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Research article

Fate of aerobic granular sludge in the long-term: The role of EPSs on the clogging of granular sludge porosity

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ABSTRACT

This work aims to investigate the stability of aerobic granular sludge in the long term, focusing on the clogging of the granular sludge porosity exerted by the extracellular polymeric substances (EPSs). The effects of different cycle lengths (short and long-term cycle) on the granular sludge stability were investigated. Results obtained outlined that during the short duration cycle, the formation and breakage of the aerobic granules were continuously observed. During this period, the excess of EPS production contributed to the clogging of the granules porosity, causing their breakage in the long run. During the long-duration cycle, the extended famine period entailed a greater EPSs consumption by bacteria, thus limiting the clogging of the porosity, and allowed obtaining stable aerobic granules. Reported results demonstrated that an excess in EPSs content could be detrimental to the stability of aerobic granular sludge in the long-term.

1. Introduction

Aerobic granular sludge is an innovative technology for the treatment of municipal and industrial wastewaters (Pronk et al., 2015; Val del Río et al., 2012). Due to their stratified structure, organic carbon, nitrogen and phosphorous can be simultaneously removed in the same reactor (de Kreuk et al., 2005; Di Bella and Torregrossa, 2013). Moreover, aerobic granules are constituted by a compact and dense structure resulting in an easy solid-liquid separation phase (Winkler et al., 2011). Nowadays, the main drawback of this technology is the stability of the aerobic granules in the long run. Several studies dealing with the granular sludge stability are confined to short observation periods, not exceeding few months (Long et al., 2015; Zhang et al., 2015). It is known that the aerobic granules become unstable once they reach a certain dimension (Verawaty et al., 2013). Indeed, once this occurs, these granules break and most of them are washed out, while the others aggregate again forming new granules (Pijuan et al., 2011). As a result, in the long term, the granulation becomes a dynamic process, in which granulation and degranulation continuously occur. Therefore, steady state conditions are quite difficult to obtain, and this could cause temporary worsening of the biological performances and as a consequence of the effluent quality. The cause of the granule breakage is likely related to the mineralization of their core (Lemaire et al., 2008), as well as to the clogging of their porosity that would limit the flow of nutrients and oxygen from the bulk into the inner layers (Lee et al., 2010). According to Lemaire et al. (2008) and Lu et al. (2012), the porosity clogging is related to an excess of extracellular polymeric substances (EPSs) production. Zheng et al. (2006) reported that the occurrence of anaerobic reactions in the inner layers of the granules, and the consequent accumulation of fermentative products, would be the main reason of their cracking. Obviously, both the pore clogging and the formation of anaerobic reactions are strictly connected, because the clogging of the porosity could enhance the formation of an anaerobic core. No studies in the literature clarify how the EPSs could cause the granules detriment, so up to now the clogging effect remains a hypothesis. Nevertheless, EPSs are recognized as a key factor to obtain the aerobic granulation (Tay et al., 2002; Liu and Tay, 2008; Xiong and Liu, 2013). Therefore, if on the one hand a low EPSs content does not allow to obtain the granulation, on the other hand an excess of EPSs production could limit the aerobic granules maintenance in the long-term. Consequently, it would seem that the aerobic granules stability would require an accurate balancing in terms of EPSs content.

The paper aim at investigating the stability of aerobic granules in the long term, focusing on the clogging of granular sludge porosity exerted by the EPSs. The effects of different cycle lengths

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on the granules EPSs content was evaluated, and it was referred to the granules stability in terms of physical characteristics and biological performances. Furthermore, in order to clarify the mechanism involving the aerobic granules breakage due to the porosity clogging, a conceptual model based on the experimental observations was proposed.

2. Materials and methods

2.1. Reactor and experimental set-up

A Sequencing Batch Airlift Reactor (SBAR) was operated for 740 days divided into two periods. During the period I (day 0–248), the SBAR was operated with a 3 h cycle, consisting in 10 min of influent feeding, 160 min of aeration, 5 min of settling and 5 min of effluent withdrawal, whereas, during the period II (day 249–740), the reactor was operatied with a 12 h cycle, including 10 min of influent feeding, 700 min of aeration, 5 min of settling and 5 min of effluent withdrawal. Consequently, the Hydraulic Retention Time (HRT) was 6 h during the first period and 24 h during the second. Due to the hydraulic selection pressure only particles with a settling velocity higher than 3.6 m h^{-1} were kept within the reactor. The reactor had an internal diameter of 8.6 cm with a working volume of 3.5 L, while the riser had a diameter of 5.4 cm and a height of 50 cm. The filling height was 70 cm, so the height to diameter ratio (H/D) was close to 13. The effluent was discharged using a solenoid valve located at 35 cm from the bottom of the reactor, so that the volumetric exchange ratio was 50%. Air was introduced in the reactor from the bottom by a fine bubble diffuser. The superficial air velocity was kept approximately close to 2.4 cm s^{-1} (3 L min^{-1}), using a flow meter. The organic loading rate (OLR) and the carbon to nitrogen ratio (C/N) were 3.6 kgCOD $m^{-3}d^{-1}$ and 10:1 respectively, and were maintained constant also during the period II by adjusting the COD and NH_4-N concentration in the feed. The TSS concentration was maintained approximately at 12 ± 2 g L^{-1} by daily purging a known amount of mixed liquor volume. Consequently, the sludge retention time resulted approximately close to 30 days. Therefore, the main operating conditions were constant for both periods except the cycle length and the HRT. The SBAR was inoculated with activated sludge collected from Palermo's municipal wastewater treatment plant, and fed with acetate-based synthetic wastewater in accordance with Beun et al. (2002).

The main operating conditions and the feeding wastewater characteristics are summarized in Table 1.

2.2. Analytical methods

All of the chemicals-physical analyses (COD, NH_4-N, NO_3-N, NO_2-N, TSS and VSS) were performed according to standard methods (Apha, 2005). The size of the granules was measured by a high speed image analyses sensor (Sympatec Qicpic) that provided the particle size distribution and the granulometric curve. The granulation rate was evaluated as the percentage of particles with diameters over 600 μm in accordance with Liu et al. (2014).

The EPSs content of the aerobic granules was measured at the end of both feast and famine periods. Furthermore, the dissolved oxygen concentration within the bulk was regularly monitored during the cycle in order to identify the end of the feast period. The end of the feast period, and the subsequent beginning of the famine, was easily identified in terms of oxygen consumption, which rapidly decreased in few minutes after that the organic substrate was almost entirely degraded (Di Bella and Torregrossa, 2013). Extracellular polymeric substances extraction was carried out according to the Heating Method (Le-Clech et al., 2006). In this work the total EPSs content (EPS_T), have been assumed as the sum of protein and polysaccharides, so it was related to the volatile suspended solid (VSS) concentration. Lastly, polysaccharides were determined according to the phenol–sulfuric acid method with glucose as the standard (DuBois et al., 1956), whereas the proteins were determined by the Folin method, with bovine serum albumin as the standard (Lowry et al., 1951). The EPSs production and consumption were evaluated as the difference between their content at the end of the famine and the feast phases.

COD analyses were also performed in the supernatant of mixed liquor at the end of the feast phase to evaluate the maximum velocity of the organic substrate depletion, as well as in the effluent.

The density and hydrophobicity of the granular sludge have been determined using the Dextran Blue method described by Beun et al. (2002) and according to the method described by Rosenberg (1984), respectively. Settling properties have been evaluated by measuring the sludge volume index (SVI) after 5 min (SVI_5) and after 30 min (SVI_{30}). The porosity of the aerobic granules was evaluated by measuring their water content. This was measured by the percentage difference between the weight of a certain amount of granules separated from the mixed liquor after centrifugation at 4000 rpm (30 min), and after drying in oven at 105 °C as far as the constant weight was reached.

After the experimental day 475, the frequency of analyses was reduced, and for 6 months the granules stability was periodically monitored only through microscopic observations. Then, at the 700th day, the reactor performances and the granular sludge physical properties were analyzed again in order to investigate whether modifications occurred during that period.

3. Results and discussions

3.1. Analyses of EPSs production/consumption

The EPSs content was measured at the end of both feast and famine phases. Thus, it was possible to calculate their production during the substrate abundance period (feast), and their consumption during the starvation (famine). For the whole experiments, SMPs concentration was approximately close to zero, so in the following they will not be taken into account in the EPS amount. The evolution of EPSs production and consumption during the feast and famine phases are shown in Fig. 1. During the first experimental period, the protein concentration in the feast phase ($PN_{,F}$) significantly fluctuated, while the polysaccharide content ($PS_{,F}$) remained almost constant (Fig. 1a).

At the beginning of the period I, the protein content significantly increased due to the new operating parameters that contributed to create metabolic stress conditions for the microorganisms (Zhu et al., 2012b). However, due to the biomass washout that generally occurs during the first stages of the granulation process,

Table 1
Summary of the main operating conditions and feeding wastewater characteristics.

Period	Cycle length [hours]	Settling time [min]	HRT [hours]	Air flow velocity [cm sec^{-1}]	OLR [KgCOD $m^{-3}d^{-1}$]	COD [mg L^{-1}]	NH_4-N [mg L^{-1}]	SRT [day]
I	3	5	6	2.4	3.6	900	90	30
II	12	5	24	2.4	3.6	3600	360	30

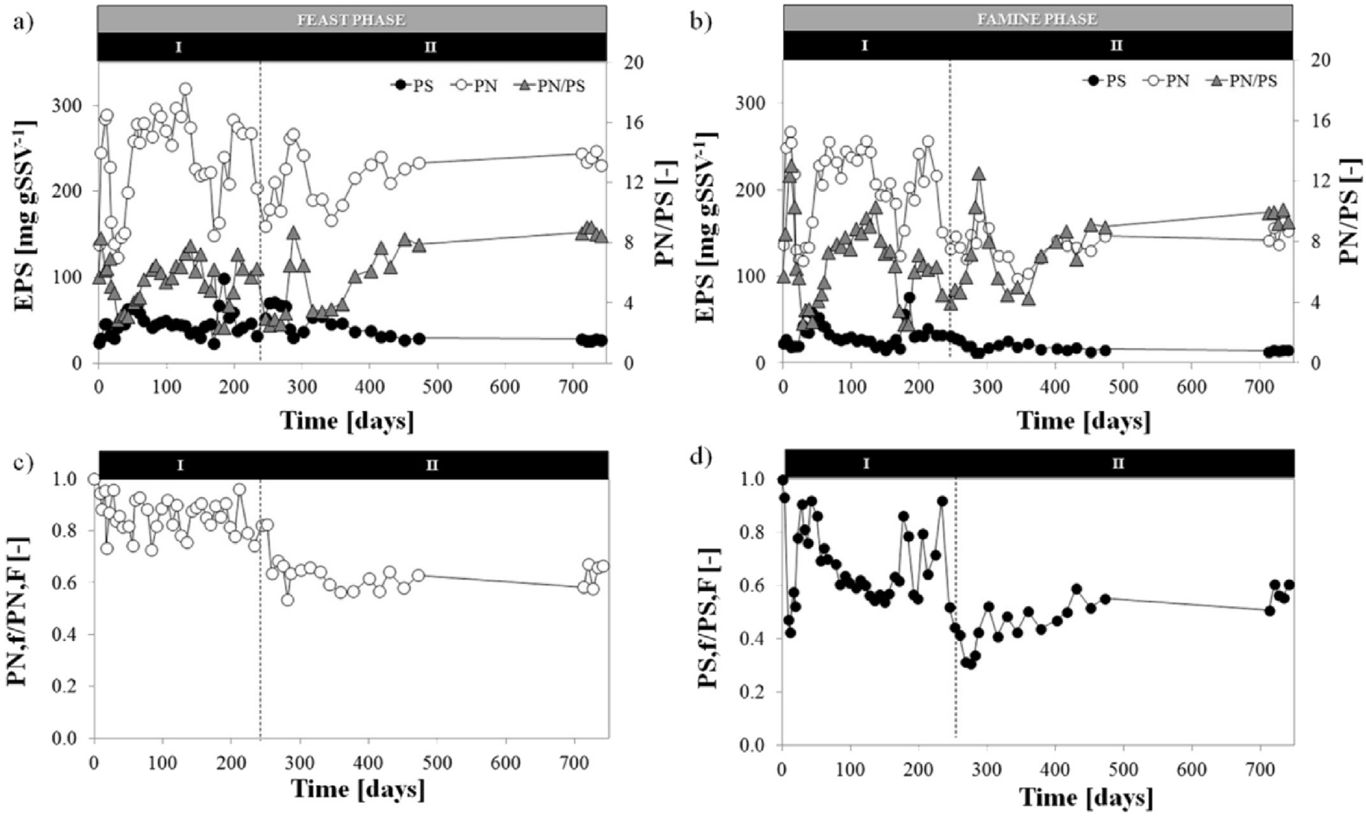

Fig. 1. Trend with time of proteins (PN), polysaccharides (PS) and their ratio (PN/PS) during the feast phase (a) and famine (b); trend of proteins (c) and polysaccharides consumption during the famine phase (d).

substrate availability for biomass unit increased. Furthermore, due to the excess of organic substrate, the bacteria produced a lower amount of storage products, hence the protein content rapidly decreased. When hydraulical selection allowed the increase of VSS concentration within the reactor, the organic substrate became to be limiting; as a consequence bacteria increased the production of EPSs mainly in forms of proteins. Then, the protein content reached a steady-state value, and the maturation of granular sludge occurred (Adav et al., 2008; Zhu et al., 2012a). However, the majority of the studies limit the observation period up to the reaching of the maturation. Consequently, scarce information about what happen in the long-run are available. The content of the EPSs remained quite during about 80 days; thus it dropped down to a minimum at the 170th day. Hereafter, the trend of EPSs replicated what observed in the early stages of this period. Every time a pseudo-steady-state value was reached, the EPSs content decreased. As a result, steady-state conditions were not basically reached.

Conversely, in the second experimental period the EPSs trend during the earlier days resulted similar to the beginning of the first period. However, once the plateau was reached the EPSs content remained stable at these values until the end of the experiment.

It is worth to notice that the EPS changes were related to the COD loading variation, and in particular with the food/microorganism ratio. Infact, for equal COD concentration, the lower was the VSS concentration, the higher the COD loading per unit of biomass. Consequently, due to the higher substrate availability, bacteria were stimulated to produce more EPS mainly in the form of proteins.

The EPSs analyses performed at the end of the famine period ($PN_{,f}$ and $PS_{,f}$), allowed to point out interesting observations regarding their consumption during the endogenous respiration (Fig. 1b). Referring to the period I, the protein content at the end of the famine period ($PN_{,f}$) was similar to the feast. By comparing the ratio between the protein content at the end of the famine phase and the protein content at the end of the feast ($PN_{,f}/PN_{,F}$), approximately close to 80–85%, a consumption of 15–20% on average was observed (Fig. 1c). Conversely, the polysaccharides consumption resulted increased. In detail, the $PS_{,f}/PS_{,F}$ ratio fluctuated from 50% to 85%, hence polysaccharides consumption ranged from 15% to 50% (Fig. 1d). Results indicated that consumption of polysaccharides occurred earlier than consumption of proteins, likely due to their simpler molecular structure.

During the second period, as bacteria spent a longer time in endogenous respiration conditions, the EPSs consumption increased. The polysaccharides consumption ranged from 50% to 70%, whereas, in contrast with the previous period, the proteins were significantly degraded. Indeed, for the whole period II, its content decreased by 50% on average at the end of the famine phase.

The EPSs composition, expressed in terms of proteins to polysaccharides ratio (PN/PS), modified according to the proteins and polysaccharides production and consumption (Fig. 1a, b). Specifically, in certain periods, the aerobic granules enriched proteins, while in others, the PN/PS ratio decreased. The periodical variation of the EPS concentration observed in the period I did not occured during the second, confirming that in the latter steady-state conditions were reached.

The comparison of the results obtained in the two periods indicated that the EPSs production and consumption, increased according to the reaction cycle length. When bacteria might face a long starvation period, on the one hand they needed to produce more storage products, but on the other hand, they used these products as carbon and energy source for the endogenous respiration. Overall, the EPSs and protein content reduced when the cycle length increased.

3.2. Analyses of biomass metabolic activity

The EPSs production is strictly related to the biomass metabolism. Although this relationship is apparently expected, it was not in-depth analyzed in the literature. A detailed discussion about this topic could better explain some unclear aspects. From now on, when EPSs data will be discussed, the EPSs content will be referred to the protein fraction because the polysaccharide was significantly lower and almost constant.

As previously discussed, during the period I, results outlined the cyclical alternation of high and low PN production. Particoularly, four different phases were identified. As aforementioned, the PN content firstly started to increase (38th day-phase I) until it reached a plateau (65th day-phase II) that was more or less prolonged. At the end of this plateau, the PN content started to decrease (125th day-phase III) until a new minimum value was reached (171th day-phase IV). Then, this trend occurred again until the end of the first period. The duration of the feast phase, the DO concentration during the feast phase and the velocity of the organic substrate depletion (v_H) measured during these days are reported in Table 2.

The results showed that in the first experimental period the feast phase length and the v_H fluctuated according to the PN content changes. After the 38th day, when the PN content started to increase, the feast phase endurance was about 25 min, and the v_H was close to 5.25 mgCOD gVSS^{-1}d^{-1}. At the beginning of the plateau (65th day) the feast phase endurance was 40 min, and the v_H reduced to 3.36 mgCOD gVSS^{-1}d^{-1}. At the 125th day, the duration of the feast phase significantly increased up to 90 min, and the v_H dropped by up to 1.44 mgCOD gVSS^{-1}d^{-1}. Lastly, at the 171st day, the duration of the feast phase reduced to 50 min, and the v_H significantly increased up to 4.18 mgCOD gVSS^{-1}d^{-1}. The increase in the feast phase duration is generally related to the lost of the aerobic granule stability, because it would favor the proliferation of filamentous bacteria (De Kreuk et al., 2005).

During the second period, only three phases have been identified, because the EPSs reduction after the plateau did not occurred. In contrast with the previous period, the feast phase duration was generally longer, because the influent concentration of COD was higher. The feast phase lasted 90 min on average in all three the phases, and the v_H slightly ranged from 4.69 mgCOD gVSS^{-1}d^{-1} to 5.80 mgCOD gVSS^{-1}d^{-1}, and thereby remained at this level until the end of the experiments. The velocity of the organic substrate depletion was higher with respect to the previous period, due to the higher substrate concentration in the influent. Overall, in the second period, both feast phase duration and velocity of organic substrate depletion remained quite stable, confirming the greater stability of the granules. Furthermore, an indirect correlation between the bacteria metabolic activity and the protein content of the granules during first period was outlined. Li et al. (2008) observed that the increase in the EPSs production slightly transformed the aerobic granules in jellylike structure, characterized by a viscous and dense film at their surface that limited the penetration of the oxygen and the nutrients in the inner layers. It is possible that the occurrence of a such phenomena led to the reduction of bacterial metabolic activity. The results indicated that the strong fluctuations of EPSs content observed especially during the first period, was due to a modification of bacterial metabolism, likely due to the reduction of the granule active fraction.

3.3. Granules physical characteristics

In Fig. 2 the trends of the granules physical characteristics (hydrophobicity, density, VSS/TSS and SVI$_5$ and SVI$_{30}$) are depicted.

The trend vs time of the granules hydrophobicity was similar to that of the EPSs protein fraction (Fig. 2a). The hydrophobicity significantly fluctuated during the first period, whereas during the second it reached a steady-state value. The relationship between the EPSs and the hydrophobicity is related to the composition of the extracellular polymeric structure. As aforementioned, the protein content was 5–8 times greater than those of the polysaccharide. Proteins are hydrophobic substances, so the aerobic granules were more hydrophobic due to proteins abundance within the granules. Overall, in the first period the hydrophobicity was higher on average. Despite the remarkable fluctuations, the pseudo steady-state value in the period I was close to 94%, whereas in the period II it was close to 88%. This difference was certainly related to the different protein content, which was lower in the period II with respect to the previous. However, although hydrophobicity was lower in the period II, the values were significantly higher compared with a conventional activated sludge (Chao et al., 2014).

The trend vs time of density was similar to that of hydrophobicity (Fig. 2a). As the other case, the density significantly fluctuated in the first period, whereas it stabilized in the second. It is worth to notice that the maximum values of the density corresponded to those of the hydrophobicity, indicating the relationship between these two parameters.

The trend of VSS/TSS ratio is shown in Fig. 2b. Almost for the whole period I, the VSS/TSS ratio gradually decreased, indicating a progressive increasing in inert material within granules, so the mineralization of their structure (Adav et al., 2010). In accordance with Isanta et al. (2012), the inert fraction within the granules generally increased in the long run due to the precipitation of inorganic salts in their core. Overall, at the end of the period I, the VSS/TSS slightly increased in contrast with the density. The results suggested that the excessive thickening of bioaggragates represented a barrier for the mass transport phenomena within the granules. In the period II, the VSS/TSS stabilized approximately at 75%, indicating that the inert fraction in the granules was lower with respect to the previous period, and consequently, the active fraction of the granules was likely bigger. These results confirmed

Table 2
Data of EPSs, velocity of organic substrate depletion and the duration of the feast phase, during the first and the second experimental period.

Period	Day	Feast phase length [min]	DO [mg L^{-1}]	EPS [mgPN gVSS^{-1}]	v_H [mgCOD gVSS-^{1}d^{-1}]
I	38	25	6.5	131	5.25
I	65	40	5.5	256	3.36
I	125	90	6.0	324	1.44
I	171	50	6.3	155	4.18
II	250	90	5.8	165	5.80
II	290	94	6.1	269	4.89
II	470	88	5.9	221	5.06
II	740	82	6.2	229	4.69

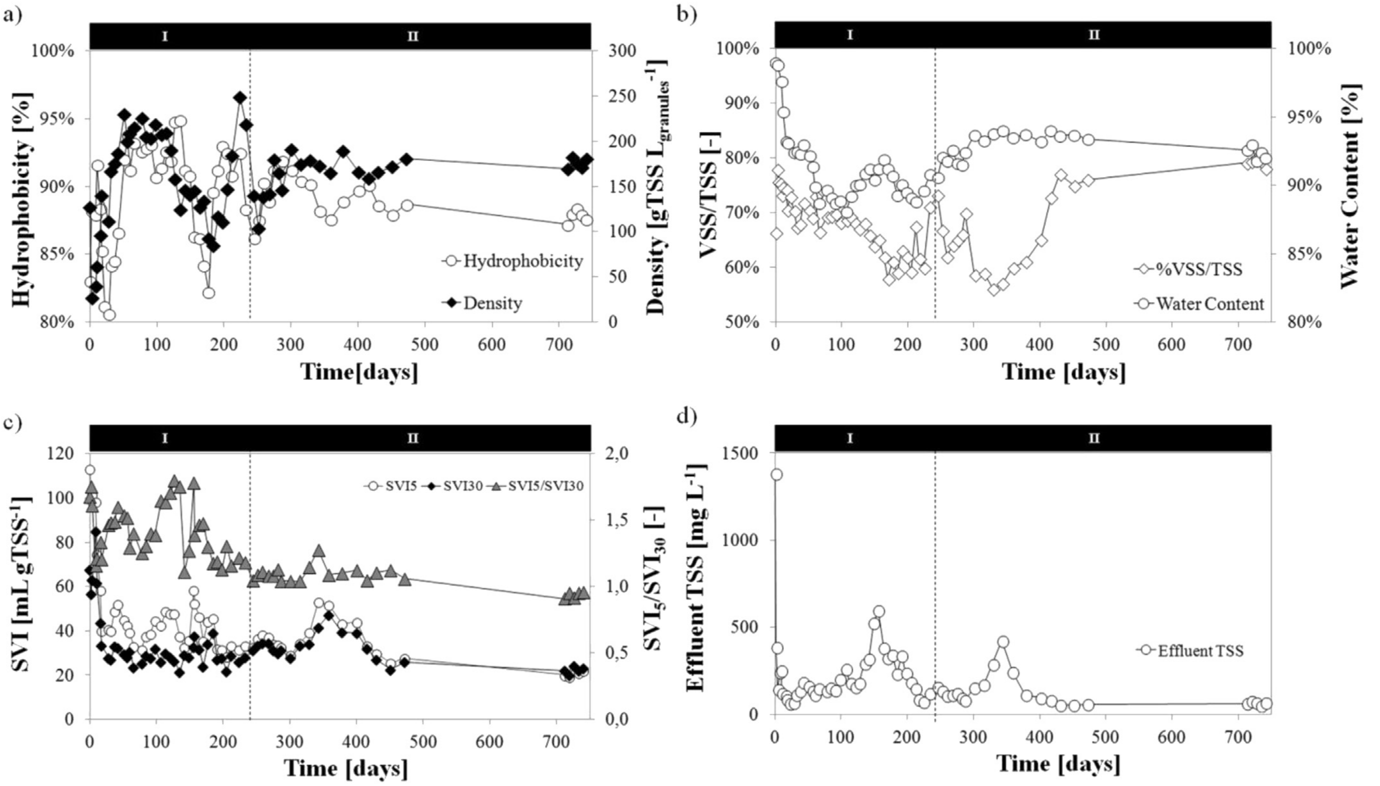

Fig. 2. Time course of hydrophobicity and granules density (a), volatile and total suspended solid ratio (VSS/TSS) and water content of the granules (b), SVI_5,SVI_{30} and their ratio (SVI_5/SVI_{30}) (c), total suspended solids (TSS) in the effluent (d).

the higher biomass respiration activity that was observed at the end of the first period.

As showed in Fig. 2a, the water content of the granules fluctuated during the whole period I. At the beginning, water content drastically reduced from 99% (value of the seed sludge) to 89%, then, it ranged between 88 and 92% until the end of the period I. Also in this case, the minimum of the water content corresponded to the higher values of EPSs content. During the period II, the water content was higher than the previous period, and it constantly remained close to 94% until the end of the experiment.

The settling properties of the aerobic granules in terms of SVI are depicted in Fig. 2c. The values of SVI_{30} were approximately close to 30–40 mL gTSS L^{-1} and remained quite stable at this value probably due to the constant application of the hydraulic selection pressure (Fig. 2c). Conversely, the SVI_5 fluctuated according to the density. In detail, SVI_5 increased when density decreased, and vice versa, so the minimum values of the SVI_5 corresponded to the minimum of the density. The ratio between the SVI_5 and the SVI_{30} is considered a good indicator for the granulation process, because values close to the unit indicate that the sludge is basically granular (Schwarzenbeck et al., 2004). Only during certain phases in the first period, and for the majority of the second, this ratio was close to the unit, confirming that the total transformation from flocculent to granular sludge, and the achievement of steady state-conditions, occurred mainly during the period II.

The structural stability of the aerobic granules was evaluated by measuring the total suspended solid concentration in the effluent (Fig. 2d). Indeed, as in the influent wastewater there were no suspended solids, their presence in the effluent could derive only from granules breakage. At the beginning of the period I, the concentration of TSS in the effluent was very high, probably due to the hydraulic selection pressure. Except this period, in which the solids washout was not attributable to the granules breakage, two peaks of concentrations at the 150th day and at 340th day were observed. This occurred in correspondence to the minimum values of density, hydrophobicity and EPSs as well, indicating that in these periods the granules were more susceptible to breakage.

3.4. Granules sizes distribution

The variability of the granule physical characteristics strongly affected their sizes and the granulation rate. Their trends with time were in accordance with those of density, EPSs and hydrophobicity, confirming the importance of these parameters for the granule structural integrity.

The time course of the granulation rate and the granule size distribution in specific days of both periods are reported in Fig. 3.

During the period I, the full granulation was achieved after 120 days, at the end of which the aerobic granules had an average size (D_{50}) approximately close to 1.3 mm. The aerobic granules remained stable for about 40 days, then most of them crushed and the granulation rate dropped down to 55%. During the following 30 days, the granulation rate slightly increased up to 80%, then dropped down again to 40% at the end of the period I. Overall, the trend of the granulation rate during the first period was the result of the lack of steady state conditions, and for this reason, the aerobic granules did not reach a real maturation phase. Detailed granulometric analyses were carried out on the 90th day, 120th day, 175th day and 205th day (Fig. 3b). On the 90th day the aerobic granules had moderate dimensions and the majority of them had sizes close to 0.7 mm. On the 120th day, when the full granulation was achieved, the granulometric analyses showed the homogeneous nature of the sludge. Compared with the previous size distribution, in this case the d_{10} and d_{60} significantly increased, that meant that the

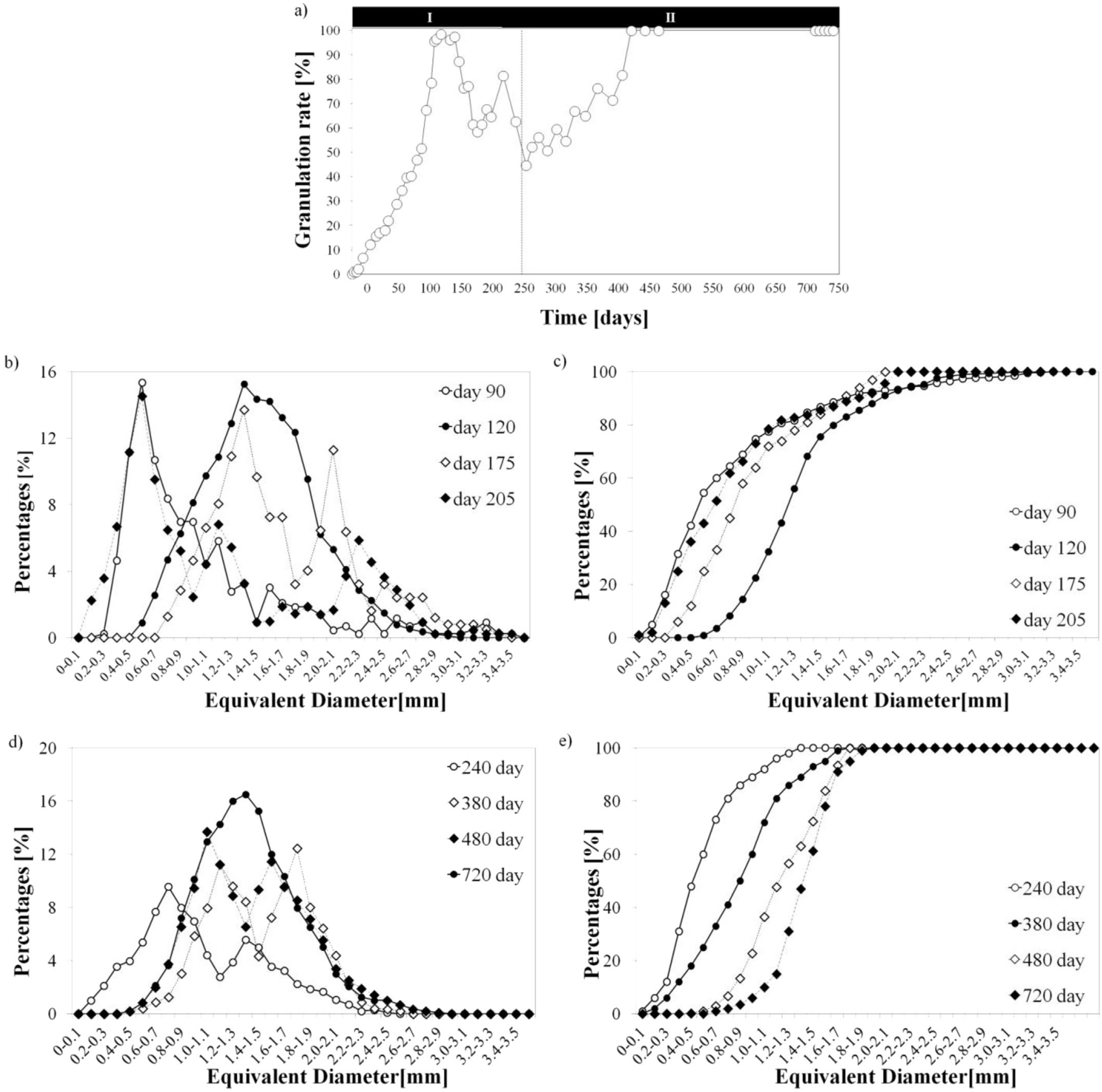

Fig. 3. Time course of granulation rate (a) granules size distribution during the period I (b) and period II (d), and granulometric curves in period I (c) and II (e).

smaller aggregates began growing and thus the beginning of the granular sludge maturation. When the granulation rate began to reduce at the 175th day, the granulometric analyses highlighted the formation of two distinct peaks, in correspondence of 1.4 mm and 2 mm, suggesting that with respect to the previous observation, some granules continued to grow, whereas some others began breaking. Slightly, the sludge evolved into a heterogeneous mixture of small and big granules. On the 205th day, the two peaks previously observed further separated, so the sludge heterogeneity increased. At the same time, a new peak was observed in correspondence of 2.2 mm. This new peak was likely attributable to the formation of new granule, whereas the others were likely related to the aged granules, more susceptible to breakage phenomena. Overall, from the 100th day onward, the granulation process evolved into a dynamic process according to which continuously new granules formed and the oldest broke. In this case, the breakage phenomena prevailed over the formation of new granules, so steady-state conditions were not reached.

The detailed granulometric analyses were carried out during the second period on the 240th day, 380th day, 480th day and 720th day. At the beginning of the period II (240th day), the sludge was a mixture of small granules and flocculent sludge. In the size distribution two distinct peaks were observed, that corresponded respectively to the flocculent (0.4 mm) and the granular fraction

(1.5 mm). On the 380th day, both peaks increased and slightly shift into bigger diameter, which meant that the granules were growing and their number within the reactor was increasing. On the 480th day, the size fractions below 0.6 mm disappeared because their sizes increased in the meantime. Therefore, the granulation rate early reached the 100% and the sizes of bioaggragates stabilized, indicating the complete maturation of the granular sludge. Both the size distribution and the microscopic observation noted the heterogeneous nature of the sludge, hence granules with sizes close to 1.2 and over 2 mm were simultaneously noticeable in the reactor. The granules remained stable for over 6 months, and at the end of the experiments (720th day) the granule size distribution resulted very homogeneous, with an average diameter close to 1.5 mm.

Comparing the results obtained in both experimental periods, interesting observations concerning the effect of the cycle length on the granulation time can be made. The slope of the granulation rate with time was higher in the period I, although the best conditions for the granulation, concerning the seed sludge, occurred in the second period (Long et al., 2015; Pijuan et al., 2011). As previously discussed, the EPSs content and the hydrophobicity were higher in the first period, and this certainly favored a rapid granulation. However, the results obtained in this study demonstrated that the same operating conditions that led to a rapid granulation, caused the instability of the aerobic granules in the long-term. In the second period, the lower EPSs content delayed the formation of granules, but at the same time it allowed obtaining more stable mature granules.

3.5. *Nutrients removal efficiency*

One of the main issues related to the aerobic granular sludge technology is the achievement of steady-state conditions in terms of nutrients removal. Generally, the achievement of satisfactory nutrient removal efficiencies occurs after granules maturation is reached; that circumstance is due to the fact that bacteria operating the nitrogen removal are slow-growing microorganisms, hence they require steady-state conditions for growing within the granules.

The organic carbon and nitrogen removal efficiencies are reported in Fig. 4.

During the period I, the COD removal efficiency fluctuated as a result of general instability conditions up to now discussed (Fig. 4a). Nevertheless, the COD removal efficiency was constantly higher than 90%, mainly due to the high biodegradability of the acetate-based wastewater used as feed. For the whole period II, the COD removal efficiency was constantly close to 98%, confirming the achievement of steady-state conditions.

Nitrogen removal efficiency was significantly affected by the granules instability. In Fig. 4b total nitrogen concentration (as ammonia) in the influent, as well as ammonia concentration, nitrite and nitrite in the effluent and the total nitrogen removal efficiency are reported. During the first experimental period, nitrogen removal efficiency resulted scarce. Rarely, the removal efficiency was over 60%, whereas only after the 200th day, it stabilized approximately at 80%. During the second period, the removal efficiency was initially modest, due to the massive sludge washout that characterized the end of the previous period. Later on, once granules reached their maturation and autotrophic bacteria acclimated within granules, the total nitrogen efficiency removal rose to 90% on average.

The main nitrogen forms in the effluent were ammonia and nitrite during the first period, whereas ammonia and nitrate during the second. It is worth noticeing that during the first period, the nitrite accumulation occurred from the 70th and 130th day, while between the 175th and 200th day during the second period. In both cases, the EPSs content, density and hydrophobicity, reached their maximum values it these days. It is possible that the granules thickening limited the oxygen penetration into the inner layers of the granules, yielding thus oxygen limited the nitrification process. In the second period the nitrite were always present in the effluent, but in this case it was due to the high ammonia concentration in the influent that caused the increase in free ammonia concentration. Although the nitrogen removal efficiencies were higher during the period II, total nitrogen concentration, mostly in the forms of nitrite, in the effluent was close to 40 mg L^{-1}, suggesting that the denitritation was not complete. The reason of this was likely related to the long starvation phase. Indeed, the largest part of organic carbon was degraded during the feast phase, that lasted about 90 min, so during famine phase the endogenous carbon was the only carbon source available for denitrification. Denitritation kinetic was too slow by using endogenous carbon as electron donor, and likely the carbon availability was not enough compared with the nitrate amount to be reduced. Therefore, likely carbon was a limiting factor for denitrification during the period II.

3.6. *Effects of the EPSs on the granules stability*

Obtained results in both experimental periods indicated a substantial difference in the granule physical characteristics. During the first period the granules physical characteristics hardly fluctuated, revealing an intrinsic weakness of the bioaggragates. Conversely, during the second period the granules showed a greater structural stability, that allowed to achieve their maturation.

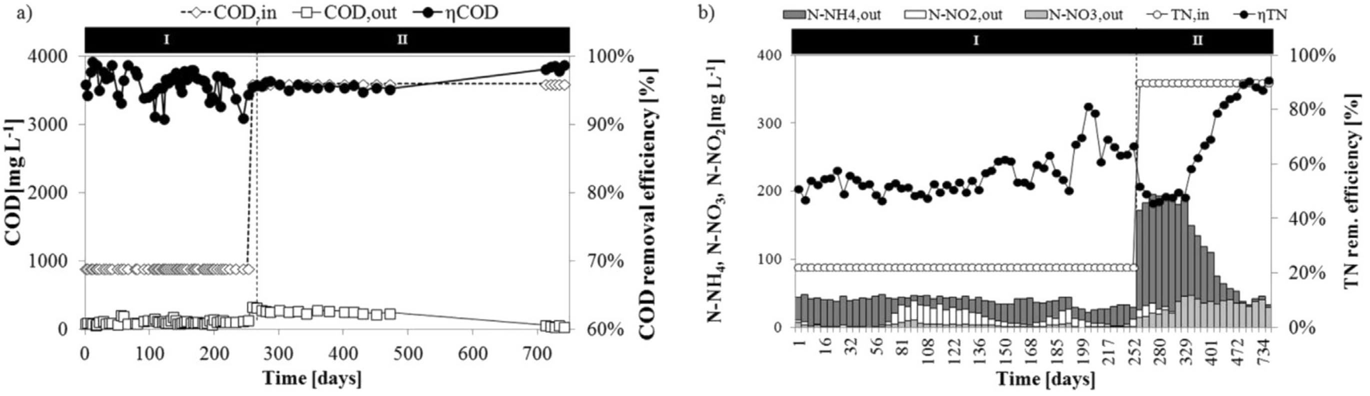

Fig. 4. Influent, effluent COD concentration and removal efficiency (a); influent, effluent nitrogen concentration and total nitrogen removal efficiency (b).

Results up to now discussed, indicated that the granules formation and breakage occurred several times, according to the EPSs trend (Fig. 1a). If on the one hand the EPSs are considered a crucial parameter for the granulation process (Lu et al., 2012), on the other hand, other authors stated that the EPSs were one of the main causes of the granular sludge breakage in the long-run (Chen et al., 2014). Authors suggested that EPSs resulted in the progressive occlusion of the granules porosity, limiting in such a way the nutrient transport from the bulk into the inner layers of the granules, and consequently the death of the bacterial cells (Lemaire et al., 2008). Results obtained in this study allowed to better explain this occurrence.

The relationships shown in Fig. 5 indicated as the main physical characteristics of the aerobic granules depended on the EPSs content. Because EPSs were mainly constituted of proteins that are hydrophobic substances, a greater amount of exopolymers produced a corresponding increase in the granular sludge hydrophobicity (Fig. 5a), in the granules density (Fig. 5b) and lastly in their settling capacities (SVI_5) (Fig. 5c). The greater tickening and the high hydrophobicity limited the penetration of nutrients inward the granules as far as the inner layers did not have enough substrate and slowly began to mineralize themselves. As a result, the VSS to TSS ratio decreased with EPSs content increasing (Fig. 5d). The combination of the physical changes caused the clogging of the granular sludge porosity. Consequently, the water content decreased with EPSs increasing (Fig. 5e). Overall, if on the one hand EPSs are fundamental for aerobic granulation, on the other hand, an excess of EPSs production seems to determine more harms than benefits in the stability of the aerobic granules. With regard to this, it is worth to notice that the relation between the concentration of TSS in the effluent and the EPSs content showed two opposite trends (Fig. 5f). Such aspect indicated that the beneficial role of EPSs on the aerobic granular sludge stability was limited within a relatively small concentration interval. This means that, if on the one hand low EPSs amounts were not enough to form the aerobic granules, on the other hand, an excess of EPSs production had detrimental effects on their stability. Consequently, in both conditions the granules resulted more susceptible to breakage and the steady-state was not actually achieved.

The microscopic analyses allowed to identify four different phases during the first experimental period (Fig. 6). The phase I was characterized by the formation of the first bioaggragates during the first 30 days. The phase II started with the increase in EPSs production and finished when the plateau of the EPSs profile was reached. In this phase, the size of the granules increased and their maturation was starting to occur. The phase III corresponded to the first plateau in the EPSs concentration profile (EPSs content almost constant). The microscopic observations allowed to note that progressively a viscous, gelatinous film was developed at the granule surface (Fig. 6). During this phase, the EPSs likely started to clog up

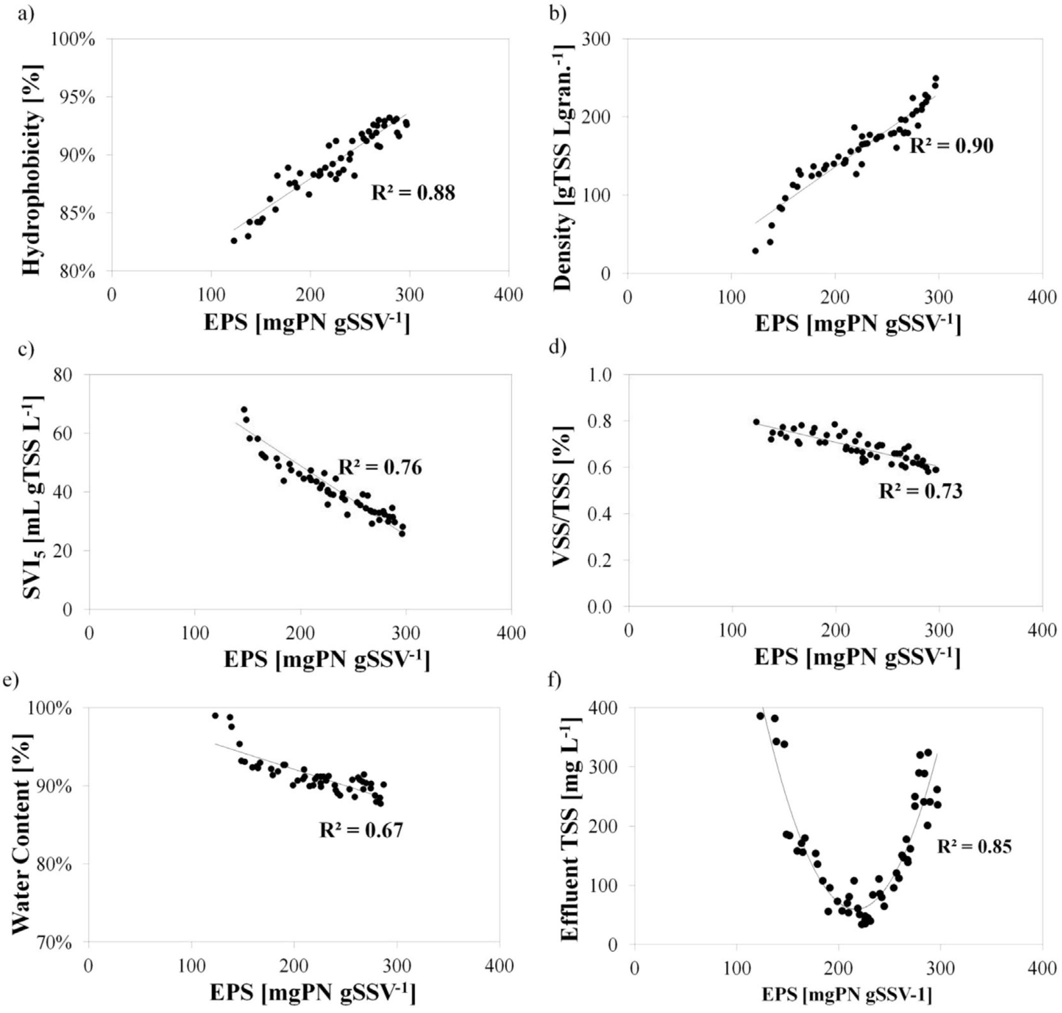

Fig. 5. Relationships between the EPSs content and the density (a), SVI_5 (b), Water Content (c), hydrophobicity (d), VSS to TSS ratio (e) and effluent TSS concentration (f).

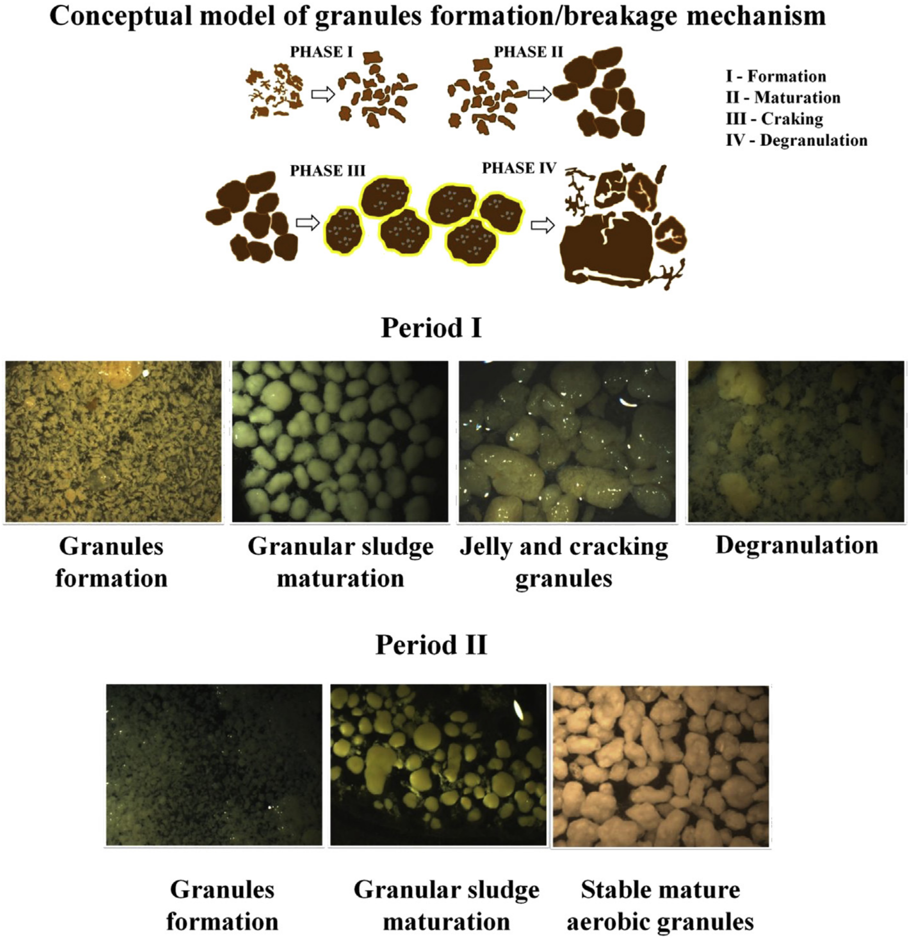

Fig. 6. Conceptual model of granules breakage due to the clogging of the pororisy and pictures of aerobic granules in each phase. Pictures in the period 1 are referred to the 90th, 120th, 175th, 205th day (from left to right), while in the period 2 to the 240th, 380th and 720th day.

the pores thus limiting the diffusion of the nutrients. To confirm this, the lowest values of the water content, which is related to the granules porosity, were observed exactly in correspondence to the first EPSs plateau. The reduction of the porosity created a barrier to mass diffusion phenomena within the granules, causing their partialization into two sections: the first in close contact with the bulk, received enough substrate so it was biologically active, whereas the second, closer to the core, did not received enough nutrients so gradually mineralized. Without external substrate supply, the only carbon and energy source for the bacteria in the inner layers were the EPSs, which were gradually degraded, independently of the availability of substrate in the bulk.

The EPSs reduction caused the decrease in the sludge hydrophobicity, hence the auto-adhesion capability gradually failed and granules began to break. Such phenomena occurred during the phase IV, at the end of the EPS plateau, so corresponding to the drop of the EPSs content. During this phase, the mixed liquor evolved into a hybrid system of both flocculent and granular sludge. It is worth to notice that the majority of those granules that disintegrated belonged to the d_{90} fraction. This meant that the mass transport within these granules, which were obstructed due to the clogging of porosity, found another barrier associated with the large thickness of the aggregates.

All these factors affected the biological performances. The organic substrate was easily biodegradable, hence it did not resulted insignificant decrease in the COD removal efficiency. In contrast, the granules instability severely affected the nitrogen removal efficiencies. The clogging of the granules pores limited the oxygen diffusion into their inner layers, so nitrification resulted likely oxygen limited. Consequently, during the period I, significant accumulations of nitrite were periodically observed. This occurred whenever the EPSs content, as well as hydrophobicity and density, reached the maximum values, so when the clogging of the granules pores reached its apical level. When the EPSs content decreased, the porosity unclogged, and finally full nitrification was achieved.

During the second experimental period, when the maturation of granules was reached, no breakage and stability loss events occurred. After the complete granulation, the aerobic granules remained stable until the end of the experiment. Although the EPSs production was higher compared with the previous period, their consumption was significantly more substantial due to the longer starvation phase. Indeed, during the famine phase, most of the 50% of proteins were degraded, so the average EPSs content was approximately lower than 30% compared with the period I. In this case, the EPSs consumption limited the clogging of pores, and this allowed the nutrient penetrating into the inner layers of the aerobic granules. In accordance with previous observation, the metabolic activity was higher compared with the period I, because the active fraction of the aerobic granules was greater. Unlike the previous experimental period, after the phase II microscopic analyses did not highlight the formation of jellylike granules. Moreover, even if the granules were morphologically irregular and their size distribution was more heterogeneous, they were structurally more stable than those of the previous.

4. Conclusions

The long term stability of aerobic granular sludge in an SBAR operated for 720 days was investigated. Specifically, the presumed role of EPSs on the clogging of granules pores was investigated, in short and long reaction cycles. The results discussed in this work demonstrated that a shorter duration of the reaction cycles (Period I) resulted in a faster enrichment of EPSs, which means a shorter start-up period. Furthermore, it was demonstrated that under the same conditions, the clogging of the granules pores and as a consequence, the loss of their structural stability, occurred in the long-run. On the other hand, lengthy cycles (period II) delayed the formation of the granules, but on the whole, they allowed obtaining granules structurally more stable in the long run. It's conceivable that, for speeding-up the start-up phase, but at the same time to avoid the occurrence of process malfunctions, the best solution could be applying short reaction cycles during the start-up phase, then prolonging the cycle duration once complete granulation was achieved.

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