Water Research 85 (2015) 158–166

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres

Effect of particulate organic substrate on aerobic granulation and operating conditions of sequencing batch reactors

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ARTICLE INFO

Article history:
Received 1 April 2015
Received in revised form
17 August 2015
Accepted 18 August 2015
Available online 20 August 2015

Keywords:
Aerobic granules
Particulate organic substrate
Selection pressure
Simultaneous fill-and-draw operation

ABSTRACT

The formation and application of aerobic granules for the treatment of real wastewaters still remains challenging. The high fraction of particulate organic matter (X_S) present in real wastewaters can affect the granulation process. The present study aims at understanding to what extent the presence of X_S affects the granule formation and the quality of the treated effluent. A second objective was to evaluate how the operating conditions of an aerobic granular sludge (AGS) reactor must be adapted to overcome the effects of the presence of X_S. Two reactors fed with synthetic wastewaters were operated in absence (R1) or presence (R2) of starch as proxy for X_S. Different operating conditions were evaluated. Our results indicated that the presence of X_S in the wastewater reduces the kinetic of granule formation. After 52 d of operation, the fraction of granules reached only 21% in R2, while in R1 this fraction was of 54%. The granules grown in presence of X_S had irregular and filamentous outgrowths in the surface, which affected the settleability of the biomass and therefore the quality of the effluent. An extension of the anaerobic phase in R2 led to the formation of more compact granules with a better settling ability. A high fraction of granules was obtained in both reactors after an increase of the selection pressure for fast-settling biomass, but the quality of the effluent remained low. Operating the reactors in a simultaneous fill-and-draw mode at a low selection pressure for fast-settling biomass showed to be beneficial for substrate removal efficiency and for suppressing filamentous overgrowth. Average removal efficiencies for total COD, soluble COD, ammonium, and phosphate were 87 ± 4%, 95 ± 1%, 92 ± 10%, and 87 ± 12% for R1, and 72 ± 12%, 86 ± 5%, 71 ± 12%, and 77 ± 11% for R2, respectively. Overall our study demonstrates that the operating conditions of AGS reactors must be adapted according to the wastewater composition. When treating effluents that contain X_S, the selection pressure should be significantly reduced.

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1. Introduction

Aerobic granular sludge (AGS) has been extensively investigated during the last decades due to its great potential for wastewater treatment. However, most studies on aerobic granulation have been performed in laboratory-scale sequencing batch reactors (SBR) using readily biodegradable (S_S) synthetic substrates containing mainly acetate and glucose as carbon sources. Only few studies reported successful granule cultivation with real wastewater, such as malting (Schwarzenbeck et al., 2004), brewery (Wang et al., 2007), dairy (Schwarzenbeck et al., 2005), swine slurry (Figueroa et al., 2011), soybean-processing (Su and Yu, 2005), and domestic wastewater (De Kreuk and Van Loosdrecht, 2006; Ni et al., 2009; Liu et al., 2010; Coma et al., 2012; Su et al., 2012; Wagner and Costa, 2013; Rocktäschel et al., 2015; Wagner et al., 2015). These studies have indicated that the kinetics of aerobic granulation when using real wastewaters are different from the ones obtained with synthetic wastewaters. This may be due to the fact that real

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http://dx.doi.org/10.1016/j.watres.2015.08.030
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wastewater, either from domestic or industrial origin, often contains diverse carbon sources along with a multitude of organic and inorganic compounds and also particulate matter (Lemaire et al., 2008). For example, the fraction of the particulate organic matter (X_S) in pre-settled domestic wastewaters usually accounts for around 40–60% of the total organic matter (Kappeler and Gujer, 1992; Orhon and Çokgör, 1997; Koch et al., 2000). Therefore, synthetic wastewaters composed only by S_S cannot truly represent real and complex wastewaters. As a consequence, the formation and application of aerobic granules for the treatment of real wastewaters still remains challenging.

Previous studies suggested that the presence of X_S in the wastewater results in aerobic granules with filamentous outgrowths on their surfaces (Schwarzenbeck et al., 2004, 2005; De Kreuk et al., 2010; Peyong et al., 2012; Figueroa et al., 2015). The development of filamentous and finger-type structures can be explained by the existence of substrate gradients inside the sludge aggregates due to diffusion limitation (Martins et al., 2004; De Kreuk et al., 2010; Weissbrodt et al., 2012). Under these conditions, filamentous bacteria have a higher outgrowth kinetic because they grow preferentially in one direction and not in three directions as the floc-forming bacteria inside the aggregates (Martins et al., 2004, 2011). Particulate matter is mainly hydrolyzed at the surface of the granules. The readily biodegradable substrates produced by hydrolysis are then utilized locally, enhancing substrate gradients inside the granules and thus stimulating the outgrowth of filamentous structures (De Kreuk et al., 2010). The higher accumulation of X_S on the finger like filamentous granule structures will further enhance this phenomenon of irregular substrate uptake over the granule (De Kreuk et al., 2010). Nevertheless, to what extent the presence of X_S affects the overall granulation process (e.g. kinetics of granules formation) and substrate degradation rate still remains unclear.

Aerobic granular sludge was successfully cultivated in a lab-scale reactor treating malting wastewater with a high content of particulate organic matter (Schwarzenbeck et al., 2004). The removal X_S by aerobic granules resulted from two different mechanisms (Schwarzenbeck et al., 2004): (1) during initial granule formation and growth, particulate substrates adsorbed onto the biofilm matrix of the granules; (2) for the completely granulated sludge bed with mature granules, X_S is removed as a result of the presence and metabolic activity of a dense protozoa population covering the granules' surface. De Kreuk et al. (2010) demonstrated using starch as a model particulate substrate that X_S is mainly removed by adsorption at the granule surface, followed by hydrolysis and consumption of the hydrolyzed products. Unlike soluble substrates, particulate organic matter cannot pass through cell membranes and need to undergo extracellular hydrolysis prior to adsorption (Orhon and Çokgör, 1997). The hydrolysis processes is responsible for the conversion of particulate into readily biodegradable substrate that can serve as a necessary carbon source for denitrification or biological phosphorus removal (Morgenroth et al., 2002). Therefore, nutrient removal can be limited by the extent and kinetics of hydrolysis processes of the particulate organic matter (Morgenroth et al., 2002). If hydrolysis of the organic substrate in the particulate form is a slow process, it can then be hypothesized that the reactor operation should be adapted so that the hydrolysis rate and ultimately the granule formation are encouraged.

The present study therefore aimed at (i) better understanding to what extent the presence of X_S in the wastewater affects granule formation and the reactor performances and at (ii) evaluating how the operating conditions of an AGS reactor can be adapted to overcome the effects of the presence of X_S in the wastewater. Two SBRs fed with synthetic wastewater were operated in parallel over several weeks in absence (R1) or presence (R2) of particulate substrate. Different operating conditions were applied in order to evaluate their effects on granulation and treatment performances. The physical properties of the sludge (size fraction of granules, sludge volume index, etc.) as well as the microbial activities (COD, nitrogen and phosphorus removal) were followed.

2. Materials and methods

2.1. Experimental set-up

Two reactors of 10.2 L (0.12 m internal diameter and 1.2 m height) were used for cultivation of aerobic granules in absence (R1) or presence (R2) of particulate substrate. Synthetic substrates were used in order to operate the reactors under controlled conditions (stable influent composition). The reactors were operated in SBR mode with cycles of 4 h and at a volume exchange ratio of 50%. The pH was monitored but not controlled. During the aeration phase, air was continuously introduced at a superficial upflow velocity of 1.2 cm s^{-1}. The solids retention time (SRT) was not controlled and was calculated taking into account the amount of biomass washed-out during the supernatant removal.

R1 was inoculated with 5 L of conventional activated sludge taken from an aeration tank of a municipal wastewater treatment plant operated for full biological nitrogen and phosphorus removal (ARA Thunersee, Switzerland). The seeding sludge had a total suspended solids concentration (TSS) of 3.4 g_{TSS} L^{-1} and a sludge volume index (SVI_{30}) of 103 mL g_{TSS}^{-1}. The SBR cycle of R1 was divided into the following phases: 60 min of anaerobic feeding from the bottom of the reactor, 167 min of aeration, 8 min of settling, 3 min of effluent withdrawal, and 2 min of idle period. After 25 days of acclimation with a synthetic wastewater containing only soluble substrate (S_S), half of the sludge volume from R1 was added in R2 and the two systems were started in parallel with the same biomass concentration (3.5 g_{TSS} L^{-1}).

2.2. Experimental approach

The experimental approach was divided in four different phases to evaluate the effect of operating conditions on granulation and reactors performances (Table 1). In *Phase I*, the effect of the presence or absence of X_S on the aerobic granulation was evaluated. The SBR cycle consisted of 60 min of anaerobic feeding from the bottom of the reactor, 167 min of aeration, 8 min of settling, 3 min of effluent withdrawal, and 2 min of idle period. The effluents were extracted at the half of the maximum working heights of the reactors, resulting in operations with variable working volume. Biomass with settling velocity larger than 4.5 m h^{-1} was therefore selected. This value corresponds to the height of supernatant (0.6 m) and the settling time (8 min).

Phase II aimed at evaluating to what extent an extension of the anaerobic phase favors the formation of the granules and the reactor performance. The anaerobic period of R2 was therefore extended to 90 min, with 60 min of anaerobic feeding and 30 min of anaerobic reaction without mixing. Due to some technical failures, the operation of R1 had to be discontinued during *Phase II*. R1 was re-inoculated with the same inoculum and operated under the same conditions previously applied in *Phase I*, in order to reestablish the biomass characteristics. No data are thus presented for R1 during *Phase II*.

In *Phase III*, the effect of an increased selection pressure for fast-settling biomass, *i.e.* granules, was investigated. The settling time in both reactors was therefore reduced from 8 min to 4 min in order to wash-out the flocs with the effluent and to retain in the reactor only aggregates with good settling ability, *i.e.* granules. As a result, biomass with settling velocity larger than 9.0 m h^{-1} was selected.

Table 1
Details of the operating conditions applied in R1 (fed with soluble substrate – S_S), and in R2 (fed with a mixture of soluble and particulate substrates – $S_S + X_S$).

Label	Studied effects	Operation period (days)	SBR volume	Cycle phases				
				Anaerobic feeding (min)	Anaerobic period (min)	Aerobic period (min)	Settling (min)	Effluent withdrawal + idle (min)
Phase I	Presence/absence of X_S on aerobic granulation	0–66	Variable	60	–	167	8	5
Phase II	Extended anaerobic period in R2 ($S_S + X_S$)	66–109	Variable	60	R1 – 0 R2 – 30	R1 – 167 R2 – 137	8	5
Phase III	Increased selection pressure for fast-settling biomass	109–176	Variable	60	R1 – 0 R2 – 30	R1 – 171 R2 – 141	4	5
Phase IV	Constant reactor working volume (simultaneous fill-and-draw)	176–250	Constant	90	–	146	4	–

Simultaneous fill-and-draw phases were applied in both reactors during *Phase IV* in order to investigate the impact of operating at constant working volume. After the settling period the reactors were directly fed with the influent wastewater from the bottom resulting in a simultaneous discharge of the clean supernatant in the upper part of the reactors, such as described in hydraulic transport investigations of Weissbrodt et al. (2014). The duration of the fill-and-draw phase for both reactors was 90 min and the wastewater was applied in an upflow velocity of 0.5 m h^{-1}. The selection pressure for fast settling biomass applied in *Phase IV* was thus much lower than in the previous phases.

2.3. Substrate composition

The reactors were fed with synthetic wastewater that contained starch and acetate/hydrolyzed peptone as carbon sources of particulate and soluble organic matter, respectively. The synthetic medium of R1 consisted of soluble substrate only (S_S) with the following composition: 500 mg_{COD} L^{-1} of chemical oxygen demand (COD) (73% of acetate and 27% of hydrolyzed peptone); 50 mg_N L^{-1} of ammonium nitrogen (in form of NH_4Cl); 5 mg_P L^{-1} of phosphorus (60% of K_2HPO_4 and 40% of KH_2PO_4). The synthetic medium of R2 consisted of a mixture of soluble and particulate substrates ($S_S + X_S$) with the following composition: 500 mg_{COD} L^{-1} (36% of acetate, 14% of hydrolyzed peptone, and 50% of particulate starch); 50 mg_N L^{-1} (in form of NH_4Cl); 5 mg_P L^{-1} (60% of K_2HPO_4 and 40% of KH_2PO_4). The medium storage vessels that contained particulate starch were continuously stirred to maintain this substrate in suspension. In each cycle, 0.4 L of the synthetic media was mixed together with 4.7 L of tap water in an intermediary tank prior to feeding in the reactors. The characteristics of the substrates are summarized in Table 2. The composition of the synthetic wastewaters was selected to simulate the substrate concentrations of C, N, and P usually found in low strength municipal wastewaters.

2.4. Analytical methods

Influent and effluent concentrations of total and soluble chemical oxygen demand (tCOD and sCOD, respectively), ammonium (NH_4^+–N), nitrite (NO_2^-–N), nitrate (NO_3^-–N), and phosphate (PO_4^{3-}–P) were determined spectrophotometrically by using standard test kits (Hach-Lange, Germany). Total suspended solids (TSS) and sludge volume index (SVI) were analyzed according to Standard Methods (APHA, 2005). The SVI was measured with a mixed liquor sample taken at the end of the aerobic phase. The SVI_{10} and SVI_{30} were determined by measuring the biomass volume after a settling period of 10 and 30 min, respectively. Since granules settle significantly faster than activated sludge flocs, the SVI_{30}/SVI_{10} ratio was used in order to better assess granule formation, as suggested by De Kreuk et al. (2007). Particle size fractions of the sludge were measured using different sieves with decreasing mesh openings of 0.63 mm, 0.25 mm, and 0.1 mm. Sieving of the different sludge fractions provides information about the granule diameter. However, sieving does not provide information about the surface shape of the granules (filamentous or not). A sludge volume of 200 mL was sampled from the reactors at the end of the aeration phase and gently mixed with 800 mL of tap water in order to prevent the build-up of a cake at the sieve surface. The sieving procedure was performed as described in Bin et al. (2011). The morphology of the flocs and granules from the reactors was regularly observed using a stereomicroscope (Olympus SZX10, Japan).

3. Results

3.1. Effect of the absence or presence of X_S on aerobic granulation (Phase I)

The changes in the biomass settling properties and TSS concentrations measured in the reactors and effluents are presented in Fig. 1. In R1 (fed with soluble substrate – S_S), the SVI_{30} remained nearly stable and lower than 50 mL g_{TSS}^{-1} throughout the whole

Table 2
Compositions of the synthetic wastewaters fed into Reactor 1 (S_S) and Reactor 2 ($S_S + X_S$).

Parameters	Unit	Reactor 1	Reactor 2
Substrate form	–	Soluble (S_S)	Soluble and particulate ($S_S + X_S$)
Carbon source	–	Acetate and hydrolyzed peptone	Acetate, hydrolyzed peptone, and particulate starch
Total COD	mg tCOD L^{-1}	496 ± 42	494 ± 38
Soluble COD	mg sCOD L^{-1}	488 ± 43	253 ± 18
Ammonium nitrogen	mg NH_4^+–N L^{-1}	50 ± 5	52 ± 3
Phosphorus	mg PO_4^{3-}–P L^{-1}	5.0 ± 0.6	4.7 ± 0.5
Total organic loading rate	kg tCOD m^{-3} d^{-1}	1.5 ± 0.1	1.5 ± 0.2
Soluble organic loading rate	kg sCOD m^{-3} d^{-1}	1.5 ± 0.1	0.8 ± 0.1
Nitrogen loading rate	kg NH_4^+–N m^{-3} d^{-1}	0.11 ± 0.01	0.13 ± 0.01
Phosphorus loading rate	kg PO_4^{3-}–P m^{-3} d^{-1}	0.02 ± 0.00	0.01 ± 0.00

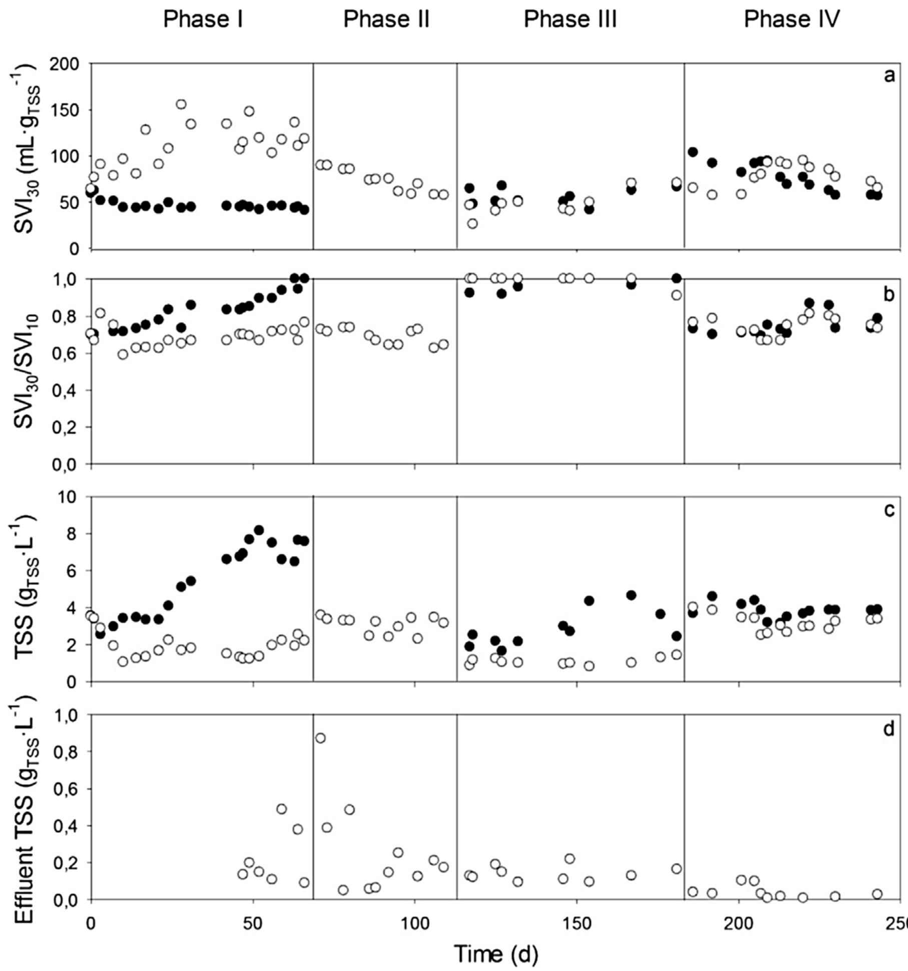

Fig. 1. Effects of operating conditions on (a) SVI_{30}, (b) SVI_{30}/SVI_{10} ratio, (c) TSS in the reactor, and (d) TSS in the effluent of R1, fed with S_S (●), and R2, fed with $S_S + X_S$ (○). The granulation process was sequentially investigated in absence or presence of X_S (*Phase I*), with an extended anaerobic phase in R2 (*Phase II*, note results for R1 are not available), with an increased selection pressure for fast-settling biomass (*Phase III*), and at constant reactor working volume (*Phase IV*).

Phase I (Fig. 1a). The TSS concentration in the reactor increased and reached a stable value of 7.0 ± 0.6 g_{TSS} L^{-1} after 50 days of operation (Fig. 1c). At this time, the average TSS concentration in the effluent amounted to 40 ± 2 mg_{TSS} L^{-1} (Fig. 1d) and the solids retention time (SRT) stabilized around 55 days. A co-existence of flocs and granules was observed during the whole *Phase I*. The fraction of granules (aggregates with d > 0.25 mm) was dominant after 60 days of operation and stabilized around 54 ± 7% of the total biomass (Fig. 2a). The SVI_{30}/SVI_{10} ratio approached a value of 1 at this period (Fig. 1b), indicating a major presence of granules in the sludge.

On the other hand, the settling properties of the biomass were highly affected by the presence of X_S in the wastewater of R2 (fed with a mixture of soluble and particulate substrates – $S_S + X_S$). The SVI_{30} increased (up to 150 mL g_{TSS}^{-1}) and washing-out of sludge occurred, resulting in a sharp decrease of the biomass concentration inside the reactor from 3.4 to less than 1.5 g_{TSS} L^{-1} (Fig. 1c). After 50 days of operation, the SVI_{30}, SVI_{30}/SVI_{10} ratio, TSS in the reactor, and fraction of granules (d > 0.25 mm – Fig. 2a) stabilized around 119 ± 14 mL g_{TSS}^{-1}, 0.71 ± 0.03, 2.0 ± 0.4 g_{TSS} L^{-1}, and 21 ± 1%, respectively. The residual TSS concentration in the effluent was significantly high during the whole experiment, varying between 55 and 490 mg_{TSS} L^{-1}. The SRT was usually lower than 4 d as a result of the continuous loss of biomass with the treated effluent. The presence of X_S in the wastewater also influenced the morphology of the biomass. The granules grown on S_S (Fig. 3a) in R1 had a spherical and defined outline, while irregular and filamentous structures could be observed at the surface of the granules cultivated in the presence X_S in R2 (Fig. 3b).

Stable elimination of carbon was achieved throughout the operation of R1, with an average efficiency of 85 ± 11% for tCOD and 96 ± 2% for sCOD (Fig. 4a). In R2, though the sCOD removal was nearly stable, with an average efficiency of 92 ± 2%, the tCOD removal varied substantially between 25 and 83% during the whole operating period, with an average value of 62 ± 18% (Fig. 4b). High ammonium removal was obtained in R1, with an average removal efficiency of 94 ± 8%, while only 14 ± 8% was removed in R2. Both reactors performed phosphorus removal as a result of the application of alternating anaerobic and aerobic conditions during the SBR cycles. However, the presence of X_S in the feed of R2 was correlated to lower phosphate uptake (50 ± 22%) than obtained under the sole presence of acetate in the wastewater (72 ± 13%), as in the case of R1.

3.2. Effect of an extended anaerobic period (Phase II)

The anaerobic period in R2 was extended to 90 min (60 min of anaerobic feeding and 30 min of anaerobic reaction without mixing) to enhance the utilization of the particulate substrate under anaerobic conditions. This led to an increase in the biomass concentration and in the total fraction of granules to around

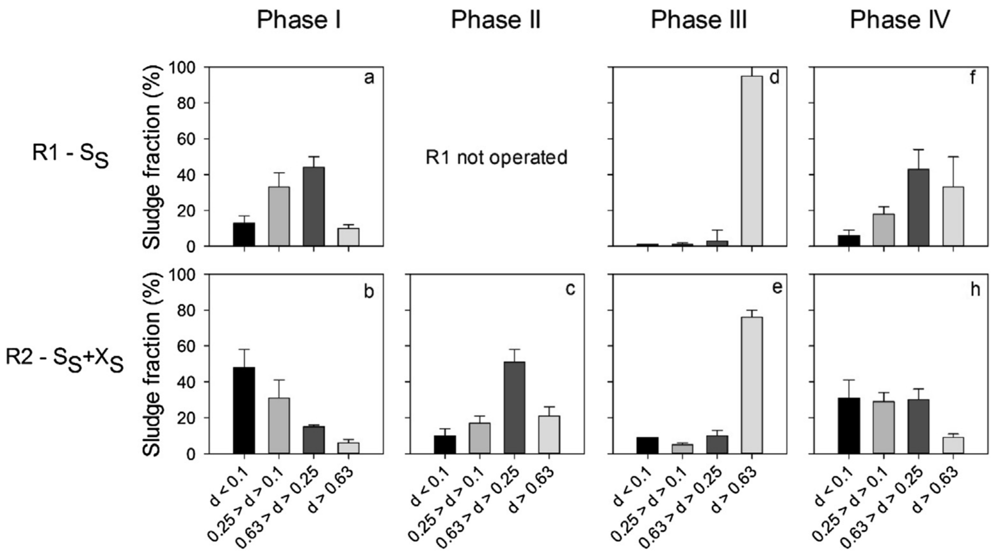

Fig. 2. Effects of operating conditions on the size distribution of biological aggregates in R1, fed with S_S, and in R2, fed with $S_S + X_S$: granulation process in absence or presence of X_S (*Phase I*), extended anaerobic phase in R2 (*Phase II*, note results for R1 are not available), increased selection pressure for fast-settling biomass (*Phase III*), and constant reactor working volume (*Phase IV*). The x-axes displays the classes of particle diameters according to measurements conducted with mesh openings of 0.10, 0.25, and 0.63 mm. The sludge fractions are presented as averages values over the last three weeks of each phase.

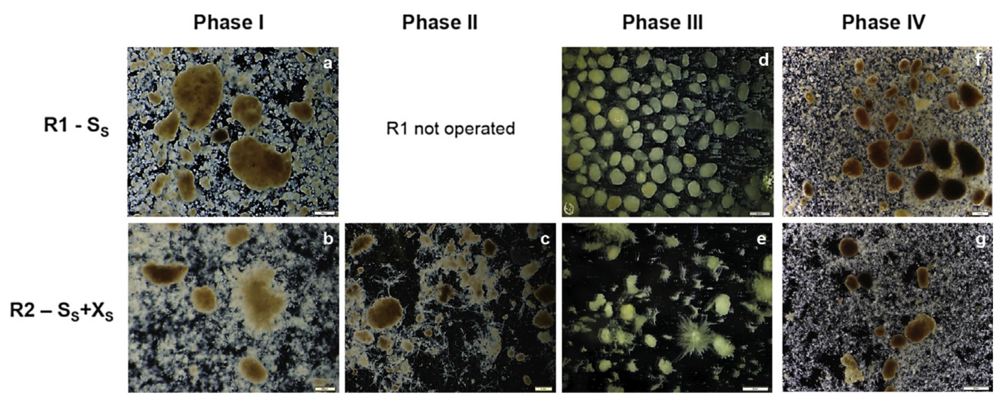

Fig. 3. Stereomicroscopic images of aerobic granules cultivated in R1, fed with S_S, and in R2, fed with $S_S + X_S$, for the different operating conditions applied: granulation process in absence or presence of X_S (*Phase I*), extended anaerobic phase in R2 (*Phase II*, note results for R1 are not available), increased selection pressure for fast-settling biomass (*Phase III*), constant reactor working volume (*Phase IV*). The biomass samples were taken from the reactors during the last week of each phase. Size bars represent 0.5 mm (a, b, and c), 1 mm (d), and 2 mm (e, f, g, and h).

3.0 ± 0.4 g_{TSS} L^{-1} and $73 \pm 8\%$, respectively (Figs. 1c and 2c). Although the SVI_{30}/SVI_{10} ratio remained at a similar level as in *Phase I* (around 0.70), lower SVI_{30} values down to 60 mL g_{TSS}^{-1} resulted from the increase of the anaerobic period length (Fig. 1a). Solids were however still present in a considerable amount of around 250 ± 246 mg_{TSS} L^{-1} in the effluent (Fig. 1d), and therefore the SRT remained usually lower than 4 d. Stereomicroscopy observations revealed that the aerobic granules became less irregular, but that the presence of filamentous organisms in the bulk liquid increased (Fig. 3c). The improvement of the granular sludge characteristics in terms of biomass concentration, settling ability, and morphology was nevertheless not followed by an increase in nutrient removal (Fig. 4c). Removal efficiencies for tCOD, ammonium, and phosphorus were indeed slightly lower during *Phase II* than during *Phase I*, with average values of $55 \pm 19\%$, $13 \pm 8\%$, and $46 \pm 29\%$, respectively. As mentioned previously, R1 had to be re-inoculated and restarted due to some operational problems that occurred in the beginning of *Phase II*.

3.3. Effect of an increased selection pressure for fast-settling biomass (Phase III)

Aerobic granules grew in both reactors under a settling time of 8 min. However, a decrease of the sedimentation time to 4 min was required to select only for the granular sludge. This selection pressure caused a wash-out of the slow-settling sludge and only the aggregates with good settling ability were retained in the reactor. As a result of the biomass wash-out, TSS decreased in both reactors down to 2.8 ± 1.0 g_{TSS} L^{-1} in R1 and 0.8 ± 0.2 g_{TSS} L^{-1} in R2 (Fig. 1c). The quality of the effluents, in terms of solids concentration, ranged between 34 and 91 mg_{TSS} L^{-1} for R1 and between 95 and 220 mg_{TSS} L^{-1} for R2 (Fig. 1d). The SRT in R1 varied around 14 d while in R2 it was usually lower than 3 d. The increase of the selection pressure for fast-settling biomass led to an improvement of SVI_{30} and SVI_{30}/SVI_{10} ratio, particularly in R2 (Fig. 1a and 1b). A SVI_{30}/SVI_{10} ratio of around 1 was reached in both reactors. Average SVI_{30} values of 56 ± 9 and 50 ± 14 mL g_{TSS}^{-1} were measured in R1 and R2, respectively. The fraction of granules increased substantially

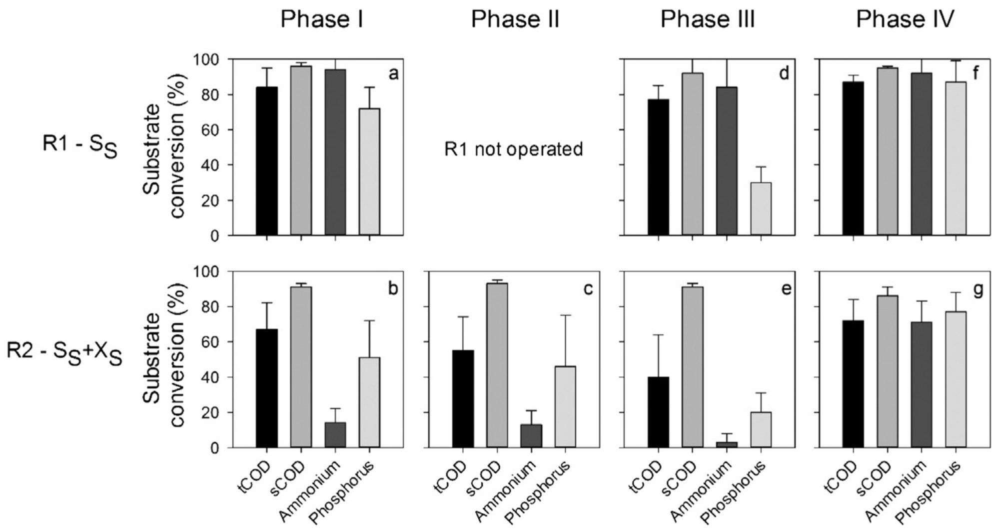

Fig. 4. Effect of operating conditions on substrate conversion in R1, fed with S_S, and in R2, fed with $S_S + X_S$: granulation process in absence or presence of X_S (*Phase I*), extended anaerobic phase in R2 (*Phase II*, note results for R1 are not available), increased selection pressure for fast-settling biomass (*Phase III*), and constant reactor working volume (*Phase IV*). The conversion efficiencies are presented as averages values over the last three weeks of each phase.

and reached 97 ± 1% in R1 (Fig. 2d) and 85 ± 2% in R2 (Fig. 2e). The aerobic granules had an almost uniform size (d > 0.63 mm) with a nearly spherical shape and a yellowish color in R1 (Fig. 3d). In R2 the granules still had a considerable irregular and filamentous structure (Fig. 3e). The substrate conversion capacity for both reactors was lower than in the previous phases, especially for nitrogen and phosphorus removal. The average efficiencies for the removal of tCOD, sCOD, ammonium, and phosphorus amounted to, respectively, 77 ± 8%, 92 ± 8%, 84 ± 22%, and 30 ± 9% in R1 (Fig. 4d) and 40 ± 24%, 91 ± 2%, 3 ± 5%, and 20 ± 11% in R2 (Fig. 4e).

3.4. *Effect of operating at constant reactor volume (Phase IV)*

The short settling time applied during *Phase III* resulted in a considerable concentration of solids in the effluent, especially for R2. This continuous biomass wash-out prevented the accumulation of solids inside the reactors, which in turn affected the overall removal performance. For this reason, the operation of the reactor was changed in *Phase IV* from variable to constant working volume, *i.e.*, simultaneous influent fill and effluent draw (so-called fill-and-draw phase) after the settling phase. The synthetic wastewaters were then injected from the bottom of the reactors at a low up-flow velocity of 0.5 m h^{-1}. The selection pressure was thus significantly reduced compared to *Phase III*, in which biomass with settling velocity lower than 9 m h^{-1} was removed together with the supernatant. Operating the SBRs at constant volume and low wastewater up-flow velocity helped to reduce the concentration of solids in the effluents of both reactors (Fig. 1d). Average values of 11 ± 10 mg_{TSS} L^{-1} for R1 and 42 ± 26 mg_{TSS} L^{-1} for R2 were achieved. The lower selection pressure for fast-settling biomass also led to a higher fraction of flocs inside the reactors (Fig. 3f and 3g). However, filamentous structures were no longer observed in R2. The concentration of solids and the fraction of granules were, respectively, 3.7 ± 0.4 g_{TSS} L^{-1} and 76 ± 6% in R1 (Figs. 1c and 2f) and 3.2 ± 0.4 g_{TSS} L^{-1} and 39 ± 8% in R2 (Figs. 1c and 2g). The corresponding SRTs were larger than 15 d and 7 d for R1 and R2, respectively. Monitoring the dynamic change in the different sludge fractions indicated that: (i) the fraction of granules first decreased from 84% to 8% following the change in the reactor operation, and then (ii) steadily increased from 6% to more than 50% in around 70 d. The sludge settleability was also affected by the presence of flocs. In R1, the SVI_{30} increased in the beginning of the experiment and stabilized around 58 ± 3 mL g_{TSS}^{-1}. This parameter increased more gradually in R2, up to an average value of 71 ± 6 mL g_{TSS}^{-1}. Working at constant volume led to an improvement of the reactors performance (Fig. 4f and 4g). The average removal efficiencies for tCOD, sCOD, ammonium, and phosphorus increased, respectively, to 87 ± 4%, 95 ± 1%, 92 ± 10%, and 87 ± 12% in R1, and to 72 ± 12%, 86 ± 5%, 71 ± 12%, and 77 ± 11% in R2.

A summary of the results obtained for the different operating conditions can be found in Table S1 in the supporting information.

4. Discussion

4.1. *Formation and morphology of aerobic granules fed with wastewater containing particulate organic substrate*

The present study displayed that the type of substrate, namely readily biodegradable soluble (S_S) and slowly biodegradable particulate (X_S), impacts significantly the morphology of the granules as well as the whole granulation process. The aggregates developed in the presence of X_S exhibited filamentous structures, which in turn affected the settling ability and the accumulation of biomass inside the reactor. The growth of filamentous organisms at the surface of the granules after switching the feed water from synthetic to real domestic wastewater was previously noticed (Peyong et al., 2012). The influence of the presence of particulate substrates (starch) on the morphology of pre-cultivated aerobic granules in a pilot system fed with municipal wastewater was also reported by De Kreuk et al. (2010). However, to what extent the presence of X_S affects the granulation process (*e.g.*, kinetics of aerobic granule formation from flocs) has never been evaluated. In R1 (S_S only), after 52 d of operation, the SVI_{30}/SVI_{10} ratio was higher than 0.9 while in R2 ($S_S + X_S$) this ratio was always lower than 0.7. A SVI_{30}/SVI_{10} ratio close to 1 accounts for full granulation of the biomass. Furthermore, the fraction of granules reached only 21% in R2, while in R1 this fraction was of 54%. The aerobic granulation process is thus much slower in the presence of X_S. This may explain why the formation of AGS in reactors fed with real wastewaters usually takes longer when compared to that of reactors fed with synthetic wastewaters containing only S_S. Several studies performed with real municipal wastewater indeed reported periods of 140 up to

400 days to achieved more than 80% of granulation (Ni et al., 2009; Liu et al., 2010; Wagner and Costa, 2013; Wagner et al., 2015). On the other hand, Liu et al. (2010) have stated that aerobic granules cultivated in lab-scale reactors using synthetic wastewater containing only S_S are usually formed in 2–4 weeks and reach the steady state in 1–2 months.

The treatment of wastewaters that contain particulate organic substrates thus requires an adaptation of the operating conditions. An extension of the anaerobic period was therefore applied in order to enhance the hydrolysis of X_S in R2. This led to the formation of more compact granules with a better settling ability ($SVI_{30} = 58 \pm 5$ mL g_{TSS}^{-1}) and to a higher biomass concentration inside the reactor (TSS $= 3.0 \pm 0.4$ g_{TSS} L^{-1}). However, filamentous structures were still present in the bulk liquid suggesting that the applied conditions (60 min of anaerobic feeding +30 min of anaerobic idle period) were not sufficient to hydrolyze all the particulate starch from the wastewater. Furthermore, the fraction of granules (d > 0.25 mm) was higher in the presence of X_S (73%) than with S_S only (54%), although microscopic observations showed opposite results (Fig. 3c). A filamentous surface contributes to the existence of granules with larger diameter but with a fluffy structure (Peyong et al., 2012). Therefore, filamentous suspended growth can likely leads to an incorrect determination of the granule fraction since these organisms had an open structure and were probably retained in the sieves with the mesh openings of 0.63 and 0.25 mm.

During *Phase I* and *Phase II* the biomass was always a mixture of granules and flocs in both reactors. The co-existence of both types of biomass is often reported in AGS reactors treating real (Ni et al., 2009; Liu et al., 2011; Liu and Tay, 2012; Wagner and Costa, 2013; Wagner et al., 2015) and synthetic (Wan et al., 2009; Mañas et al., 2011) wastewaters. To maintain a stable operation of the AGS reactor, the fraction of flocs should be kept at a low level (<35% of total biomass) by stimulating the growth of granular sludge or by physically discharging the flocculent sludge (Liu and Tay, 2012). Shortening the settling time from 8 to 4 min favorably selected for granular sludge as dominant form of biomass in the reactors by significantly washing-out the fraction of flocculent sludge. However, the continuous wash-out of flocs resulted in a low biomass concentration inside the reactors. No biomass accumulation was observed under these conditions, indicating that the growth of aerobic granules was limited. Furthermore, the amount of solids present in the effluents remained high, especially in the presence of X_S. Based on our results, it can be noted that the application of a strong selection pressure for fast-settling biomass in order to achieve full granulation is not an adequate operating condition when treating wastewaters containing X_S. Achieving successful granulation in the presence of X_S requires the reduction of the selection pressure.

At variable volume the effluent was extracted at the half of the maximum working heights of the reactors. After settling, all the biomass that remained above this port was washed-out with the effluent. Biomass with settling velocities greater than 4.5 m h^{-1} (*Phase I* and *II*) or 9 m h^{-1} (*Phase III*) was thus selected. At constant volume, the reactors were directly fed with the influent wastewater from the bottom resulting in a slow discharge of the effluent in the upper part of the reactors. The upflow velocity of the wastewater thus determined the selection pressure during the anaerobic feeding. In *Phase IV*, biomass with settling velocity larger than 0.5 m h^{-1} was selected, which is much lower than in *Phases I*, *II*, and *III*. Operating the SBRs at constant volume with a reduced selection pressure for fast-settling biomass limited the wash-out of the biomass (flocs) at the end of each cycle. This resulted in a larger accumulation of flocs and therefore in a higher biomass concentration. The operation of AGS reactors at constant volume was previously reported (De Kreuk and van Loosdrecht, 2004; Giesen et al., 2013). Under such an operation mode, a low wastewater upflow velocity results in a plug-flow regime behavior of the liquid across the settled bed (Weissbrodt et al., 2014). The granules in the lower part of the settled bed will then face a higher substrate concentration than the flocs in the upper part of the settled bed (De Kreuk and van Loosdrecht, 2004). Preferential substrate uptake by granules leads therefore to the establishment of granular sludge over floccular sludge in the system. However, a significant fraction of flocs remained in R2 despite the rather low SVI_{30} and although no filamentous organisms were observed at the granules surface and/or in the bulk liquid. The more regular morphology of the biomass might be related to enhanced hydrolysis of X_S as a result of longer anaerobic period and reduced selection pressure.

4.2. Substrate conversion

The quality of the treated effluent was also affected by the presence of X_S in the wastewater. The substrate removal in R2 was always substantially lower than in R1. Nutrient removal was more pronounced in R1 probably as a result of the higher biomass concentrations maintained in this reactor (higher SRT). The proliferation of filamentous organisms in R2 indeed led to a constant wash-out of fluffy biomass produced in the presence of X_S. Therefore, the overall SRT was usually lower than 4 d in R2 during *Phases I*, *II*, and *III*. The growth of polyphosphate accumulating organisms (PAOs) typically requires SRTs larger than 3 d at 20 °C (Henze et al., 2008). The SRT maintained in R2 during *Phases I*, *II*, and *III* thus corresponds to the minimum SRT required for the PAO growth. SRT values lower than 4 d are, however, too low to support the growth of ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). Larger SRTs are usually required for the growth of AOB and NOB (6–8 days at 20 °C to achieve full nitrification) (Henze et al., 2008). The low SRT in *Phases I*, *II*, and *III* is therefore directly responsible for the partial ammonium removal. In *Phase IV*, reducing the selection pressure helped to increase the SRT in R2 to values larger than 7 d. In turn, the increase of the SRT in R2 directly enhanced the substrate conversion (ammonium and biological phosphate removal). Thus, the presence of X_S in the influent indirectly influenced the substrate conversion through its influence on the floc wash-out and thus on the SRT. One might however notice that the SRTs that were calculated in our study mainly correspond to the SRT of the slow-settling biomass, *i.e.*, the biomass that was washed-out with the supernatant. The specific SRT of the granule fraction that was selectively retained in the reactor was larger than the values provided by these calculations. Over long-term, slow-growing organisms might preferentially grow within the granules. Thus, nitrogen and phosphorus removal would be expected despite the low overall SRT of 4 d.

Another possible explanation for a lower substrate removal in the presence of X_S is the inefficient COD removal under anaerobic conditions, and the subsequent degradation of the residual COD under aerobic conditions. If COD is not completely stored under anaerobic conditions, nutrient removal during the aerobic phase will be reduced (Bassin et al., 2012). Samples taken immediately after the beginning of the aerobic phase (data not shown) displayed that the anaerobic uptake of soluble COD was incomplete in R2 during *Phases I*, *II*, and *III*, while in R1 this substrate was mostly consumed during the anaerobic feeding. This may be related to the low rate of X_S hydrolysis rates under anaerobic conditions. The hydrolysis process converts slowly biodegradable particulate substrate into readily biodegradable soluble compounds. As a relatively slow process, hydrolysis is the rate-limiting step for the biodegradation of organic carbon and therefore it is generally the decisive step triggering the quality of the effluent (Insel et al., 2003). When operating at constant reactor volume (*Phase IV*), COD was mainly consumed during the anaerobic fill-and-draw period. These results suggest that the anaerobic uptake of particulate matter was improved at low wastewater upflow velocity during the fill-and-draw period, leading to a better nutrient removal. Limited biological dephosphatation and simultaneous nitrification and denitrification (SND) were previously observed in AGS reactors due to the leakage of COD into the aerobic period (De Kreuk et al., 2010; Weissbrodt et al., 2012). However, denitrification process was not the focus of our study and was therefore not evaluated.

4.3. Relevance of the findings for practical applications

Lab-scale AGS reactors fed with synthetic substrate containing only soluble organic matter are usually operated at variable volume with a rapid effluent withdrawal and a high selection pressure for the fast-settling biomass (sedimentation time usually <5 min). Our results demonstrated that such operating conditions cannot directly be transposed to the operation of reactors fed with wastewaters containing X_S. The key factor triggering the formation of regular and dense aerobic granules with a high substrate removal capacity in the presence of X_S seems to be related to the establishment of an operating mode where the uptake of particulate matter under anaerobic conditions is enhanced. In our study, this was achieved by decreasing the selection pressure for fast-settling biomass and applying a simultaneous fill-and-draw mode. Future works should nonetheless investigate different wastewater upflow velocities during feeding. This should allow for an evaluation of the balance between the fractions of granules and flocs inside the reactor while assuring a good quality of the treated effluent, especially regarding denitrification and biological phosphorus removal.

5. Conclusions

(i) Forming aerobic granules when treating wastewaters containing particulate organic matter (X_S) requires: (1) reducing the selection pressure for fast-settling biomass, and (2) applying a longer anaerobic period to favor the hydrolysis of X_S and the organic substrate utilization. Conventional operating conditions applied when treating wastewaters that contain only S_S (*e.g.* high selection pressure) cannot be directly transposed when treating wastewaters that contain X_S.

(ii) The aerobic granular sludge reactor fed with X_S was always composed of a substantial fraction of flocs. A high selection pressure for fast-settling biomass (settling time of 4 min) helped to maintain the fraction of flocs considerable low (<15%). However, this led to a continuous wash-out of the flocs, resulting in partial substrate conversion. Increasing the anaerobic period and reducing the selection pressure for fast-settling biomass enhanced the substrate conversion and favored the granulation process. Despite the presence of X_S in the influent, very good settling properties were achieved under these conditions: low SVI_{30} (around 70 mL g_{TSS}^{-1}) and high SVI_{30}/SVI_{10} ratio (>0.8).

Acknowledgments

This work was conducted during a scholarship (grant number 19002-12-2) financed by CAPES Foundation – Brazilian Federal Agency for Support and Evaluation of Graduate Education within the Ministry of Education of Brazil.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2015.08.030.

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