were reported by De Kreuk et al. (2010) who showed that aerobic granules grown on wastewaters rich in particulate and slowly biodegradable substrate gradually evolved toward irregular and unstable structure. These authors observed abundant presence of filamentous bacteria structure on the outer layer. The cause was identified to be the hydrolysis of the slowly biodegradable substrate previously adsorbed on the granules surface. This led to a continuous availability of substrate during the aeration period and consequently an extended feast period. Therefore, the presence of substrate micro-gradient distribution within the granular structure favoured the growth of filamentous organisms that caused the development of irregular shaped granules (Martins et al., 2004). The authors suggested that a longer anaerobic feeding period could favour the hydrolysis of more complex substrate and avoid the development of irregularity in granular structure. However, Wagner et al. (2015) demonstrated that at long anaerobic feeding period (90 min) filamentous structure were still present. The filamentous development and the instability of granular sludge must therefore be avoided by different means than anaerobic feed duration. Val del Río et al., (2012) demonstrated that the proper balance of feast/famine conditions inhibits the proliferation of filamentous microorganisms and favors the growth of floc-forming bacteria capable of substrate storage

To ensure complete degradation of particulate and slowly biodegradable organic matter, and promote a proper balancing of the feast and famine periods, an extended famine regime was imposed by increasing the cycle length in Period II.

In a matter of weeks the granular structure fully recovered. Granular sludge, in fact, was observed to be very homogeneous, with an average diameter close to 1.5 mm, 1.2 mm and 1.0 mm in R1, R2 and R3 respectively. At steady state, mixed liquor solids concentration was close to 2.9 gTSS L<sup>-1</sup> in R1 and R2 with 2.04 gTSS L<sup>-1</sup> in R3. The VSS/TSS ratio was approximately close to 90% in all the reactors. Compared with the previous period, the granules resulted on average slightly smaller size, because a lower OLR was provided (Di Bella and Torregrossa, 2013). The granules did not show irregular structure and were stable for the entire period, indicating that the new operating conditions were more appropriate for their stability.

### 3.2 Organic carbon removal

In Period I, when stable granular sludge was achieved, the three reactors were able to remove more than 80% of TCOD, 85% of the SCOD and 65% of the PCOD. In Period II, SCOD removal efficiencies were comparable to the previous period, while TCOD and PCOD removal was slightly higher. The particulate COD removal efficiency averaged 87%, 83% and 81% for R3, R2 and R1, respectively. The longer reaction time ensured a greater efficiency mainly because of the increase of the HRT. Hence, bacteria had more time to hydrolyze and degrade the particulate matter leading to a significant improvement in the PCOD removal efficiency. Moreover, since the mechanism of the PCOD removal required its adsorption on the granules, their structural stability improved the adsorption efficiency. The higher PCOD removal efficiency of R3 is due to a smaller granular structure and therefore higher surface area compared to the other reactors. Figure 1 shows the trends of SCOD and PCOD concentrations, measured in the mixed liquor supernatant, during the cycle in both experimental periods.

[INSERT FIG.1]

During both experimental periods, the SCOD was mainly degraded between the first and second hour in Period I and Period II, respectively (Fig. 1 a and c). Concerning the PCOD concentration, in Period I it remained stable for an hour after aeration and slowly decreased within the next 30 min (Fig. 1b). Removal continued up until 4h, after that the PCOD removal rate slightly decreased. PCOD also remained stable for the first hour of aeration during Period II. Compared to the previous period, PCOD removal occurred more rapidly in the first part of the aerated phase. For the remainder of the cycle PCOD was removed at a similar rate to Period I. Overall, the kinetics of the PCOD removal were characterized by two different slopes. In particular, the first part of the curves that were characterized by a higher slope (Fig. 1b and 1d) likely represented the particulate matter adsorption on the granules surface, followed by the slow and gradual hydrolysis of the adsorbed material. The second part of the curves were instead characterized by a significantly lower removal rate, that identified the conclusion of the feast period and the beginning of the famine (de Kreuk et al., 2010).

In Period I, the COD removal rate changed after 4h in all the reactors, while in Period II after 8h in R1 and R2 while after 5h in R3. Hence, the famine period lasted less than 2h in Period I and approximately 4-7h in Period II.

### 3.3 EPS variation during the cycle

The specific EPS content was measured through the reaction cycle. Samples were taken more frequently at the beginning of the aeration period (every 15 min) until 1 h corresponding with the plateau of soluble COD in the bulk solution. Afterwards, the sampling time intervals were 2 h until the cycle end. Figure 2 reports the Period I and Period II typical not-bound EPS (NB-EPS) and tightly bound EP (TB-EPS) profiles within the cycle in R1, R2 and R3.

## [INSERT FIG.2]

During Period I (Fig.2a), three different phases in the TB-EPS profiles were observed. At first an increasing trend in TB-EPS was found in all three reactors. This trend followed the SCOD consumption profile and lasted until the 45<sup>th</sup> min of the cycle. The extracellular polymers were therefore produced by microorganisms metabolism uptaking part of readily biodegradable SCOD into storage as TB-EPS. The rate of SCOD converted into intracellular polymers was not evaluated in this study. Afterwards, another increasing trend was observed (from the 75<sup>th</sup> min to 4h) which represents the hydrolysis and uptake of PCOD into TB-EPS storage for further metabolic degradation (Wagner et al, 2015). After 4h, the TB-EPS content started decreasing because bacteria in the absence of external carbon source consumed EPS to produce energy for their metabolism. This time coincided with the beginning of the famine phase.

In Period I, TB-EPS profiles revealed a short starvation time and the feast phase lasted for 4 h on average. In general, an appropriate feast phase might have a duration no longer than 20-30% of the entire cycle length (López-Palau et al., 2012). In Period I, 60% of the cycle was occupied by feast phase and this explains an improper feast/famine balance leading to unstable granular structure. Therefore, the famine phase was not long enough to support the granular structure over filamentous microorganisms development causing the progressive deterioration of the spherical shape (Corsino et al., 2016b).

The NB-EPS (Fig.2b) profile indicated that a significant increase of its concentration occurred during the anaerobic feeding period (40 min) in all three reactors, likely deriving from the TB-EPS hydrolysis. In literature, it is reported that anaerobic conditions could influence the production of EPS. In particular, the uptaken EPS in the sludge would decrease under anaerobic conditions (Sheng et al., 2010). The activated sludge flocs tend to deteriorate under oxygen limitation due to resuspension and/or the hydrolysis of EPS. Therefore, the increased NB-EPS fraction is detrimental

for the granules' stability. For these reasons, the anaerobic feeding duration of 40 min in Period I was not increased in the second part of the study.

During Period II (Fig.2c,d), three similar trends in the TB-EPS concentration to Period I were observed. The extended feast phase accounted for about 6h in R1 and R2, while 4h in R3.

Therefore, the famine phase lasted 6h in R1 and R2, while was approximately 8h in R3. A greater difference between maximum and minimum values of the TB-EPS concentrations compared to period I was observed, indicating that a longer starvation phase stimulated bacteria to degrade more extracellular matrix. Compared with Period I, different operating conditions and longer cycle length allowed in Period II to have better balance in feast/famine distribution within the reaction time. As previously discussed, this ensured a better stability of the aerobic granules structure that were characterized by an outer surface being more regular and round-shaped compared to the granules in Period I.

### *3.4 EPS characterization and composition*

In this study the extracellular polymeric substances were fractionated into TB-EPS and NB-EPS. In general, the EPS have different characteristics and functions in the aerobic granules structure (Yan et al., 2015). The TB-EPS are responsible for granular sludge structure and morphology. However, the not-bound fraction (NB EPS) affects only the surface properties of the granules and is not responsible for any specific structural characteristic (Sheng et al., 2010). In this study the loosely-bound EPS was considered as not-bound EPS. In Fig. 3a the average TB-EPS content in period I and II is compared.

**[INSERT FIG.3]**

Figure 3 a shows the average TB-EPS content in the three AGS reactors in Period I and Period II.

Under the same cycle length conditions, no significant relationship between the EPS content and the OLR was observed. In Period I, the EPS content ranged between 160 mg EPS gVSS<sup>-1</sup> (R1) and 200 mg EPS gVSS<sup>-1</sup> (R3). Overall, the EPS content slightly increased as OLR decreased but a different trend was observed in Period II where the EPS content was comparable in all three reactors ranging from 75 mg EPS gVSS<sup>-1</sup> (R2) to 90 mg EPS gVSS<sup>-1</sup> (R1). However, by comparing the results in Period I and Period II, a significant reduction in EPS distribution was observed. The EPS concentration decreased as much as 50% from Period I to Period II and in the latter the distribution did not have a correlation with the different OLR applied within the same period. It can be hypothesized that the reduction in the influent biodegradability due to the lower BOD influent concentration in Period II, decreased the bacterial EPS production.

Figure 3b shows the average ratio between the NB-EPS and TB-EPS during the experiment in R1, R2 and R3. In Period I and Period II, the not-bound EPS fraction increased with the increased OLR. In R1, the not-bound fraction of the total EPS was close to 30%, while it was approximately 20% and 12% in R2 and R3, respectively. The correlation could be related to the bacteria metabolic activity that causes a higher EPS hydrolysis in the starvation period during endogenous respiration. The different operating conditions in Period II resulted in an increase of the NB-EPS in R1 and R2 over that of Period I, with decrease in R3. The minimization of EPS content in solution resulted in an efficient production/consumption that optimized the granular sludge structure. Therefore, operating at longer aeration periods reduced the NB-EPS and promoted granular sludge stability. The difference in EPS fractionation and amount of proteins (PN) and polysaccharides (PS) is shown in Figure 3c. It was found that the protein fraction was much more abundant compared to carbohydrates and PN/PS ratio ranged between 8 and 15 during both period. The PN/PS ratio increased with the cycle length and was observed increasing as the OLR decreased. A high PN/PS ratio is shown to be beneficial for the granular structure analyzed under stereomicroscope. The proteins enhance the sludge hydrophobicity leading to an improvement in cells self-adhesion

capacity, which promotes granular aggregation (Zhang et al., 2011). For these reasons, R3 presented the best structural physical characteristics of granular biomass.

Figure 3d reports the ratio between the EPS consumed during the famine phase and the EPS produced during the feast phase. In Period I, only 37% of the EPS on average were consumed by microbial activity independently from the OLR applied. However, in Period II an expected significant increase in EPS consumption (68% on average) was observed with an extended starvation phase in all reactors, confirming that famine condition actually occurred in this period. In R1 about 60% of the EPS were consumed during the famine period, while it was higher in R2 and R3 (70% on average) suggesting a negative correlation between EPS degradation and OLR. The long starvation period and the lack of available substrate for microbial activity lead to a greater bacterial utilization of stored organic material.

Overall, the reactor with lower OLR (R3) resulted to have the least amount of NB-EPS and higher content of TB-EPS both mainly protein based. Evidence in structural stability confirm that extended famine conditions and low OLR contributed to the health and stability of the granular structure which is most suitable in treating industrial wastewater.

### 3.5 Substrate conversion to EPS

Extracellular polymeric substances are produced from bacterial metabolism and secreted outside the cell walls. Heterotrophic microorganisms are able to convert the organic substrate into various forms of EPS. Many factors affect the EPS production such as substrate availability and type of organic material (e.g., soluble or particulate), growth phase status, operating conditions (e.g., OLR, SRT), and shear forces (Sheng et al., 2010). In this study during the cycles two different EPS production/consumption phases were noted. The first, from the beginning of the cycle starts including anaerobic feeding to minute 45 in Period I and the 75<sup>th</sup> minute in Period II, while the second occurred approximately in the middle of the cycle. Kinetic tests on COD confirmed that at the beginning of the cycle more than 90% of the soluble COD was removed. Therefore, in this first

case, the EPS was produced from the microbial utilization of the readily biodegradable substrate present in soluble form. In the second phase, the EPS production was attributed to consumption of particulate substrate. Particulate matter previously adsorbed on the granules' surface was hydrolysed and became available for EPS production.

Figure 4 presents specific TB-EPS produced per unit of soluble COD (Fig.4a) and particulate COD (Fig.4b) removed.

**[INSERT FIG.4]**

The amount of soluble COD converted to EPS rose with the increase in cycle length and the decrease of OLR. During Period I, the TB-EPS production for unit of soluble COD removed was at its minimum in R1 ( $20 \text{ mgTB-EPS gVSS}^{-1} \text{mgSCOD}^{-1}$ ), more than double in R3 ( $60 \text{ mgEPS gVSS}^{-1} \text{mgCOD}^{-1}$ ) and was about  $35 \text{ mg EPS gVSS}^{-1} \text{mgCOD}^{-1}$  in R2. In Period II, however, a significant increase (almost doubling) in specific EPS production, maintaining the same relationship with OLR, was observed.

It was found that the increasing of the famine period and the reduction of OLR led bacteria to produce more storage products as TB-EPS (Fig. 3a). Under extended starvation period (Period II), the microorganisms in the granules core tend to produce more storage products to sustain metabolic activity while the substrate availability is lower. For these reasons, supported also by microscopical structural evidence, the stability of AGS is strictly related to EPS production and composition. It was concluded that low OLR and long reaction cycle, stimulate the production of EPS and optimizes its consumption.

The EPS production per unit of particulate COD removed confirmed that the R3 reactor, at the lowest organic loading, is more efficient in TB-EPS storage. The adsorption process is related to the surface area of the granules. The smaller the granules are, as microscopic observation in R3 revealed, the higher is the surface area available for particulates to sorb and hydrolyze. For this



reason, the amount of particulate COD removed in general increases with decreasing granule size. The EPS production from particulate COD decreased in Period II, when the cycle was extended from 6 h to 12 h, but a no definitive explanation cannot be offered as to why the decrease happened. One explanation may be offered that that lower bacterial metabolic activity reduced the capability to hydrolyze the substrate and therefore negatively affected EPS production.

#### 4. Conclusions

The effects of extended famine conditions within the SBR cycle and different OLR on the stability of AGS treating brewery wastewater were studied. The following conclusions were made:

- 6 hour cycle (Period I) produced unstable granules independently from the OLR because the feast/famine phases were not balanced;
- During the 12 hour cycle (Period II) stable granules were achieved because of a more proper distribution of the feast/famine periods. The granules developed at lower OLR were structurally more compact than at higher loadings, due to the highest EPS content, the lowest not-bound EPS fraction and the highest PN/PS.

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### Figure caption

Fig.1: Soluble and particulate COD profiles during a cycle in one representative day in Period I (a, b) and Period II (c, d).

Fig.2: Specific tightly bound EPS (TB-EPS) and not bound EPS (NB-EPS) profiles during the cycle in one representative day in Period I (a, b) and Period II (c, d).

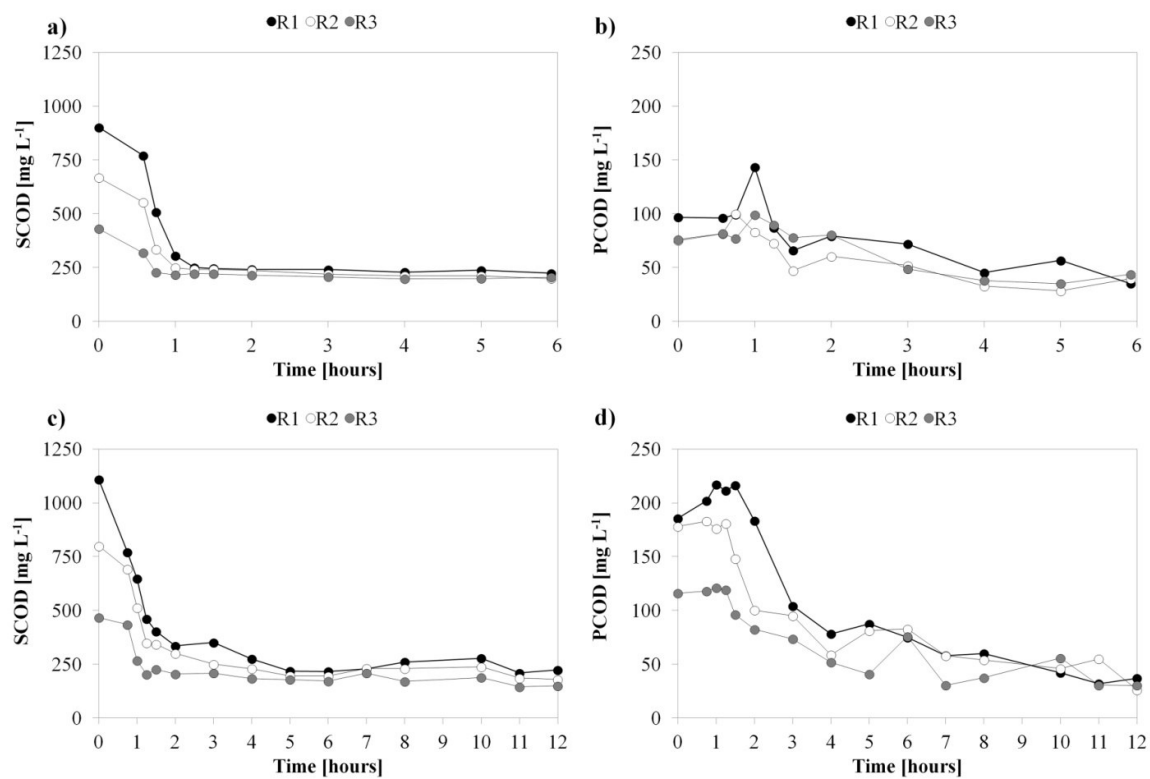
Fig.3: Average specific tightly-bound EPS content (a); not-bound and tightly bound EPS ratio (b); proteins to polysaccharides (PN/PS) ratio (c); ratio of tightly-bound EPS consumed to total TB-EPS produced (d); in R1, R2 and R3.

Fig.4: Specific TB-EPS production per unit of removed soluble COD (a) and particulate COD (b).

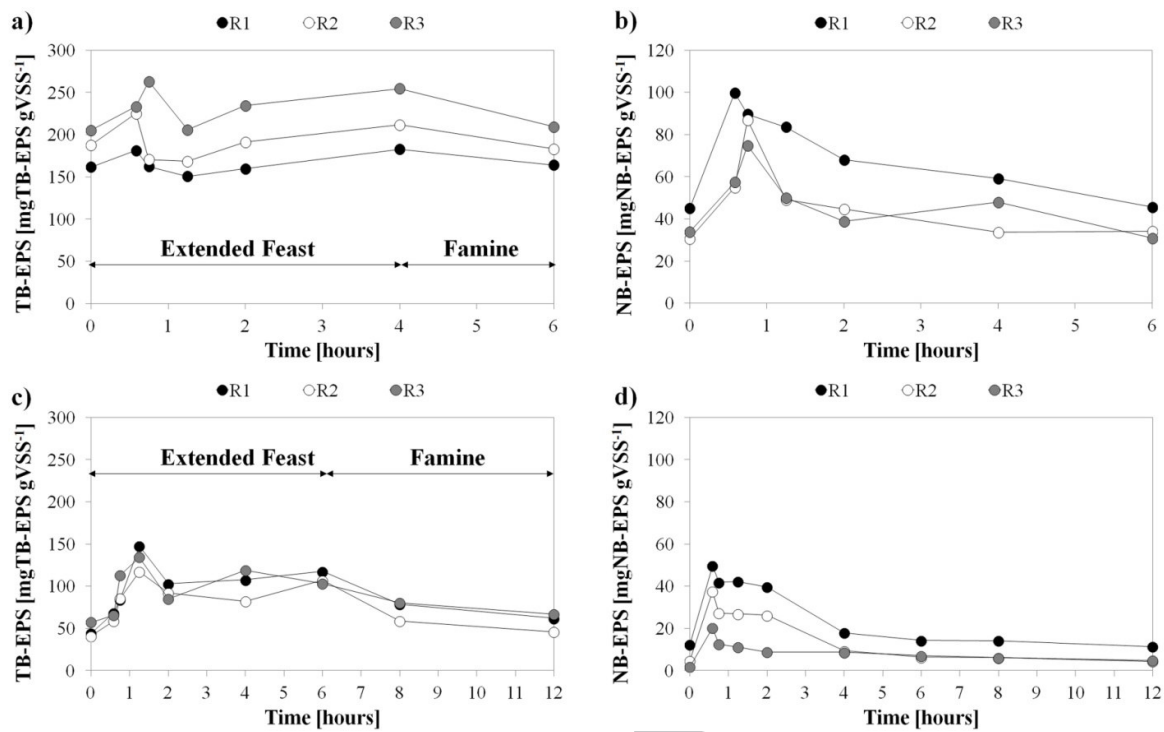
#### **Table legend**

Tab.1: Operational parameters.

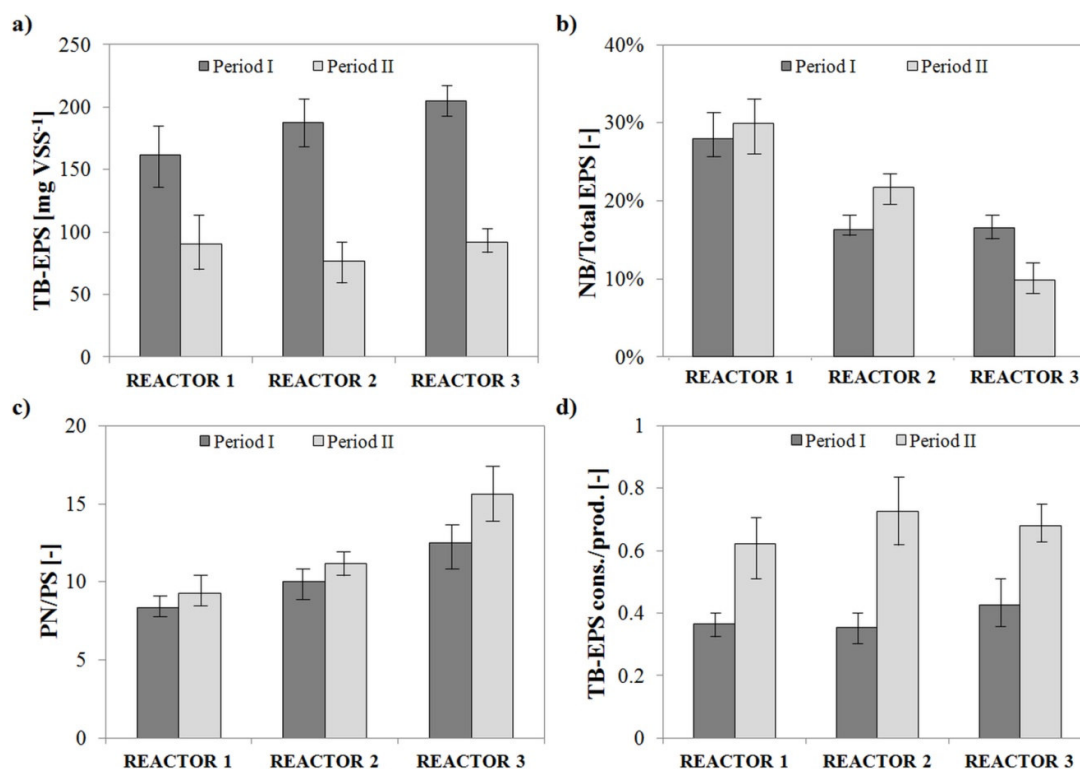
Tab.2: Averaged raw influent composition.



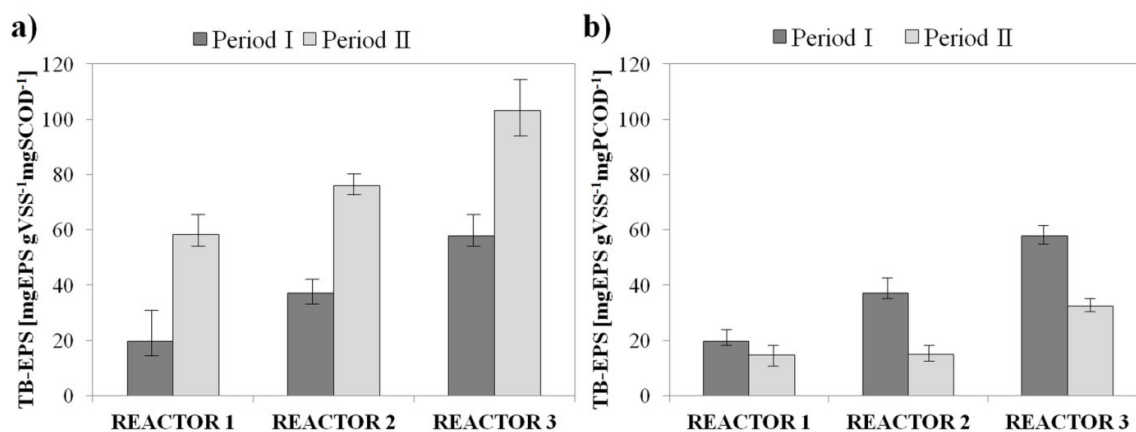
**Fig.1:** Soluble and particulate COD profiles during a cycle in one representative day in Period I (a, b) and Period II (c, d)



**Fig.2:** Specific tightly bound EPS (TB-EPS) and not bound EPS (NB-EPS) profiles during the cycle in one representative day in Period I (a, b) and Period II (c, d)



**Fig.3:** Average specific tightly-bound EPS content (a) ; not-bound and tightly bound EPS ratio (b); proteins to polysaccharides (PN/PS) ratio (c); ratio of tightly-bound EPS consumed to total TB-EPS produced (d); in R1, R2 and R3.



**Fig.4:** Specific TB-EPS production per unit of removed soluble COD (a) and particulate COD (b)



**Tab.1:** Operational parameters

	Parameter	R1	R2	R3	Units
Period I	Cycle length	6	6	6	h
	VER	0.75	0.5	0.25	-
	OLR	4.1	2.8	1.4	kg COD m <sup>-3</sup> d <sup>-1</sup>
	HRT	8	12	24	h
	Settling Time	4.5	3	1.5	min
Period II	Cycle length	12	12	12	h
	VER	0.75	0.5	0.25	-
	OLR	2.1	1.5	0.8	kg COD m <sup>-3</sup> d <sup>-1</sup>
	HRT	16	24	48	h
	Settling Time	4.5	3	1.5	min

VER: Volumetric Exchange Ratio; OLR: Organic Loading Rate; HRT: Hydraulic Retention Time;

**Tab.2:** Averaged raw influent composition

	Parameter	Values	Units
Period I	TCOD	1409	mg L <sup>-1</sup>
	SCOD	1199	mg L <sup>-1</sup>
	PCOD	210	mg L <sup>-1</sup>
	BOD <sub>5</sub>	800	mg L <sup>-1</sup>
	TN	136	mg L <sup>-1</sup>
	TP	20	mg L <sup>-1</sup>
Period II	TCOD	1345	mg L <sup>-1</sup>
	SCOD	1100	mg L <sup>-1</sup>
	PCOD	245	mg L <sup>-1</sup>
	BOD <sub>5</sub>	620	mg L <sup>-1</sup>
	TN	112	mg L <sup>-1</sup>
	TP	20	mg L <sup>-1</sup>

TCOD = Total Chemical Oxygen Demand; SCOD = Soluble Chemical Oxygen Demand; PCOD = Particulate Chemical Oxygen Demand;

BOD<sub>5</sub> = Biochemical Oxygen Demand; TP = Total Phosphorous; TN = Total Nitrogen

**Highlights**

- Granular sludge stability in the treatment of brewery wastewater was studied.
- Short duration cycle produced unstable granules independently from the OLR.
- Extended famine conditions and low OLRs improved the AGS structure.
- A proper feast/famine balance is crucial for maintaining stable aerobic granules.