Particulate substrate retention in plug-flow and fully-mixed conditions during operation of aerobic granular sludge systems

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ABSTRACT

Particulate substrate (XB) is the major organic substrate fraction in most municipal wastewaters. However, the impact of XB on aerobic granular sludge (AGS) systems is not fully understood. This study evaluated the physical retention of XB in AGS sequencing batch reactor (SBR) during anaerobic plug-flow and then aerobic fully-mixed conditions. The influence of different sludge types and operational variables on the extent and mechanisms of XB retention in AGS SBR were evaluated. XB mass-balancing and magnetic resonance imaging (MRI) were applied. During the anaerobic plug-flow feeding, most XB was retained in the first few cm of the settled sludge bed within the interstitial voids, where XB settled and accumulated ultimately resulting in the formation of a filter-cake. Sedimentation and surface filtration were thus the dominantXB retention mechanisms during plug-flow conditions, indicating that contact and attachment of XB to the biomass was limited. XB retention was variable and influenced by the XB influent concentration, sludge bed composition and upflow feeding velocity (vww). XB retention increased with larger XB influent concentrations and lower vww, which demonstrated the importance of sedimentation on XB retention during plug-flow conditions. Hence, large fractions of influent XB likely remained suspended during aerobic fully-mixed conditions, where XB then preferentially and rapidly attached to the flocs. During fully-mixed conditions, increasing floc concentrations, longer mixing times and larger XB concentrations increased XB retention. Elevated XB retention was observed after short mixing times < 60 min when flocs were present, and the contribution of flocs towards XB retention was even more pronounced for short mixing times < 5 min. Overall, our results suggest that flocs occupy an environmental niche that results from the availability of XB during aerobic fully-mixed conditions of AGS SBR. Therefore, a complete wash-out of flocs is not desirable in AGS systems treating municipal wastewater.

1. Introduction

Our understanding of the effect of particulate organic substrate (XB) on the formation, operation and overall process performance of aerobic granular sludge (AGS) remains limited, despite many full-scale installations (Derlon et al., 2016; Ali et al., 2019). Prior studies suggested that XB might have several different effects on the behaviour and performance of AGS systems: (1) floc formation and reduced settleability (Wagner et al., 2015b; Derlon et al., 2016; Layer et al., 2019), (2) longer start-up duration (Wagner et al., 2015a; Layer et al., 2019), (3) reduced nutrient removal capability (De Kreuk et al., 2010; Jabari et al., 2016; Guimarães et al., 2018), and (4) deterioration of effluent quality due to an increased effluent solids concentration (Roktášek et al., 2015; Van Dijk et al., 2018). However, the link between these observations and the presence of XB in the influent wastewater (WW) is not well understood yet. Research on the overall impact and utilisation pathways of XB on AGS systems is therefore necessary.

XB represents a major fraction of the organic substrate present in municipal WW (typically > 50%) (Metcalf and Eddy, 2014). Hydrolysis of XB is required prior to its utilisation, which often is considered the rate limiting step in biological WW treatment (Margroth et al., 2002). In AGS systems, an anaerobic feeding phase of 1–2 h duration - most of the time as plug-flow - is typically applied (Pront et al., 2015b). However, such period of plug-flow feeding is likely too short to allow for full hydrolysis of XB (Jabari et al., 2016; Guimarães et al., 2018).
Therefore, it is suspected that some XB could “leak” into aerobic conditions in AGS operation. The presence of organic substrate in aerobic conditions favours the growth of finger-type granules (De Kreuk et al., 2010; Pronk et al., 2015a) or can even result in process breakdown due to granule breakage (Sturm et al., 2004). However, finger-type granules are rarely observed in AGS systems treating municipal WW (Pronk et al., 2015b; Derlon et al., 2016). Rather, a noticeable growth of flocs is actually observed, so that flocs represent a substantial fraction of 10–20% of the AGS formed during treatment of municipal WW (Wagner et al., 2015a; Derlon et al., 2016; Layer et al., 2019; Pronk et al., 2015b). The presence of flocs in AGS is now acknowledged in full-scale installations (Van Dijk et al., 2018), despite their origin is not well understood. AGS is therefore step-by-step seen as hybrid system, where biofilm (granules) and suspended biomass (flocs) coexist (Layer et al., 2019). Understanding the connection between influent XB and the presence and role of flocs is therefore required.

XB degradation is a three step process: (1) physical contact to biomass (physical XB retention), (2) initiation of enzymatic hydrolysis after contact to biomass, and (3) further utilisation of hydrolysis products as readily biodegradable substrate (SB) in anaerobic (fermentation, storage), anoxic (denitrification) or aerobic (direct oxidation, storage) processes. The present study focuses specifically on physical retention of XB. Several aspects might hamper physical retention of XB in AGS in comparison to conventional activated sludge systems: (1) distinct hydraulic conditions during anaerobic plug-flow feeding followed by aerobic fully-mixed conditions in SBR operation and (2) the presence of both biofilms (granules) and suspended biomass (flocs) in AGS systems treating municipal WW. Fig. 1 illustrates the possible pathways of XB retention during anaerobic plug-flow feeding (Fig. 1A) and aerobic fully mixed conditions (Fig. 1B). Plug-flow feeding from the bottom of the reactor into the settled AGS bed could limit attachment through restricted contact between influent XB and biomass. If XB then re-suspends during fully mixed conditions, it is hypothesized that flocs would have a competitive advantage in capturing XB due to their much increased adsorption capacity (Andreadakis, 1993) (Fig. 1B). Understanding when, i.e., during plug-flow or fully-mixed conditions, and where, i.e., by flocs or at the granules surface, XB is retained therefore needs to be clarified. In addition, it should be clarified what external factors (operational, influent WW) influence XB retention in AGS SBR operation.

The main objective of this study was to better understand the fate of XB during AGS SBR operation and ultimately to get insights about the presence and role of flocs in AGS systems. Therefore, the focus was on evaluating (1) to what extent XB is retained during anaerobic plug-flow feeding and then during aerobic fully mixed conditions, (2) to what extent is the retention of XB affected by operational variables (upflow feeding velocity vww, mixing time) and influent composition on XB retention in AGS SBR operation, and ultimately (3) to provide insights about the presence and role of flocs in AGS systems. Therefore, several different retention tests under plug-flow and fully-mixed conditions were conducted. Different types of biomass or mimics of biomass (AGS fed with acetate/propionate, glass beads, AGS fed with municipal WW, activated sludge flocs) and the effect of different vww during plug-flow feeding and mixing time during fully-mixed conditions were tested. Real municipal WW particles were used as XB source, and COD mass-balances were conducted to quantify the extent of XB retention in the different experiments. In addition, magnetic resonance imaging (MRI) was used to identify the mechanisms of XB retention during plug-flow feeding.

![Fig. 1. Hypothesized fate of XB during (A) anaerobic plug-flow and (B) aerobic fully mixed conditions. Case (A) illustrates the hypothesized transport and retention pathways of XB during anaerobic plug-flow feeding (retention through sedimentation and surface filtration without much contact or attachment to biomass). Under fully mixed conditions (B) it is hypothesized that XB preferentially attaches to flocs rather than granules.](image-url)
2. Materials and methods

2.1. Experimental approach

Both plug-flow, MRI and fully-mixed tests were conducted to evaluate the fate of XB in AGS SBR operation (Table 1). Primary effluent WW of the WW treatment plant (WWTP) of Eawag (Dübendorf, Switzerland) was used as the source of XB during all tests. Anaerobic or anoxic redox conditions were kept during all tests in order to minimize degradation of XB.

2.2. Experimental set-up

2.2.1. Plug-flow tests

Plug-flow tests were conducted to quantify XB retention during anaerobic plug-flow feeding (Fig. 2A). Tests with different sludge beds of similar height (13 cm) were first conducted: empty bed (no biomass), activated sludge flocs, AGS fed with municipal WW (named “AGS Eawag”), and glass beads (2 mm) (see images of the different sludge in Supplementary Information Figs. S1A and C). Different vww (1.0–5.0 m h⁻¹) and XB influent concentration (variable) were tested (Table 1). Tests with variable bed height (0–20 cm) were then conducted to better understand the distribution of XB retention over the sludge bed height (Table 1). Low and medium vww (1.25 and 2.5 m h⁻¹) and filter-bed composed of glass beads (d = 2 mm) were tested. In parallel to the tests with real WW XB as influent, blank plug-flow tests were conducted (tap water injection instead of real WW) to account for XB loss from the filter-bed during feeding. Columns with 2.5 and 5 cm inner diameter (working volume of 393 and 1963 mL, height of 82 and 100 cm, respectively) were used. The volume-exchange ratio (VER) was 1.3 during the plug-flow tests and the sludge volume after 30 min of settling (SV₃₀) was 130 mL L⁻¹. Very high VER >1.0 was used to make sure that some influent WW would exit the column through the effluent. The procedure of the plug-flow tests was as follows:

Step 1: Addition of 300 mL of biomass to 1 L glass beakers, with a target total suspended solids (TSS) concentration of 4 gTSS L⁻¹.

Step 2: 1st tap water injection from the bottom of the column to a targeted bed height of 13 cm (fixed sludge bed height tests) or variable from 0.5 to 20 cm (variable sludge bed height tests) after 20 min of settling. A settling duration of 20 min was sufficient to ensure a complete settling of the sludge during all tests. Supernatant removal above settled sludge bed using drainage ports.

Step 2: 1st tap water injection from the bottom of the column using a peristaltic pump to refill the column (Heidelberg, Germany). Second settling phase (20 min). Tap water was injected to refill the reactor, in order to mimic simultaneous fill-drain mode (constant volume operation), typically applied in full-scale AGS systems during feeding.

Step 3: Injection of 500 or 2500 mL (for small and large column, respectively)

Step 4: 2nd tap water injection, drainage and collection of column supernatant.

Step 4: Collection of 50 mL of supernatant 9 cm underneath the water surface.

2.2.2. Fully-mixed tests

Fully-mixed tests were conducted to analyse XB retention under fully-mixed conditions, representative of the aerobic phase of AGS systems (schematic Fig. 2B). The approach was based on Modin et al. (2015) and Jimenez et al. (2005). The influence of sludge composition, mixing time and influent XB concentration was evaluated (Table 1). The sludge was composed of different ratios of large granules (>1 mm) 0–100% and flocs 100–0% in increments of 25% (see images of the different sludge in Supplementary Information Figs. S1B and D). Mixing times of 0.5, 5, 10, 60, 180 min and variable influent XB concentrations were evaluated. All fully-mixed tests were conducted for a defined sludge composition. Blank fully-mixed tests were in addition conducted (tap water instead of real WW) to account for XB loss from biomass. The procedure of the fully-mixed tests was as follows:

Step 1: Addition of 300 mL of biomass to 1 L glass beakers, with a target total suspended solids (TSS) concentration of 4 gTSS L⁻¹.

Step 2: Mixing for 0.5, 5, 10, 60 or 180 min. The mixing velocity gradient (G) was set to 3.3 s⁻¹ using an apparatus with propellers, similar to the G-values maintained during aeration in the long-term lab-scale experiments performed at Eawag (Layer et al., 2019; Supplementary information S2).

Step 3: Settling for 30 min in order to separate biomass and supernatant.

Step 4: Collection of 50 mL of supernatant 9 cm underneath the water surface.

2.3. Analytical methods

TSS was quantified using standard methods (Apha, 2005). Sludge was separated using sieves of 0.25 mm (to separate flocs < 0.25 mm from granules > 0.25 mm) or 1 mm (to separate large granules > 1 mm from small granules, flocs and debris). Sieving of the different sludge fractions was performed by gently pouring the sludge into the sieve, and then washing the sieve with additional tap water. The particles retained by the sieve were collected by back-washing the cake that formed on the sieve with tap water. Size fractions were then quantified using TSS measurements. Total and soluble COD was measured using cuvette tests (LCK 114, 314, Hach-Lange, Germany, Kits). XB was defined as the

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| Hydraulic condition | Specific question addressed | Independent variables |
|---------------------|-----------------------------|-----------------------|
| Plug-flow test       | Extent of XB retention during anaerobic plug-flow feeding? | Filter-bed composition (activated sludge flocs, real AGS, large granules, glass beads, or no biomass) |
|                     | Effect of sludge bed type, vww and wastewater composition on XB retention? | Uplow velocity within the reactor (vww = 1.0–5.0 m h⁻¹) |
|                     | XB retention distribution over bed height? | Fixed (13 cm) or variable sludge bed height (0–20 cm) |
| MRI                 | XB retention during plug-flow feeding: attachment or sedimentation in interstitial void space? | - |
| Fully-mixed test    | - Does XB attach to flocs, granules or both? | Biomass type (increasing fractions of activated sludge flocs (0–100%) and large granules (100-0%) in 25% increments) |
|                     | - Effect of mixing time on XB retention? | Mixing time (0.5–180 min) |
Fig. 2. Schematic of procedure and sampling points during plug-flow tests (A) and fully-mixed tests (B). Underlined and bold measurement points indicate tests with WW addition, green measurement points indicate blank-tests without WW addition. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
difference between total and soluble COD, measured after filtration at 0.45 μm using membrane filters (Macherey Nagel, Nanocolor Chromatil membranefilter GF/PET 0.45 μm, Germany). Samples were collected in 50 mL vials and homogenized for 1 min at 10'000 rpm (Ultra-Turrax, Ika, Germany) prior to total COD measurement. In our study, Xb refers to all COD fractions larger than 0.45 μm, including biodegradable and unbiodegradable fractions of particulate COD and possibly a fraction of the colloidal COD (Levine et al., 1985).

2.4. Calculations

COD mass-balances were performed to calculate Xb retention (%) during plug-flow tests (Eqs. (1) and (2)) and fully-mixed tests (Eqs. (3) and (4)). The mass-balance of plug-flow tests takes into account mass of Xb from influent, effluent, supernatant and is corrected for the mass of Xb that is detached during the tests (from blank plug-flow tests), Eqs. (1) and (2), Fig. 2A.

\[
f_{Xb,PF,retained} = \frac{M_{Xb,retained} - M_{Xb,non-retained} + M_{Xb,attached} \times 100\%}{M_{Xb,retained}}
\]  
\[
f_{Xb,mix,retained} = \frac{X_{b,in} \cdot V_{in} - X_{b,eff} \cdot V_{eff} - X_{b,col} \cdot V_{col} + X_{b,eff,ret} \cdot V_{eff,ret} + X_{b,col,ret} \cdot V_{col,ret}}{X_{b,in} \cdot V_{in}} \times 100\% 
\]

where \(X_{b,in}\) is the Xb influent concentration and \(V_{in}\) is the injected volume into the column, \(X_{b,eff}\) is the Xb effluent concentration, \(V_{eff}\) is the effluent volume, \(X_{b,col}\) is the Xb concentration in the column supernatant, \(V_{col}\) is the volume of the column supernatant, \(X_{b,eff,ret}\) is the detached Xb concentration in the effluent during blank plug-flow tests, \(V_{eff,ret}\) is the effluent volume during blank plug-flow tests, \(X_{b,col,ret}\) is the detached Xb concentration of the column supernatant during blank plug-flow tests and \(V_{col,ret}\) is the volume of the column supernatant during blank plug-flow tests.

The fully-mixed tests mass-balance takes into account the mass of Xb which was added via WW, supernatant after a certain mixing time and is corrected for detaching mass of Xb from blank fully-mixed tests, Eqs. (3) and (4), see Fig. 2B.

\[
f_{Xb,mix,retained} = \frac{M_{Xb,retained} - M_{Xb,non-retained} + M_{Xb,attached} \times 100\%}{M_{Xb,retained}}
\]  
\[
f_{Xb,mix,retained} = \frac{X_{b,in} \cdot V_{in} - X_{b,eff,ret} \cdot V_{eff,ret} + X_{b,sup,ret} \cdot V_{sup,ret}}{X_{b,in} \cdot V_{in}} \times 100\% 
\]

where \(X_{b,in}\) is the Xb concentration of the primary effluent WW added and \(V_{in}\) is the volume of the primary effluent WW added to the beaker at \(t = 0\) min (0.7 L), \(X_{b,sup}\) is the Xb concentration of the supernatant after mixing for a given time and additional 30 min of settling, \(V_{sup}\) is the supernatant volume (1 L), \(X_{b,sup,ret}\) is the Xb supernatant concentration during blank fully-mixed tests, and \(V_{sup,ret}\) is the supernatant volume during the blank fully-mixed tests (1 L).

The Reynolds number was calculated according to Eq. (5).

\[
Re = \frac{vd}{\nu}
\]
3. Results

3.1. Retention of XB during the anaerobic plug-flow feeding of AGS systems

3.1.1. How is XB retention influenced by influent WW composition, \( v_{\text{wW}} \) and biomass type in plug-flow conditions?

XB retention was evaluated during plug-flow tests (Fig. 3). XB retention during plug-flow conditions varied between 10 and 90%. The concentration of XB in the influent WW had major impact on XB retention. Biomass composition and applied \( v_{\text{wW}} \) influenced XB retention to a lesser extent.

Increasing XB concentrations significantly increased XB retention \((p = 2.48 \times 10^{-7})\), independent of biomass composition or applied \( v_{\text{wW}} \). Specifically, high XB influent concentrations > 600 mg L\(^{-1}\) resulted in XB retention > 60%. Biomass composition also affected XB retention during plug-flow conditions \((p = 1.28 \times 10^{-3})\). In absence of a filter bed (blank test) 10–52% of influent XB were retained (Fig. 3A). In presence of a filter bed, overall XB retention is increased to > 60% on average (Fig. 3B–D). In addition, lower \( v_{\text{wW}} \) in general resulted in higher XB retention \((p = 0.022)\).

3.1.2. How is XB distributed over the bed height during plug-flow conditions?

A main question is where does the retention of XB occur within the settled bed of AGS during plug-flow feeding? Results from the plug-flow tests with variable sludge-bed heights indicated that a gradient of XB retention over the bed height existed (Fig. 4). Hereby, large amounts of XB were retained at the bottom of the settled sludge bed. The larger was the upflow feeding velocity during the plug-flow feeding, the deeper was the penetration of XB and hence the lower was the gradient of XB retention within the settled sludge bed. Low \( v_{\text{wW}} \) of 1 m h\(^{-1}\) led to increased XB retention at the bottom of the sludge bed. Almost 70% of final XB retention occurred within the first 0.5 cm. On the other hand, higher \( v_{\text{wW}} \) of 2.5 m h\(^{-1}\) during feeding increased the penetration depth of XB, thus resulting in a more homogeneous distribution of XB within the bed. The first 0.5 cm of the settled sludge bed retained 30% of the final retention in this case. Overall higher XB retention at \( v_{\text{wW}} = 1 \) m h\(^{-1}\) were likely the result of a higher influent XB concentration compared to the run at \( v_{\text{wW}} = 2.5 \) m h\(^{-1}\), which were 292 and 201 mg L\(^{-1}\) for \( v_{\text{wW}} = 1 \) and 2.5 m h\(^{-1}\), respectively. The Reynolds numbers were 0.6 and 1.4 for \( v_{\text{wW}} \) of 1 and 2.5 m h\(^{-1}\), respectively.

3.1.3. Does XB attach to granules surface or accumulate within interstitial voids of the sludge bed during plug-flow conditions?

Our results from plug-flow tests helped to quantify the extent of XB retention during plug-flow feeding and its spatial distribution over the height of the sludge bed. A major aspect is however to better understand if XB is attached to the settled biomass after feeding, or if it simply accumulated within the bed without much contact. MRI tests were thus conducted to evaluate the spatial

![Fig. 3. Retention of XB in percent COD during plug-flow feeding for different influent XB concentrations and different \( v_{\text{wW}} \) (1.0–5.0 m h\(^{-1}\)) with different biomass compositions: A) Blank (no sludge bed), B) Glass beads (diameter 2 mm), C) AGS Eawag and D) Flocs.](image-url)
distribution of $X_B$ within the settled granular sludge bed during anaerobic plug-flow feeding. Results from MRI tests demonstrated that $X_B$ accumulated within the interstitial voids in the first few cm of the settled sludge bed, and that $X_B$ accumulation was actually affected by both sedimentation and surface filtration (Fig. 5, Fig. 6).

Most $X_B$ accumulated within the first 13 mm in vertical direction after the 1st feeding (Fig. 5A, white colour, Fig. 6AB). Granules were pushed by the applied flow, creating channels and resulting in void space (Fig. 5A). Moreover, $X_B$ hardly distributed horizontally within the granule bed. Instead, $X_B$ was located mostly in the bottom of the chamber and additionally occupied the void space in vertical direction extending the inlet (Fig. 5A). Only minimal distribution of $X_B$ in the x- and y-direction occurred despite the rather narrow chamber of the MRI, and no wall-effects were visible. After the 2nd feeding $X_B$ occupied even more of the void space and was distributed along the whole height of the chamber (Fig. 5B). Occupation of the void space by $X_B$ was indicated by large white-coloured areas/volumes surrounding the preferential flow channel, created by the inlet flow in the centre of the column after the 1st and 2nd feeding (Figs. 5 and 6A). The Reynolds number during MRI tests was 0.1 assuming a granule diameter of $d = 1.0$ mm.

**Fig. 4.** $X_B$ retention in percent COD during plug-flow feeding at different locations through a sludge bed composed of glass bead (2 mm) at $v_{wv} = 1$ and 2.5 m h$^{-1}$. Primary effluent WW was composed of $X_B = 292$ mg L$^{-1} (v_{wv} = 1 \text{ m h}^{-1})$ and $X_B = 201$ mg L$^{-1} (v_{wv} = 2.5 \text{ m h}^{-1})$.

**Fig. 5.** Quantified images after first (A) and second WW feeding (B). Quantified images are 2D sections out of the 3D measurements. $X_B$ particles (white), granules (grey), water filled void space (dark grey) and exterior parts (black) can be differentiated.
3.2. How is $X_b$ retained during fully-mixed conditions?

If large fractions of influent $X_b$ are retained within the settled sludge bed during anaerobic plug-flow feeding but not binding to the granules, it is then likely that $X_b$ re-suspends and becomes available for attachment in aerobic fully-mixed conditions for both flocs and granules in AGS systems. Fully-mixed tests were thus conducted to better understand where $X_b$ does attach during mixed conditions, i.e., to granules or flocs (Fig. 7). Results from the fully-mixed tests indicated that an increasing floc fraction in the AGS significantly increased $X_b$ retention during fully-mixed conditions in AGS systems ($p < 1.0E-05$), specifically in the first 60 min of mixing. Additionally, longer mixing times as well as higher influent $X_b$ concentrations significantly increased $X_b$ retention in AGS systems (all $p < 1.0E-05$).

Over 50% of the final $X_b$ removal was achieved during the first 30 s of mixing if flocs were present in the biomass (Fig. 7). Reduced $X_b$ retention was observed after 30 s in absence of flocs (>$20\%$ less $X_b$ retention by 100% Granules, Fig. 7AB). However, the longer the mixing time was, the smaller were the differences in overall $X_b$ retention between the different biomass compositions. After 3 h of mixing, $X_b$ retention was $60–85\%$ among all biomass compositions and floc fractions were less important towards overall $X_b$ retention ($p = 0.14$) (Fig. 7AB). It must be noted that the total biomass concentration during fully-mixed tests was held constant, independent of different granule-flocs fractions, which further highlighted the impact of flocs on $X_b$ retention in mixes of granules and flocs. Influent $X_b$ concentration also contributed to the overall level of $X_b$ retention. The increased $X_b$ influent concentration of fully-mixed test B (196 mgCOD L$^{-1}$; Fig. 7B) led to overall higher $X_b$ retention, independent of mixing time or biomass composition, when compared to the lower $X_b$ influent concentration of fully-mixed test A (94 mgCOD L$^{-1}$; Fig. 7A).

4. Discussion

4.1. $X_b$ accumulates within the sludge bed during plug-flow feeding but does not attach to granules

Our first main result is that $X_b$ accumulated predominantly within the voids at the bottom of the settled sludge bed, thus indicating that $X_b$ retention was governed by sedimentation and surface filtration during plug-flow feeding (Figs. 3–6). If $X_b$ retention was governed by sedimentation and surface filtration, it is then likely that only a minor fraction of $X_b$ is actually in contact with the granules during anaerobic plug-flow feeding (Figs. 5–6; Ranzinger et al., 2020).

We propose that $X_b$ retention through sedimentation and surface filtration during plug-flow feeding of AGS systems is a 3-step process, consisting of (1) channel formation, (2) settling of $X_b$ and (3) surface filtration. Firstly, influent flow causes slight redistribution of granules, which locally enlarges void space and then forms channels in upward direction within the settled sludge bed. Secondly, influent $X_b$ settles within the channels. The channels are progressively filled up by the settling of $X_b$, ultimately resulting in a filter-cake. Thirdly, influent $X_b$ is then strained by the filter-cake and surface filtration occurs (Maroudas and Eisenklam, 1965). With continuing influent WW injection, the filter-cake consisting of $X_b$ is being pushed upwards. Attachment of $X_b$ to the granules during plug-flow feeding is thus limited. However, our results do not allow us to conclude about actual contact between filter-cake and the granule surface, since the resolution of MRI is too coarse (Ranzinger et al., 2020). A minor fraction of $X_b$ could thus be in contact to the granules during anaerobic plug-flow feeding.

Our results also indicated that the influent $X_b$ concentration and upflow velocity determined the extent of $X_b$ retention during plug-flow feeding. The upflow feeding velocities applied at pilot-scale but also in full-scale AGS system are ranging from 0.5 to 5 m h$^{-1}$, with a typical value of 2 m h$^{-1}$ when treating municipal WW (Derlon et al., 2016; Wagner et al., 2015a; Pronk et al., 2015b). The settling velocity of influent $X_b$ particles in the size range of 45–200 μm in diameter is 0.8–16 m h$^{-1}$ (specific gravity 1.2 kg L$^{-1}$) or 4.0–75 m h$^{-1}$ (specific gravity 2.0 kg L$^{-1}$) (Stokes, 1851; Levine et al., 1985; Johnson et al., 1996). The settling velocities of $X_b$ particles are in general larger than the values of upflow feeding velocities. However, the upflow velocity of the influent WW must be corrected for the porosity of the settled sludge bed, with a typical value of 0.52 (Van Dijk et al., 2020). The actual upflow velocity within the sludge bed pores therefore increases by a factor of 1.9, to values of 1.0–9.6 m h$^{-1}$. In general, the actual upflow velocities are in the same range as the settling velocities of influent $X_b$ particles.
However, large X_b particles are strongly affected by sedimentation and could therefore play an important role in the initial formation of a filter-cake at the bottom of the settled sludge bed. Smaller X_b particles that are transported through advection could then be retained through surface filtration by the filter-cake (Maroudas and Eisenklam, 1965). Higher X_b concentrations usually coincide with larger particle diameters (Sophonsiri and Morgenroth, 2004). Influent WW composed of high X_b concentrations will thus lead to increased settling of X_b at the bottom of the sludge bed and fast formation of a filter-cake during the anaerobic plug-flow feeding.

The overall particle size distribution entering the AGS SBR is determined by whether primary treatment via primary clarification or a similar filtration or sedimentation step is implemented or not (Levine et al., 1991). Colloids (particles d < 1 \( \mu \)m) are prone to diffusion, and can indeed diffuse into the granules located at the bottom of the sludge bed during plug-flow feeding (Ranzinger et al., 2020). Retention of colloidal particles is therefore governed by inherently different mechanisms compared to retention of X_b particles, which cannot diffuse into the biofilm (Polson, 1950). It must be noted that MRI tests were conducted using a very high X_b loading, in order to increase the image quality. Therefore results gained from MRI (Figs. 5–6) likely overemphasised the magnitude but not the occurrence of sedimentation and surface filtration as X_b retention mechanisms during plug-flow feeding.

We therefore propose that a large fraction of X_b is retained at the bottom of the settled sludge bed through sedimentation and surface filtration and is thus not attached to biomass. Combining limited attachment to biomass and slow hydrolysis in anaerobic plug-flow feeding conditions suggests that large fractions of X_b are not hydrolysed during anaerobic plug-flow feeding conditions (Henze and Mladenovski, 1991). Large fractions of influent X_b could therefore re-suspend once aerobic fully-mixed aerobic conditions are applied (Ranzinger et al., 2020), in analogy to particle re-suspension during backwash of granular media filters (Amirtharajah, 1985).

4.2. Large fractions of X_b are retained by flocs during fully-mixed conditions

Another main finding of our study is that X_b re-suspends during fully-mixed conditions, e.g., once aeration starts, and is available for attachment onto both granules and flocs. A main question is whether X_b will then attach preferentially to the flocs or to the granules.

During the first 60 min of mixing, the presence of flocs increased X_b retention by more than 20%, in comparison to the “100% granules” case (Fig. 7). We hypothesize that X_b retention is mostly achieved by flocs through rapid attachment, due to the very large specific surface area of flocs (flocs TSS fraction 20%, flocs-to-granules surface area ratio 939-to-1, Supplementary Information S4, Andreadakis, 1993; Mihciokur and Oguz, 2016; Jimenez et al., 2005). Granules, on the other hand, have a much smaller specific surface area and are much lower in number (Supplementary Information S4). In addition, the surface of mature granules is often rather smooth when flocs are also present in the AGS, and granules do not offer many locations for attachment in comparison to odd-shaped, ramified flocs. Reduced X_b removal and decreased X_b removal rates by biofilm systems is linked to limited active adsorption sites (Boltz and La Motta, 2007). We thus propose that flocs have a competitive advantage over granules to retain X_b.

Fig. 7. X_b retention in percent COD during fully mixed conditions displaying effects of different granule/flocs fractions for two different runs (A and B). Mixing times varied from 0.5 to 180 min. Primary effluent was used as X_b source (X_b concentration = 94 and 196 mgCOD L \(^{-1}\) for runs A and B, respectively).
through attachment during fully-mixed aerobic conditions, due to their physical structure despite their minor fraction in AGS systems treating municipal WW (10–30% TSS-based; Layer et al., 2019). If $X_B$ is attaching rapidly and preferentially to the flocs, only little $X_B$ is then left for attachment onto the granules. Attachment of $X_B$ onto the granules was much slower compared to $X_B$ attachment to mixtures of flocs and granules, or solely flocs (Fig. 7).

Previous studies indeed indicate that the contribution of biofilms to the retention and hydrolysis of $X_B$ is quite limited during fully-mixed conditions. Particles $>1$ $\mu$m are typically considered the most difficult to be removed in biofilm systems (Levine et al., 1991). In moving bed biofilm reactors (MBBR) used for the treatment of municipal wastewater, no reduction in TSS usually occurs in the MBBR stage (Ähl et al., 2006). In general, reduced hydrolysis of $X_B$ has been reported for biofilm systems in comparison to conventional activated sludge systems (Janning et al., 1998; Morgenroth et al., 2002). Actually, several studies even suggested that hydrolysis in biofilm systems is carried out in the bulk phase rather than at the biofilm surface (Rohold and Harremoes, 1993; Larsen and Harremoes, 1994a, 1994b). Those findings suggest that the contribution of biofilms to $X_B$ hydrolysis is rather small, due to the limited attachment of $X_B$ onto biofilms. AGS systems treating municipal WW are now often regarded to as hybrid biofilm systems (Layer et al., 2019). Therefore, we hypothesize that in hybrid systems such as AGS, flocs outcompete granules in $X_B$ retention through attachment once mixing is applied.

4.3. Practical implications

Attachment of $X_B$ was quite limited during anaerobic plug-flow conditions, and full retention of $X_B$ was then achieved in aerobic fully-mixed conditions. Retention of $X_B$ during fully-mixed tests were performed using very high $X_B$-to-biomass ratios (70/30 v/v), and final $X_B$ retention was $>80$% in all tests. Thus, complete removal of $X_B$ can be expected during the aerobic fully-mixed phase in full-scale AGS SBR operation. Flocs retained a large fraction of $X_B$ through rapid attachment after mixing was applied. Therefore, it is very likely that (1) $X_B$ will be fully hydrolysed within the SBR cycle (Henze et al., 2000) and that (2) the majority of hydrolysis products are consumed within the floc micro-environment, too (Martins et al., 2011). Flocs will thus always co-exist with granules in AGS systems as long as the WW contains organic substrate in the form of $X_B$. Aggressive wash-out of flocs via short settling times still is a common start-up and operational strategy in AGS SBR operation (Adav et al., 2008). We however propose that too aggressive wash-out of flocs is neither desirable nor expedient in AGS systems treating municipal WW, even at the cost of decreased settling performance (Layer et al., 2019). It is likely that too high wash-out of flocs in AGS systems treating $X_B$-rich municipal WW leads to increased $X_B$ attachment, hydrolysis and utilisation by the granules. An increased aerobic utilisation of $X_B$ by the granules would then result. Aerobic utilisation of organic substrate by the granules was linked to filamentous outgrowth, loss of nutrient removal performance and/or granule breakage and process failure, eventually (Sturm et al., 2004; De Kreuk et al., 2010; Derlon et al., 2016; Haaksman et al., 2020). To date, it is still under debate if flocs have other important functions in AGS systems like, e.g., if their contribution towards nutrient removal is significant or negligible, and whether their presence is desirable or not (Ali et al., 2019; Layer et al., 2019, 2020). Therefore, more research is required on the specific function of flocs in AGS systems treating municipal WW. $X_B$ retention can be optimised by e.g. introducing an anaerobic-mixed phase after plug-flow feeding (Layer et al., 2019). An increased attachment of $X_B$ to flocs and granules during anaerobic conditions would be the result. However, prior research has indicated that anaerobic hydrolysis of $X_B$ originating from municipal WW can be limited (Jabari et al., 2016). Thus, anaerobic $X_B$ degradation by introducing anaerobic-mixing could be limited. Another option could aim at minimising $X_B$ in the influent to the AGS stage through advanced pre-treatment such as micro-sieving or chemically enhanced pre-treatment (Sancho et al., 2019). Pre-fermentation of captured $X_B$ in primary treatment could indeed enhance AGS performance in low-strength municipal WW conditions (Yuan et al., 2020; Vollertsen et al., 2006). However, more research is required to identify feasible operational strategies and technologies to improve $X_B$ retention, degradation and utilisation in AGS-based WWTP.

Reynolds numbers calculated for plug-flow tests indicated laminar flow conditions during plug-flow feeding at lab-scale. It must be noted that turbulent flow conditions could occur during the feeding phase of a full-scale AGS SBR, depending on the design of the influent WW distribution system, e.g., due to scarce injection nozzle distribution. In such case, two distinct zones might exist, where the first zone (e.g., bottom 10–50 cm of the settled sludge bed) experiences turbulent flow conditions and could act as a fluidized bed. Within the fluidized bed attachment of $X_B$ to biomass could be possible. The second zone above the fluidized bed would experience laminar flow conditions, where similar $X_B$ retention mechanisms as observed in our study likely occur. However, to date no detailed information on full-scale AGS SBR injection hydraulics are available, and thus, considerations are highly speculative.

5. Conclusions

1. During anaerobic plug-flow feeding of AGS SBR, $X_B$ is retained within the interstitial voids of the settled sludge bed, but with minimal attachment. In the subsequent fully-mixed phase $X_B$ then attaches preferentially to the flocs.

2. $X_B$ retention results from the combined mechanisms of sedimentation and surface filtration that occur at the bottom of the settled sludge bed during anaerobic plug-flow feeding. Up to 70% of the final $X_B$ retention occurred within the first 0.5 cm of the settled sludge bed. The attachment of $X_B$ onto the granules is thus limited during anaerobic plug-flow feeding.

3. The extent of $X_B$ retention during plug-flow feeding is determined by WW composition (influent $X_B$ concentration), $V_{ww}$ and sludge bed composition. High influent $X_B$ concentrations and low $V_{ww}$ increase $X_B$ retention.

4. A large fraction of influent $X_B$ likely re-suspends during aerobic fully-mixed conditions. Rapid $X_B$ retention after 0.5–60 min of mixing occurs if flocs are present in the biomass. Therefore, $X_B$ attaches preferentially to flocs and only a small fraction of $X_B$ attaches to granules.

5. Flocs are an important biomass fraction in AGS systems treating municipal WW rich in $X_B$. Too high wash-out of flocs is not desirable in those conditions.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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