Desalination and Water Treatment
www.deswater.com
doi:10.5004/dwt.2016.11120

61 (2017) 196–205
January

The role of extracellular polymeric substances (EPS) on aerobic granules formation: comparison between a case of synthetic wastewater supply and another of industrial wastewater

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Received 17 April 2015; Accepted 6 June 2016

ABSTRACT

The paper focused on the evolution and the comparison of the extracellular polymeric substances (EPSs) content during the granulation process in two Granular Sequencing Batch Airlift Reactors (GSBAR) (3,5 L) fed with synthetic (R1) and industrial wastewater (R2). The results showed that in both the reactors the EPSs, in particular proteins (PN), were mainly produced during the *feast* phase because of the high substrate availability, especially under conditions of metabolic stress. Then, the EPSs content reduced during the *famine* period, because of biodegradation by bacteria. More in detail, during the granulation process, a greater polysaccharides (PS) consumption occurred in both reactors, whereas the PN content reduced significantly only in R2. As a result, the PN/PS ratio increased significantly during the granules formation, confirming the key role of proteins on the granulation process. In R2, the granules produced a greater amount of PN on average, and this resulted in a more rapid granulation. Nevertheless, due to the several consumption of PN during the *famine* period, the granules in R2 resulted weaker and less dense compared with the granules in R1. For this reason, the granules in R2 were more susceptible to breakage. Overall, although the industrial wastewater favored a more rapid granulation, the excessive consumption of the EPSs, led to the structural weakening of the granules.

Keywords: Aerobic granular sludge; Granulation; Extracellular polymeric substances (EPSs); Feast/famine phases; Industrial wastewater

1. Introduction

Recently, aerobic granular sludge has attracted increasing interest, because of its high metabolic activity, large granules diameter of 0.2–9 mm, remarkable settleability and high biomass retention without any support media [1]. Due to the stratification of the microbial populations within the granule, the simultaneous removal of the organic matter, nitrogen and phosphorus can be achieved in a single unit [2,3]. Moreover, because of the dense structure, microorganisms residing within the granules are protected against toxic substances which could be in the bulk [4]. In fact, the concentration of the toxic compounds within the granules is generally lower with respect to the bulk, due to diffusion phenomena that limit their penetration toward the inner layers. Lastly, compared with conventional activated sludge, aerobic granular sludge has a greater organic treatment loading capability [5]. All these advantages make the aerobic granular sludge a better option to treat industrial wastewaters compared with the conventional activated sludge.

In recent years, many extracellular polymeric substances (EPSs) related researches have focused on their role on the aerobic granules formation and structural characteristics [6]. It is well known as the EPSs play a crucial role

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Presented at EuroMed 2015: Desalination for Clean Water and Energy Palermo, Italy, 10–14 May 2015.
Organized by the European Desalination Society.

on the granules formation. In fact, the EPSs matrix formed part of the microbial mechanism of a successful aerobic granular sludge formation. Moreover, the EPSs molecules form buffering layer for microbial cells against the harsh external environment, and in this context, the analyses of the extracellular polymeric substances in the treatment of the industrial wastewater has a crucial importance. Finally, the EPSs provide carbon and energy source during bacterial starvation phases [7].

The EPSs production is affected by the influent wastewater characteristics, as well as by the operating conditions. Consequently, the formation and the characteristics of the granules could be significantly influenced by the wastewater nature. Some authors asserted that the salinity promotes the EPSs production, following a particular mechanism of adaptation by microorganisms to high salt concentrations [8,9]. In another study, the authors observed that the EPSs content in the aerobic granules treating a mixture of synthetic and petrochemical wastewater was significantly affected by the petrochemical wastewater ratio in the feed [10]. Nevertheless, there are no studies that clearly analyze the relation between the wastewater, the EPSs production and the granules formation and characteristics. Furthermore, the role of the EPSs components, like proteins (PN) and polysaccharides (PS), on the granules formation and stability is quite controversial. In fact, if on the one hand some authors reported that the PS content in EPS was more abundant than the PN [10,11], on the other hand, other authors [12,13] asserted that the PN were the main component of EPSs. This was apparently related with the wastewater nature.

In general the EPSs content modifies in a SBR cycle. In fact, in a typical SBR reaction cycle, two different phases can be identified. During the former, that is characterized by high substrate availability (*feast* phase), microorganisms degraded substrate producing EPSs, which serve as carbon and energy source for the endogenous respiration during the latter period (*famine* phase). As a consequence, the EPSs content modifies during a reaction cycle. Many authors report EPSs values during the granules formation, however the EPSs were extracted at the end of the cycle. Therefore, no information are available about their changes during the cycle, and on how these changes evolved during the granulation process and affected the granules characteristics. In addition, it is possible that proteins and polysaccharides are degraded in a different way due to their different molecular structure, resulting in the variation of PN/PS ratio. It is reasonable that if the cycle is long enough, microorganisms could consume a considerable amount of EPSs during the long starvation period. Consequently, the granules stability could be compromised. This aspect must be taken into account in the treatment of industrial wastewater. In fact, due to low influent biodegradability, the cycle length, so the starvation period, is considerable long (7–11 h).

On the basis of what above discussed, it is clear that in the literature, there is lack of information on how the characteristics of the wastewater affect the EPSs production and composition and so the granules formation and stability. For these reasons, the aim of this paper was to analyse and compare the start-up phase of two GSBARs (volume 3.5 L) fed with acetate-based wastewater (R1) and a industrial wastewater called *slops* (R2). In detail, great attention has been paid to the role of the EPSs on the granulation process and on the granule characteristics, emphasizing the differences between the synthetic and the industrial wastewater supply on the EPSs production and composition, and consequently on the granules physical characteristics.

2. Materials and methods

2.1. Pilot plant description

The experimental study was carried out in two sequencing batch airlift reactors (SBAR) [14], named R1 and R2. R1 was inoculated with activated sludge collected from conventional activated sludge plant, whereas R2 was inoculated with activated sludge collected from a Biofilm-Membrane BioReactor (BF-MBR) bench scale reactor treating slop. In this reactor the conventional activated sludge was gradually acclimated to the slops, by gradually increasing its dosage in the influent. Then, only the suspended biomass was withdrawn and inoculated in R2. R1 and R2 operated for 212 and 154 d respectively, until the full granulation was reached in both reactors. R1 was feed with acetate-based synthetic wastewater, whereas R2 with a mixture of real and simulated slop. More specifically, R2 was fed with a mixture of real slop (20% in volume) and simulated slop (80% in volume) during the first 100 d, whereas in the last 54 d, the percentage of real slop was increased to 40%. In the simulated slop, a known amount of sodium acetate was added as carbon source.

The synthetic wastewater fed in R1 had the same composition of other synthetic wastewater reported in the literature [14]. The main characteristics of the R1 and R2 feeds are summarized in Table 1.

Two identical sequencing batch airlift reactor (SBAR) was used in this study. The reactors were column-type (internal diameter of 8.6 cm) and both had an internal cylinder working as riser (50 cm high, 5.4 cm internal diameter,

Table 1
R1 and R2 influent characteristics

Parameter	R1	R2	
		Period I	Period II
COD, mg L^{-1}	900 ± 15	1350 ± 50	1850 ± 30
BOD_5, mg L^{-1}	850 ± 20	1150 ± 50	1250 ± 15
TOC, mg L^{-1}	380 ± 10	550 ± 25	650 ± 10
$N–NH_4$, mg L^{-1}	90 ± 5	105 ± 5	110 ± 5
N_{tot}, mg L^{-1}	90 ± 5	115 ± 10	115 ± 5
P_{tot}, mg L^{-1}	25± 2	25 ± 3	25 ± 3
TPH, mg L^{-1}	–	6.8 ± 1.5	13.5 ± 2
Aromatic hydrocarbons, mg L^{-1}	–	1.18 ± 0.25	2 ± 0.25
Chloride, mg L^{-1}	225	25000	25000
Pb, mg L^{-1}	–	< 2	< 2
Cu, mg L^{-1}	–	< 2	< 2
Mn, mg L^{-1}	–	< 2	< 2
Zn, mg L^{-1}	–	< 2	< 2
Surfractant (SDBS), mg L^{-1}	–	30 ± 3	30 ± 3

bottom distance 5 cm). The air was introduced via a fine bubble aerator at the base of the reactor (5 L min^{-1}). Each reactor had a working volume of 3.5 L and a filling height of 60 cm. The effluent was discharge using an solenoid valve placed at 30 cm from the bottom of reactor, so the volumetric exchange ratio (VER) was 50% in each cycle. The reactors operated with different cycle length. Particularly, R1 was operating on a 3 h per cycle, included 10 min of influent feeding, 160 min of aeration, 5 min of settling and 5 min of effluent withdrawal. In R2 instead, due to lower influent biodegradability, a longer cycle length was imposed, so that the cycle length would not represent a limitation factor for the biodegradation of the recalcitrant compounds in the slop. For this reason, R2 was operating on a 12 h per cycle, included 10 min of influent feeding, 700 min of aeration, 5 min of settling and 5 min of effluent withdrawal.

Except for the cycle duration, the main operating conditions were the same for both reactors. A not too high hydraulic selection pressure was applied, in order to taking into account the effect of the salinity on the buoyancy in R2. More in detail, the hydraulic selection pressure was such that only the particles with a settling velocity higher than 3.6 m h^{-1} were kept within the reactors. Nevertheless, the value of the hydraulic selection pressure was in line with the literature reference values [4,6,8]. Due to the different COD concentrations in the influent media, the F/M ratio was balanced controlling the suspended solid (SS) concentration within the reactors. The C/N ratio was kept close to 10:1. The dissolved oxygen (DO) concentration was not controlled, consequentially its concentration ranged from 4 to 8 mg L^{-1} during the aeration phase. Cycling SBR operations has been automatically handled by a programmable logic controller (PLC). The main operating conditions are summarized in Table 2.

2.2. Analytical procedures

All the chemical–physical analyses (COD, BOD, NH_4 N, NO_3–N, NO_2–N, PO_4–P, TSS, VSS) were performed according to the standard methods [15]. At first, the size of the granules was measured by laser diffraction (Malver Mastersizer®, 2000 series) with tap water as the suspension medium and standard optical parameter. Hereafter, when the granules size exceeded 2 mm, their dimensions were analyzed by digital image capture by means a stereo-microscope; the granules size distribution was obtained by measuring a significant number of particles (approximately 500) using an image analyzer [3]. The granulation rate was evaluated as the percentage of the particles with a diameter over 600 µm [16]. The EPSs content of the granules was analyzed once per week at the end of both the *feast* and the *famine* period. To identify the end of the *feast* period, regularly the dissolved oxygen concentration within the bulk was monitored for all the reaction cycle. The end of the *feast* period, and the subsequent beginning of the *famine*, was easily identified in terms of oxygen consumption, which rapidly decreased after that the organic substrate was mostly degraded [3]. The EPSs extraction was carried out in accordance with the Heating Method [17]. More in detail, the mixed liquor samples were centrifuged at 5000 rpm for 5 min. The supernatant was filtered through a 0.22-µm membrane (Millipore, Fisher Scientific) to ensure a cell-free EPSs suspension, then the filtrate was used for SMPs determination. The precipitate was resuspended with deionized water and intermixed. The mixture was heat-treated for 10 min at 80°C, which let the bound EPSs extract from the sludge. The solution was cooled down to room temperature, followed by the centrifugation at 7000 rpm for 10 min. The supernatant, after filtration through a 0.22 µm membrane, was used for the EPSs determination. For each EPSs fraction (soluble and bound) the polysaccharides were determined according to the phenol–sulphuric acid method with glucose as the standard [18], whereas the proteins were determined in accordance with the Folin method with bovine serum albumin as the standard [19]. In this way the polysaccharides and proteins content of the SMPs (SMP, c and SMP, p) and of the EPSs (EPS, c and EPS, p) were determined. In this work, the total EPSs content (EPS_T), has been assumed by the sum of the protein and polysaccharides, then, their content was related to the volatile suspended solid concentration.

3. Results and discussion

3.1. Formation and performances of aerobic granular sludge

The aerobic granules developed successfully in both reactors. Nevertheless, the time required to obtain the complete granulation was different. In fact, about 210 d were necessary to obtain the full granulation of the sludge in R1, whereas in R2 it was achieved after 160 d. At the end of the cultivation period, the morphology of the aerobic granules in the reactors were quite different. The granules of R1 exhibited a regular outer shape and a white-yellow appearance, in accordance with other studies in which the aerobic granules were fed with acetate-based synthetic wastewater [20]. The granules of R2 instead, had a brownish colour and a smooth outer surface (Fig. 1). This difference was due to the different influent wastewater, which was synthetic for the former, and real (although in a minor fraction) for the latter. At the end of the cultivation phase, the granules in R2 were quite bigger with respect to the R1 granules.

In fact, the granules in R2 had a diameter of 2 ± 0.3 mm on average, whereas the granules in R1 were 1.5 ± 0.2 mm (Fig. 2a, c). The first bio-aggregates with a diameter over 0.6 mm started to be visible in R1 only after 44 d. In detail, the bulk was composed by a heterogeneous mixture of lit-

Table 2
R1 and R2 operating conditions

	Cycle length [min]	Settling time [min]	VER [%]	F/M [KgCOD Kg $VSS^{-1}d^{-1}$]	Air flow velocity [cm sec^{-1}]	C/N [–]
R1	180	5	50	0.42 ± 0.10	3.5	10/1
R2	720	5	50	0.40 ± 0.25	3.5	10/1

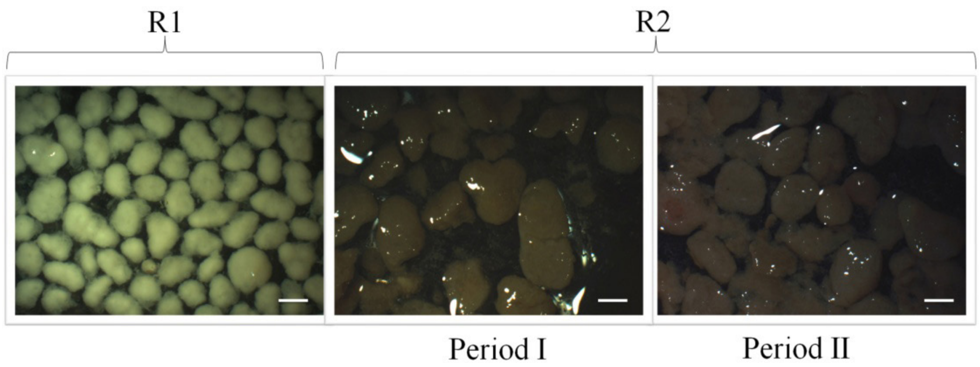

Fig. 1. Mature aerobic granules in R1 (218 d) and R2 (at 98 and 154 d) (scale bar 1 mm).

tle granules and flocculent sludge. The granulation rate in R1 ranged between 30–40% for about 190 d. Hereafter, it increased very quickly in less than 20 d to 100%, when the sludge became totally granular (Fig. 2b). In R2 large granules with diameter over 1 mm developed after 15 d. Gradually, granules with diameter of 2 ± 0.5 mm became prevailing in the reactor at 30 d, after that mature granules resulted dominant in bioreactor, with a granulation rate close to 60% (Fig. 2d). However, the granulation rate did not increase in the following days, reaching a steady-state value of 50% at the 100 d. Due to the difference in COD concentration and in the cycle duration, the volumetric load rate was higher in R1 (3.6 Kg COD $m^{-3}d^{-1}$) then R2 (1.3 Kg COD $m^{-3}d^{-1}$). To obtain the full granulation, after the 100th the volumetric loading rate was increased. In detail, the ratio of the real slop in the feed was increased to 40%, resulting in a volumetric load rate of 1.8 Kg $CODm^{-3}d^{-1}$. At first, a rapid breakdown of the granules was observed, due to the shock load as also observed by other authors [21]. This resulted in a significant reduction of the granulation rate which dropped below the 10% in 30 d. Hereafter, the sludge adapted to the new conditions and the aerobic granules developed again, reaching the total granulation after 160 d. It is possible that the increase of the volumetric load rate enhanced a rapid development of the granules. In fact, as reported in other studies, although the volumetric load rate do not have a crucial role in the granule formation, it accelerates the granulation process [22]. A volumetric load rate lower than 1.5 Kg COD $m^{-3}d^{-1}$, led likely to low granulation rates, whereas an higher value promoted a faster formation of larger granules and the complete granulation.

At the end of the cultivation period, the granules in R1 and R2 showed significant differences in terms of size, density and structural/morphologic features. As previously mentioned, the granules in R1 were slightly smaller than R2 ones. The average sizes of the mature granules were

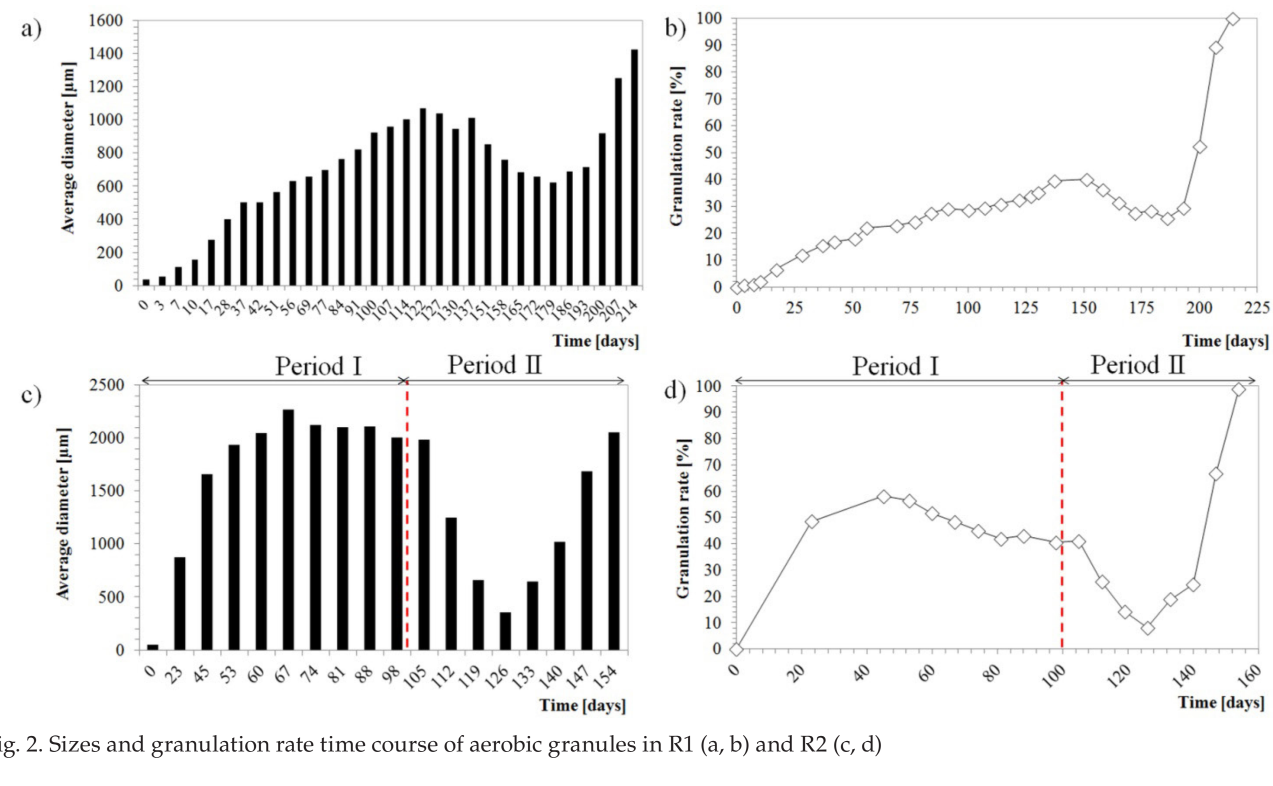

Fig. 2. Sizes and granulation rate time course of aerobic granules in R1 (a, b) and R2 (c, d)

1.5 ± 0.2 mm and 2.3 ± 0.3 mm for R1 and R2 (period I) respectively. However, the granules in R1 exhibited a significantly higher density compared with those in R2. In fact, the average density was 240 g TSS/L granules in R1, whereas in R2 it resulted significantly lower (80 g TSS/L granules). Therefore, although in R1 the granules developed more slowly and were smaller compared with R2, their structure wasmuch stronger. During the second period in R2, after that the granules crushed, a new process of granulation occurred. In this case, the granules were slightly smaller with respect to the previous period (2.0 ± 0.3 mm), but their density was significantly higher (130 g TSS/L granules). However, on the whole, in R2 the granules were smaller and less dense with respect to R1. These results confirm that the aerobic granules cultivated with acetate-based synthetic wastewater are more dense than the granules cultivated with industrial wastewater [23,24].

The TSS concentrations ranged between 10–12 g TSSL-1and 4.5–5.5 g $TSSL^{-1}$ in R1 an R2 (period I) respectively. When the complete granulation was achieved, the TSS concentrations in R1 and R2 were respectively 14 and 6.5 g $TSSL^{-1}$. BOD and COD removal efficiencies were abundantly higher than 90% in R1 and R2. When the organic load was changed in R2, the BOD/COD ratio in the influent decreased from 0.8 to 0.65. The COD efficiency removal decreased by up to 70% (between the 100th and 125th day). Contrarily, the BOD efficiency removal remained stable close to 90%. It is possible that the granules breakdown caused a lower removal of the particulate substrate. The particulate substrate was an important fraction of the organic matter in the real slop. As reported by other authors [25], the large molecules of slowly biodegradable COD are initially adsorbed on the granules surface, and then gradually hydrolyzed and finally degraded, whereas the readily biodegradable substrate, for the most part constituted by the sodium acetate of the simulated slop, was quickly oxidized in any case. The disappearance of the large granules caused the increase of the particulate organic matter concentration in the effluent, therefore, although the readily biodegradable substrate was almost completely oxidized, the overall COD efficiency removal decreased. Gradually, the COD removal efficiency increased up close to 93% when the aerobic granules aggregated again. When steady-state conditions were achieved, the total nitrogen removal efficiencies were close to 70 and 50% in R1 and R2 respectively, whereas the phosphorous removal efficiencies were approximately close to 50% in both reactors.

3.2. Comparison of the EPSs production during the granulation

The EPSs are recognized to have a crucial role in the granular sludge formation and stability. The EPSs form a thick matrix that embed microorganisms, resulting in a dense architecture of microbial cells. In addition, the EPSs molecules form buffering layer for microbial cells against the harsh external environment [7]. Several studies reported that the EPSs content changes in a typical SBR reaction cycle [4,6]. More in detail, at the beginning of the cycle, that is characterized by high substrate availability (*feast* phase), microorganisms degrade the organic substrate producing EPSs, which will serve as carbon and energy source for the endogenous respiration that characterizes the rest of the cycle (*famine* phase). However, few authors reported EPSs variation during the reaction cycle in a granular sequencing batch reactor [6]. Therefore, in the literature there is a lack of available information about the EPSs changing during the cycle, and how it evolves during the granulation process. This aspect need to be in depth analysed because it could significantly affect the granule formation and characteristics, because the EPSs content and composition could modify especially in the field of the industrial wastewater treatment. To clarify this, the proteins (PN) and polysaccharides (PS) content of the granules was analyzed at the end of both the *feast* ($PN_{,F}$ and $PS_{,F}$) and the *famine* phases ($PN_{,f}$ and $PS_{,f}$) during the whole granulation period.

Concerning R1, throughout the granulation process, the EPSs content showed a "saddle" trend, in both the *feast* and the *famine* phases, before the achievement of steady-state conditions (Fig. 3a, b).

During the earlier stages of the granulation process, a significant EPSs production was observed [8]. As a whole, the protein content was much higher compared with the polysaccharides in both the *feast* and the *famine* phases. More in detail, the content of the polysaccharides and the proteins rose quickly during the first two weeks, from 140 mg PN/g VSS and 25 mg PS/g VSS in the seed sludge, close to 280 mg PN/g VSS and 50 mg PS/g VSS. During this period, microorganisms started to adapt to the new conditions of intermittent feeding, the substrate and to the high shear forces given by the reactor geometric configuration. In such conditions, microorganisms began to regulate their metabolism producing more EPSs [6]. Consequently, the EPSs content grew rapidly and the first bio-aggregates appeared in the reactor. The granules were dispersed in a suspension of flocculent sludge, so that the granulation rate was quite moderate. Other authors reported that when the EPSs content reach the peak level, then gradually started in decreasing [8,26]. Only general explanation are given to this evidence, so this aspect is not yet clear. In the earlier stages of the granulation process, due to the severe sludge washout, the biomass concentration within bioreactor decreased, leading to increase of the F/M ratio. As a consequence, the metabolic stress conditions for microorganisms reduced, because the substrate availability increased and for this reason they produced a lower amount of EPSs. This results confirm those reported by other authors, which observed a linear correlation between the EPSs content and the TSS concentration [27]. When the poor-settling microorganisms were discharged, and the microbial community with good settling capability enriched in the reactor, the biomass concentration increased again. In contrast of what previously observed, the F/M decreased, leading to new stress conditions for the biomass. In response to the metabolic stress, bacteria started to secrete again a significant amount EPSs, so the PN and the PS content increased. In this phase, other dense and stable granules formed, and the sludge became mainly granular (maturation phase). The EPSs content maintained quite stable, with a significant prevalence of the protein fraction over the polysaccharide. At the 140th d, a long plant showdown occurred (5 d), during which sludge was neither fed nor mixed. This occurrence, caused a dramatic granules breakdown, resulted in the reduction of both the granules sizes and the granulation rate. Consequently, a severe sludge wash-out occurred when reactor

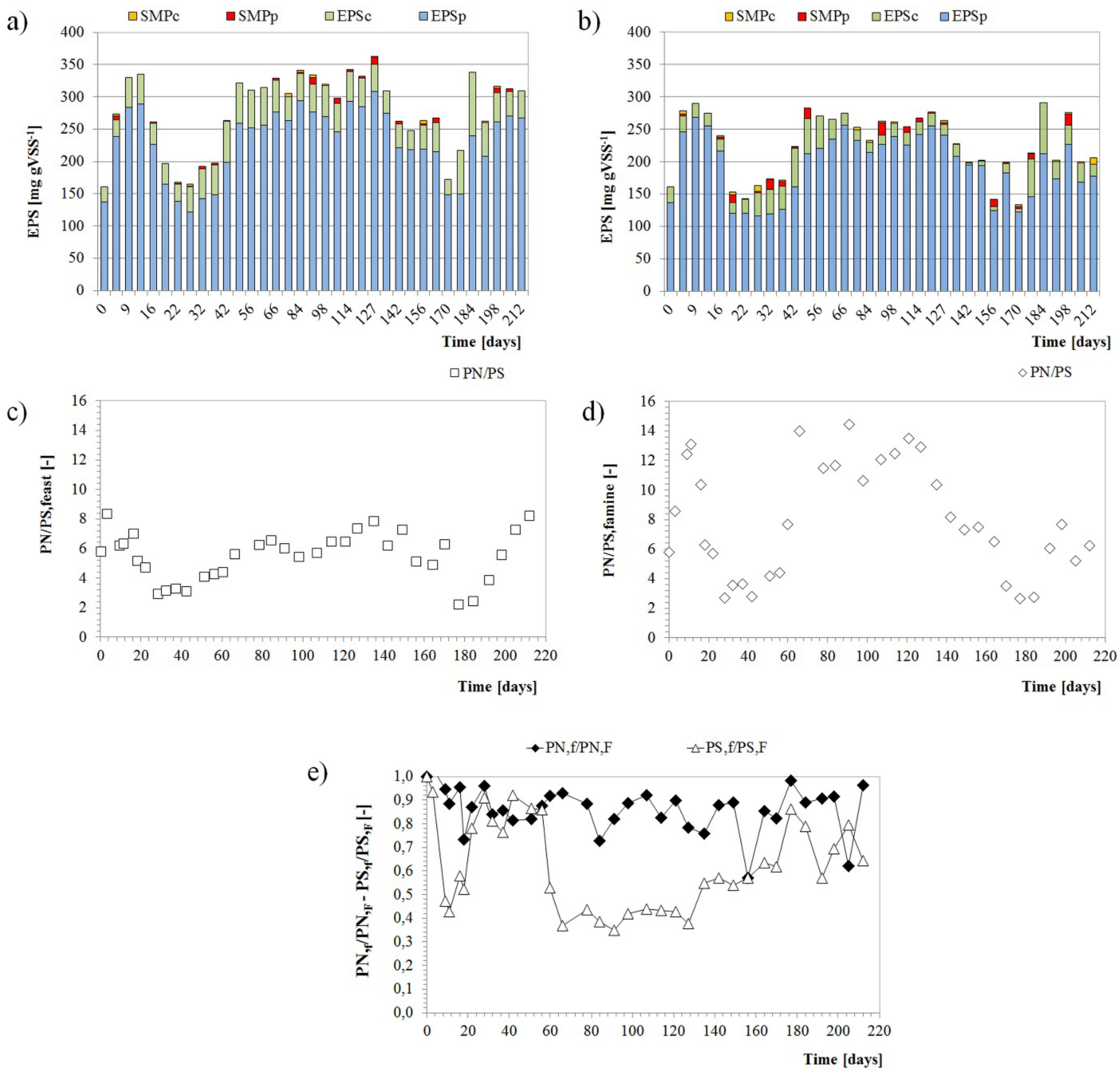

Fig. 3. Trend of the EPSs content and the PN/PS ratio during the granulation process in R1 at the end of feast (a, c) and famine phases (b, d); time course of the $PN_{,f}/PN_{,F}$ and $PS_{,f}/PS_{,F}$ ratio (e).

started up again. The F/M ratio increased in response to the reduction of the biomass concentration, therefore, as previously explained, the EPSs content reduced again. When new steady-state conditions were achieved, the EPSs content increased again, and the granulation process was finally completed.

A similar trend was observed concerning the PN/PS ratio, both in *feast* and *famine* phases (Fig. 3c, d). More in detail, concerning the *feast* phase, in the earlier stage of the granulation process, this ratio slightly increased and reached its maximum value in correspondence at the highest EPSs value measured on 16th day. Afterwards, the PN/PS ratio significantly decreased when the biomass wash-out occurred. Later, when poor-settling microorganisms were discharged, the PN/PS ratio increased slowly toward a pseudo steady-state value close approximately to 7. The partial breakdown of the granules caused a reduction in this ratio. Hereafter, the PN/PS ratio increased rapidly to steady-state conditions until total granulation was achieved. These results indicated that microorganisms produce the same amount of polysaccharides on average during the *feast* phase, independently of the stress conditions. In contrast, under metabolic stress conditions, microorganisms were stimulated to produce a higher amount of proteins, resulting in the increase of the PN/PS ratio. A similar trend was observed during the *famine* phase. However, in this case, the PN/PS ratio fluctuated in a larger range (2–14). The graph in Fig. 3e shows the ratio of the PN and the PS both evaluated at the end of the *feast* ($PN_{,F}$ and $PS_{,F}$) and the *famine* phases ($PN_{,f}$ and $PS_{,f}$) respectively. The PN_f/PN_F ratio fluctuated around a stationary value close to 0.85. This result suggest that during the starvation period microorganisms degraded about the 15% on average of the proteins. Contrarily, the PS_f/PS_F ratio significantly fluctuated during the granulation process. In fact, it reached its maximum value (0.8) when the EPSs production was minimum, and *vice versa* its minimum value (0.2) when the EPSs production was maximum. Under conditions of metabolic stress, during the *feast* phase microorganisms produced a higher amount of proteins with respect to the polysaccharides. Instead, during the *famine* phase, microorganisms degraded polymers for the endogenous respiration. Microorganisms degraded polysaccharides as a priority with respect to the proteins, due to the more simple molecular structure of the polysaccharides. As a result, the content of polysaccharides significantly reduced at the end of the *famine* phase. In contrast, the content of the proteins did not reduce, due perhaps to the short duration of the *famine* phase (about 2 h). Overall, under conditions of metabolic stress, the PN/PS ratio increased due to both proteins production and to the polysaccharides consumption during the starvation period. As reported in Fig. 3c, d, the time course of the PN/PS ratio, except for the first two weeks, resulted very similar to the trend of the granulation rate. These findings confirm those reported by other authors which observed that the increasing of the PN/PS ratio is accompanied by the formation of the aerobic granules [9]. In conclusion, at the beginning of the granulation the sludge enriched in proteins and the granules were forming. When the EPSs consumption reduced, PN/PS ratio reached a steady-state value, that implied the beginning of the maturation phase.

Concerning the soluble polymers, at the end of the *feast* phase (Fig. 3a), the SMPs release occurred occasionally, in particular when the granules reached steady-state conditions in terms of sizes. At the end of the *famine* phase instead (Fig. 3b), the SMPs release resulted more frequent, likely due to the lack of the organic substrate that induced the EPSs hydrolysis [28,29]. Overall, the SMPs concentrations was rather moderate. Therefore, it is possible that lysis phenomena did not occur and the SMPs release derived only from a partial hydrolysis of the EPSs. The SMPs were mainly constituted by proteins.

Concerning R2, the EPSs content resulted higher than R1, especially at the end of the *feast* phase. This result could be related to different influent wastewater. As reported in other studies [12,13,30], in condition of high salt concentration, microorganisms regulate their osmotic pressure to the external, involving a modification of their metabolism and consequently a higher EPSs production.

Regarding the EPSs trend, it resulted comparable with R1 (Fig. 4a, b). According to the trend observed in R1, at the end of the *feast* phase (Fig. 4a) the EPSs content increased during the first weeks, due to the micro organisms acclimation to the new conditions within the reactor. Hereafter, a significant reduction characterized the following three weeks as a consequence of the sludge washout. Finally, the EPSs content increased again and reached a stationary value when the maturation of the granular sludge occurred. After the 100th d, the ratio of the real slop in the influent wastewater increased up to 40%, so the volumetric load rate (VLR) increased. The effect of the VLR increase was the disintegration of the granules, as also observed by other authors [31]. As a result, a massive washout determined the drop of the biomass concentration and the F/M ratio increase. Consequently, as previously explained, this condition determined the reduction of the metabolic stress toward microorganisms, which produced a lower amount of EPSs. Hereafter, the EPSs content increased again, and reached a steady-state value at the end of the experimental period. The EPSs concentration reduced significantly at the end of the *famine* phase (Fig. 4b), especially until to the 40th d. During this period, both the PN f/PN F and PS f/PS F ratio resulted very low, suggesting a severe consumption of the EPSs during the starvation period (Fig. 4e). As observed in R1, microorganisms mainly degraded the polysaccharide fraction, that reduced by more than 50%. In contrast with R1, the content of proteins reduced as well due to the longer starvation phase. After the 40th d, both the PN f/PN F and the PS f/PS F ratio slightly increased, as observed in R1. There is no a unique explanation to this result. It is possible that the metabolic activity of bacteria reduced with time, hence, the bacteria needed a lower amount of substrate to maintain their metabolism. As a result, when the maturation of the granules was achieved, microorganisms degraded a lower amounts of EPSs during the starvation period. Moreover, the feed in R2 was partially constituted by particulate, slowly biodegradable and recalcitrant substrates. As reported by other authors, microorganisms are able to oxidize these kind of substrates if a long contact time with contaminants is guaranteed [25,32]. Therefore, it is possible that those compounds were firstly adsorbed on the granules surface, and subsequently hydrolysed and oxidized. The hydrolysis of the particulate substrate, as well as complex molecules like hydrocarbons, could determine an increase of the substrate availability during the *famine* phase, so that microorganisms did not need to degrade the stored substrate, resulting in the lower EPSs consumption.The PN/PS ratio evaluated at the end of the *feast* phase increased significantly in the early stage of the granulation process, according to the granulation rate (Fig. 4c). In the following days, this ratio rapidly decreased in accordance with the EPSs reduction during this period. Hereafter, it showed a slightly increasing trend, until the end of the experimental period. At the beginning of the second period, the PN/PS ratio dropped because of the reduction of the EPSs production. Afterwards, it rapidly increased up to a steady-state value at the end of the experimental period, in good agreement with the granulation rate. As previously observed in R1, the granulation was accompanied by the increase of the PN/PS ratio. These results confirm that the proteins constitute the supporting structure of the granules. Moreover, the proteins are hydrophobic compounds, so they caused the increase of the sludge hydrophobicity, favouring in this way the cell adhesion and lastly the granules formation. The values of PN/PS ratio at the end of *famine* phase resulted quite similar to the *feast* (Fig. 4d), because the proteins and the polysaccharides were equally degraded during the starvation period. In R1, the PN/PS ratio significantly fluctuated because only the polysaccharides were in the most part degraded during the *famine* period, whereas the proteins consumption resulted often negligible. Moreover, in R2, especially during the first experimental period, the PN/PS ratio continuously decreased, and only at the end of the experimentation, it reached a steady-state value approximately close to 6. The longer starvation phase, due to the longer reaction

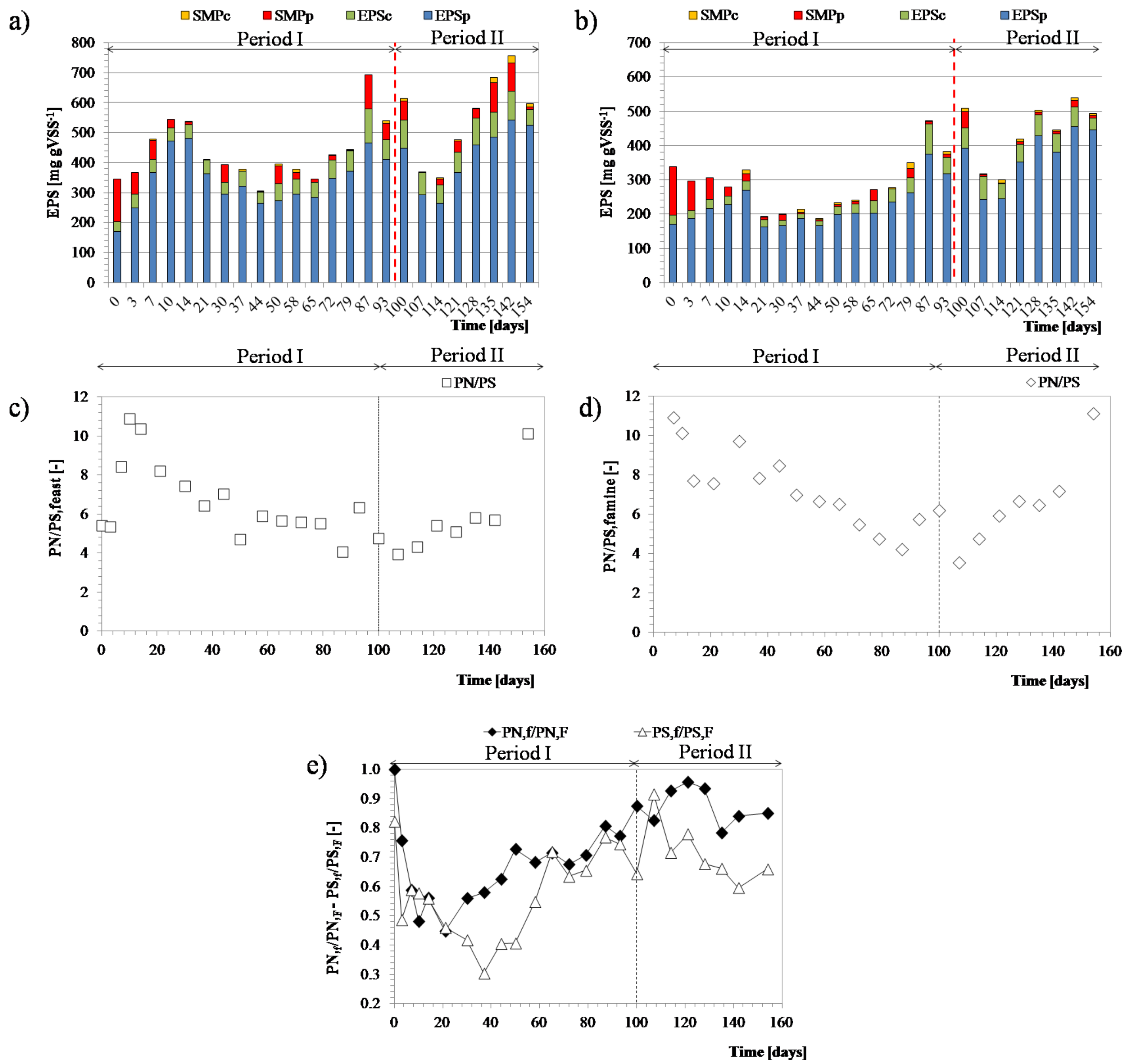

Fig. 4. Trend of EPSs content and PN/PS ratioduring granulation process in R2 at the end of feast (a, c) and famine phases (b, d); time course of $PN_{,f}/PN_{,F}$ and $PS_{,f}/PS_{,F}$ ratio (e).

cycle, determined a significant consumption of EPSs. As observed in R1, the significant consumption of the polysaccharides did not involve the granules weakening. In fact, if no proteins consumption occurred, the granules enriched in proteins and resulted structurally stronger. In contrast, the proteins in R2 reduced significantly during the starvation period. As a result, although the granules developed more rapidly in R2, their structure resulted weaker due to the massive consumption of the proteins during the starvation phase. During the second period, when the granules formed again, the consumption of the proteins during the *famine* period was lower with respect to the previous period, because of the increase of the VLR, which resulted in a major substrate availability during the long starvation phase. Microorganisms consumed mainly the polysaccharides that reduced of 40–50% on average. Consequently, the PN/PS ratio increased, and the granules enriched in proteins that improved their structural characteristics. In fact, although granules resulted slightly smaller with respect to those of the previous period, their density increased up to 130 g TSS/L granules.

With regard to SMPs, a significant amount of SMPs were observed, especially at the beginning of the granulation process in both the *feast* and the *famine* phases (Fig. 4a, b). Concerning the *feast* phase (Fig. 4a), the SMPs were in significant concentrations twice: the former at the beginning of granulation process, and the latter when the EPSs content reached its maximum values at 90 and 130 d. More in detail, during the first weeks, the SMPs release was likely due to microorganisms adaption to the new feeding and hydrodynamic conditions of the SBAR. In fact, during the first weeks, microorganisms were exposed to high shear forces that likely caused lysis phenomena. The seed sludge derived from a BF-MBR reactor. It is well know that the filtering action of the membrane determines a substantial reduction of the flocs sizes [33,34]. Therefore, the structure of the flocs resulted almost weak. Consequently, the long time exposure to the high shear forces caused the flocs detachment, leading to the release of the EPSs in the bulk in forms of soluble polymers. At 90 and 130 d, a further release of SMPs occurred. In this case, the granules were mature (90 d) or were forming (130 d). Because the granules size exceeded 2 mm, during the *feast* phase, when the substrate was rapidly removed mainly in the outer layers of the granules, both the substrate and the oxygen did not penetrate toward the core of the granules. Consequently, in the inner layers, the EPSs hydrolysis or lysis phenomena occurred, determining the SMPs release. Therefore, in this last case, the SMPs release resulted from the metabolic stress conditions, whereas in the previous case it is possible that physical phenomena were prevalent.

SMPs were also observed at the end of the *famine* phase (Fig. 4b). However, their concentration resulted generally lower with respect to the end of the *feast* phase. Unfortunately, it was not possible to clarify if during the *famine* phase further SMPs release occurred. In fact, generally the presence of SMPs at the end of the *famine* phase could be due to a long starvation phase [35]. In such conditions, firstly the hydrolysis of the EPSs, and later, the cell autolysis phenomena, could occur because of the substrate unavailability. Proteins were the main component of the SMPs, whereas the polysaccharides were missing. Likely the polysaccharides and the proteins fractions of the SMPs were both released in the liquid bulk during the reaction cycle, but microorganisms degraded the polysaccharides rapidly, therefore only proteins remained as SMPs at the end of aeration phase. After the first two weeks, SMPs gradually disappeared, and hereafter only occasionally were observed in significant concentrations.

4. Conclusions

The evolution and the comparison of the EPSs content during the granulation process in two GSBAR fed with synthetic and industrial wastewater was studied. The results indicated that the granules cultivated with industrial wastewater had a higher PNs content. During the *feast* phase the EPSs content increased, conversely it decreased during the *famine*, especially in R2. Particularly, while the aerobic granules were forming, a higher amount of PS were degraded in both reactors during the starvation phase, whereas the PN content significantly reduced only in R2, resulting in the structural weakening of the granules. When a lower EPSs consumption occurred, aerobic granules became mature and their structural strength increased. The PN/PS ratio increased good agreement with the granulation rate, which implied the key role of the protein in the aerobic granulation. In conclusion, although the industrial wastewater favored a more rapid granulation, the long reaction cycle, which was necessary to ensure a good nutrients removal efficiency, determined the excessive consumption of the EPSs, leading to the structural weakening of the granules. In these cases, the duration of the cycle should be accurately considered, to ensure on the one hand good removal efficiencies, but on the other hand to avoid the granules weakening. Obviously, this is related with the nature of the wastewater and with its biodegradability.

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