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Granulation strategies applied to industrial wastewater treatment: from lab to full-scale

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ABSTRACT

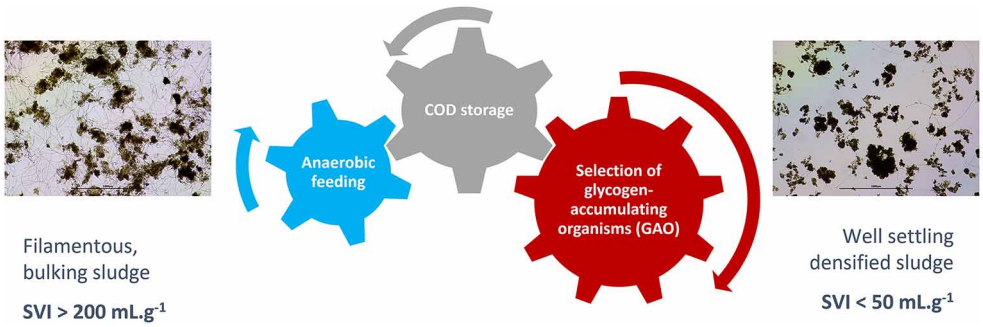
About one third of the industrial activated sludge (AS) plants worldwide suffer from bad settling sludge, often caused by filamentous bulking phenomena. The present study investigated the effectiveness of a sludge granulation/densification strategy, based only on a metabolic selection mechanism, to eliminate sludge bulking in a sequencing batch reactor (SBR) treating real industrial wastewater. The wastewater originated from a tank truck cleaning company transporting chocolate and beer. The proposed strategy involved the introduction of a slow unaerated/anaerobic feeding step in the SBR operation. On lab-scale, the new feeding strategy resulted in (1) excellent settling with a sludge volume index (SVI) decreasing from more than 300 mL·g^{-1} to 100 mL·g^{-1} and lower, (2) the elimination of sludge bulking genera and (3) the significant enrichment of glycogen-accumulating organisms (GAO), mainly *Defluviicoccus* and *Candidatus* Competibacter, and this in less than 80 days. The feeding strategy was then applied to the full-scale installation, yielding similar results: a stable average SVI of 37 mL·g^{-1} was reached after approximately 150 days. Full granulation was however not reached, which warrants further optimization. The present study shows that the proposed strategy can easily be applied in existing SBR systems to solve the problem of sludge bulking.

Key words: anaerobic selector, feeding strategy, filamentous bulking, glycogen accumulating organisms (GAO), sequencing batch reactor, storage

HIGHLIGHTS

- Filamentous bulking in industrial activated sludge was eliminated by introducing an anaerobic feeding step.
- Anaerobic feeding selected for glycogen-accumulating organisms (GAO).
- Lab-scale results were successfully translated to full-scale implementation.
- Excellent settling sludge was obtained, but full granulation was not achieved.

GRAPHICAL ABSTRACT



INTRODUCTION

Activated sludge (AS) systems represent the main biological treatment in many industrial wastewater treatment plants. In its simplest form, AS consists of an aeration tank where the biological conversions take place, followed by a secondary settler to

separate sludge from the treated water. The settled sludge is then returned to the aeration tank. In a recent survey in Flanders, Cornelissen *et al.* (2018) reported that about one third of the industrial AS plants are facing sludge separation problems due to bad settling sludge. These findings agree with more global reports on sludge bulking occurring in more than 30% of the biological treatment plants (Rossetti *et al.* 2017). Besides being linked to nutrient deficiency and oxygen limitation, sludge bulking can often be related to the operation of the AS plant, more specifically the feeding pattern (Jenkins *et al.* 2003). Slow feeding of readily degradable chemical oxygen demand (rbCOD) under aerobic conditions, without build-up of a significant organic substrate concentration, promotes filamentous growth and negatively impacts sludge settling (Martins *et al.* 2004b).

To counter sludge bulking, both curative (e.g. chlorination) as preventive solutions (e.g. introduction of a selector) have been proposed (Jenkins *et al.* 2003), the latter being preferred as they provide a longer-term solution to the bulking problem. A selector is a separate zone in the reactor that receives both the return sludge and the influent wastewater. Depending on the retention time within the selector, the sludge is exposed to high substrate concentrations and feast–famine conditions which selects for floc-forming bacteria according to the kinetic selection theory. Aerobic selectors need to be operated at high sludge loading rate, 3–4 kg COD·(kg MLSS·d)$^{-1}$, and with high dissolved oxygen (DO) concentrations to achieve the kinetic selection (Jenkins *et al.* 2003). Under anaerobic conditions, however, the main mechanism in a selector is not a kinetic but a metabolic selection, and lower sludge loading rates can be applied. Even without build-up of substrate in the selector, substrate storing organisms, such as polyphosphate and/or glycogen accumulating organisms (PAO and/or GAO) are stimulated, leading to well settling sludge without filamentous bulking (Yao *et al.* 2019). Martins *et al.* (2004a) showed the formation of well settling sludge when synthetic acetate-based wastewater was slowly fed under anaerobic conditions in the presence of phosphate. Under aerobic conditions, slow feeding of acetate led to severe filamentous bulking (Martins *et al.* 2003).

The metabolic selection of PAO and GAO also forms the main mechanism for aerobic granulation in wastewater treatment plants. PAO and GAO are referred to as 'slow-growing' organisms as they convert rbCOD to internal storage polymers such as polyhydroxyalkanoates (PHAs) under anaerobic conditions. Under aerobic (or anoxic) conditions, these PHAs are subsequently slowly degraded without electron acceptor diffusion limitations. Since the whole volume of the flocs/granules is then metabolically active, compact sludge aggregates are formed and maintained (Pronk *et al.* 2015b). In full-scale aerobic granular sludge (AGS) reactors of the Nereda® type treating domestic wastewater, full granulation is achieved through a combination of (1) PAO selection by a plug-flow anaerobic feeding regime, and (2) hydraulic selection of well settling sludge aggregates through selective withdrawal of slow settling floccular sludge (Pronk *et al.* 2015a). Regarding industrial wastewater treatment by AGS, most lab studies report the application of a pulse feed of wastewater to build up substrate during the first minutes of a cycle, followed by prolonged anaerobic mixing phase to promote PAO/GAO metabolism (e.g. Caluwé *et al.* 2017; Stes *et al.* 2019; De Vleeschauwer *et al.* 2021).

Pulse feeding of influent wastewater, i.e. feeding the influent in a few minutes time, like is often done in lab-scale studies (e.g. De Vleeschauwer *et al.* 2021), is, to the best of our knowledge, not technically feasible at full-scale. Conversion of existing full-scale installations to Nereda® type reactors with an upflow batch feeding regime, on the other hand, can be done, but at a significant investment cost. There are but a few studies reporting granulation using a slow feeding strategy applicable in practice. Meunier *et al.* (2016) reported on the successful implementation of a granulation/densification strategy to improve the sludge quality in a lab-scale sequencing batch reactor (SBR) treating phosphate-rich dairy wastewater. By introducing a slow anaerobic feeding step (90 min) in the process sequence, PAO were stimulated, filamentous bulking sludge was suppressed, and granular sludge was formed. No hydraulic selective pressure was applied. Stes *et al.* (2020) applied an anaerobic feast/aerobic famine strategy only, using slow anaerobic feeding (127 min), to form granules in a SBR treating chocolate processing effluent low in phosphorus. Stable AGS was formed when a partial static feeding step was introduced in the SBR operation.

Building upon the lab research of Meunier *et al.* (2016), Henriet *et al.* (2017) investigated the impact of an anaerobic feast/aerobic famine sequence on the sludge morphology in an existing full-scale SBR treating dairy wastewater. The full-scale plant experienced severe bulking caused by *Thiothrix* species. The fully aerobic feed step was replaced by an extended unaerated mixed feeding step (95 min), leading to the promotion of GAO and PAO, and the formation of densified granular-like sludge. Full granulation was, however, not achieved.

The present study investigates the application of this novel anaerobic feeding strategy to improve sludge settling properties in an SBR treating wastewater at a tank truck cleaning site (TTC) and experiencing severe sludge bulking. In this specific case, the wastewater originates from trucks transporting liquid food, i.e. chocolate and beer. The nutrient-deficient influent is high

in readily biodegradable chemical oxygen demand (rbCOD). An additional aim is to assess whether mere implementation of an unaerated feeding step and the associated metabolic selection would also lead to formation of AGS, as defined by de Kreuk *et al.* (2007).

METHODS

Description of the full-scale installation

The case study involves a transport and tank truck cleaning company (TTC) transporting liquid feedstuff, mostly chocolate, beer and glucose. Wastewater, resulting from the cleaning activities of tank truck interiors, is first stored in a buffer tank, then pre-treated in a chemically enhanced primary dissolved air flotation (DAF) unit, and finally biologically treated in two parallel sequencing batch reactors (SBR). At the start of each SBR cycle, the sludge volume is approximately 382 m^3 in both reactors. The average daily flow to each SBR is 40 m^3. The 24 h SBR cycle consists of 4 h aerobic feeding, 16 h aerobic reaction, 2 h settling and 2 h discharge (Table 1). During the study reported here, the average sludge loading rate, the food to microbes ratio (F/M), was 0.10 $\pm$ 0.03 kg COD·(kg MLSS·d)$^{-1}$. During aeration, the dissolved oxygen (DO) level was controlled between 1 and 4 mg·L^{-1} using an on/off control strategy. The sludge retention time (SRT) was set at 30 d. Due to excessive loss of suspended solids from the SBRs, the bio-treated effluent is post-treated by the DAF.

Lab-scale reactor set-up and operation

A fully automated double-walled lab-scale SBR with a working volume of 5.5 L and an H/D ratio of approximately 1, was operated at a controlled temperature (22 °C) for 125 days. The SBR was equipped with a mechanical mixer (RZR2020, Heidolph Instruments, Germany) to keep the sludge in suspension, a feeding pump (ES-B16VC-3, IWAKI, Japan), a discharge valve (Eriks RX10.X33.S00, ERIKS, Belgium) and an aeration disc (Aquadistri) connected to an aeration pump (Super Fish Koi Flow 60, AQUADISTRI, China). Process operation was controlled and monitored by a Siemens PLC and a custom-built LabVIEW program (National Instruments). The reactor was equipped with a luminescent dissolved oxygen (LDO) sensor (Hach Lange, USA). During aerobic phases, the DO level was controlled between 1 and 4 mg·L^{-1} using an on/off control strategy.

The reactor was seeded with activated sludge from the full-scale plant, and fed daily with 750 mL of wastewater collected from the plant after DAF treatment. The SBR cycle consisted of a feeding phase, a reaction phase and ended with settling and discharge. During the lab-scale test, the cycle duration (12 h or 24 h), the feeding regime (anaerobic or aerobic, mixed or static) and the application of an anaerobic mixed reaction phase varied (Table 1). The sludge retention time (SRT) was kept at 30 d by daily wasting of sludge from the mixed liquor.

Industrial wastewater

During the lab-scale test, 16 different influent batches were fed to the SBR. The main composition of the wastewater is summarized in Table 2. On average, the soluble COD (sCOD) represented 87% of the total COD, and the average ratio of volatile fatty acids (VFA) to total COD was 35% (on COD basis). The average ratio of influent sCOD to dissolved organic carbon (DOC) was 3.3. The nitrogen (N) and phosphorus (P) content of the nutrient-deficient influent was adapted to reach a desired COD/N/P ratio of 100/3.0/0.5 (adapted to 100/2.5/0.4 during period 5) by adding NH_4Cl and K_2HPO_4. The average pH of

Table 1 | Modes of operation of the lab-scale SBR and the full-scale SBRs (FS-SBR1 and FS-SBR2; Ana: anaerobic, Aer; aerobic)

SBR	Day (Period P)	Cycles (#·d^{-1})	Feeding regime	Feed (h)	Ana mixing (h)	Aer react	Settling + Discharge
LAB-SBR	P1: 0–7	1	Ana, Mixed	4	–	17 h 55 min	2 h + 5 min
	P2: 8–27	1	Ana, Static	3	1	17 h 55 min	2 h + 5 min
	P3: 28–64	2	Ana, Static	1.5	1	8 h 25 min	1 h + 5 min
	P4: 65–86	1	Ana, Static	3	2	16 h 55 min	2 h + 5 min
	P5: 87–125	1	Aer, Mixed	3	–	18 h 55 min	2 h + 5 min
FS-SBR1	P1: 0–158	1	Aer, Mixed	4	–	16 h	2 h + 2 h
FS-SBR1	P2: 160–250	1	Ana, Mixed	4	1	15 h	2 h + 2 h
FS-SBR2	0–250	1	Ana, Mixed	4	1	15 h	2 h + 2 h

Table 2 | Composition of the influent wastewater during the lab-scale SBR test ($n = 16$), and the applied sludge loading rate (F/M ratio) in the lab-scale SBR (LAB) and in both full-scale SBRs (FS-SBR1 and FS-SBR2)

Parameter		Min	Max	Avg. ± SD	CV (%)
COD (mg L^{-1})		2,120	6,590	4,988 ± 1,142	23
sCOD (mg L^{-1})		1,840	6,000	4,350 ± 1,066	25
F/M ratio (kg COD· (kg MLSS·d)$^{-1}$)	LAB	0.04	0.19	0.14 ± 0.04	29
	FS-SBR1	0.02	0.31	0.12 ± 0.06	47
	FS-SBR2	0.02	0.21	0.10 ± 0.03	35

the influent was 4.3 ± 0.3, so the influent pH was adjusted to 7.0 by adding sodium hydroxide (1M NaOH). The influent wastewater was kept in a fridge (at 4 °C) to prevent COD degradation.

Implementation in the full-scale plant

After the conclusion of the lab-scale experiment, the granulation strategy, i.e. the anaerobic feeding strategy, was implemented at full-scale. The implementation was done in one of the two full-scale SBRs, FS-SBR2, while FS-SBR1 acted as a reference with an unchanged fully aerobic process operation (Table 1). To this end, FS-SBR2 was first completely emptied by transferring sludge from FS-SBR2 to FS-SBR1. Then, a submerged mixer (1.5 kW) was installed, at a height of 3 m above the SBR floor, to keep the sludge in suspension during the unaerated feeding step. Finally, half of the mixed liquor from FS-SBR1 was transferred to FS-SBR2, resulting in a similar initial situation in both reactors. The new operational strategy in FS-SBR2 consisted of 4 h unaerated mixed feeding, 1 h unaerated mixing, 15 h aerobic reaction, 2 h settling and 2 h discharge.

After about 150 days of operation, the new strategy was also implemented in FS-SBR1. During a two-week period, sludge wasting from FS-SBR2 was stopped to allow sludge growth, while the loading rate to FS-SBR1 and the sludge height in FS-SBR1 were gradually lowered. An identical submerged mixer (1.5 kW) as in FS-SBR2 was installed, and FS-SBR1 was seeded with sludge from FS-SBR2. A similar unaerated feeding strategy was then applied in both FS-SBRs (Table 1).

Analytical methods

Total and soluble COD were measured with micro-COD test tubes (HI 93754A-25 and HI 93754B-25) from Hannah Instruments (Temse, Belgium). NH_4-N (HI 93715-01), NO_3-N (HI 93766-50) and PO_4-P (HI 93717-01) were measured with test kits from Hannah Instruments, after filtration over a 1.2 μm glass microfibre filter (VWR International, Belgium). The DOC was analysed with a Sievers InnovOx laboratory total organic carbon analyser. The sludge mixed liquor suspended (volatile) solids (ML(V)SS) and the sludge volume index (SVI) were measured according to the standard methods (APHA 1998). Microscopic images were taken with a MOTICBA310 microscope (Opti-service, Belgium).

Anaerobic carbon substrate removal

The removal of dissolved carbon substrate, expressed as DOC or sCOD, was evaluated via in-situ and ex-situ cycle measurements. In-situ refers to the actual substrate removal during a lab or full-scale SBR cycle, while ex-situ refers to the substrate removal capacity of sludge sampled from the full-scale SBR. The in-situ SBR cycle measurements were regularly performed to evaluate the DOC removal during the anaerobic mixing phases in the lab-scale SBR. Grab samples were taken at the end of the cycle (DOC_base), and at the start and the end of the anaerobic mixing phase (respectively DOC_start and DOC_end).

$$\text{Anaerobic DOC removal (\%)} = 100 \times \frac{\text{DOC}_{\text{start}} - \text{DOC}_{\text{end}}}{\text{DOC}_{\text{start}} - \text{DOC}_{\text{base}}}$$

The in-situ SBR cycle measurements were also performed to evaluate sCOD removal during the anaerobic mixing phases in the full-scale installation. To this end, grab samples were taken every 30 min during the anaerobic feeding phase, which happened while the sludge was in suspension. The sCOD removal at each sampling point was estimated relative to the amount of sCOD added during feeding.

$$\text{Anaerobic sCOD removal at time t (\%)} = 100 \times \frac{(\text{sCOD}_{\text{added\_t}} + \text{sCOD}_{\text{t0}}) - \text{sCOD}_{\text{t}}}{\text{sCOD}_{\text{added\_t}} - \text{sCOD}_{\text{t0}}}$$

With $sCOD_{added-t}$ the amount of sCOD added at time t during feeding (estimated based on influent sCOD concentration, reactor volume and influent flow rate), $sCOD_{t0}$ the measured initial sCOD concentration in the reactor, and $sCOD_t$ the measured sCOD concentration at time t.

Ex-situ SBR cycle measurements were regularly performed to evaluate the anaerobic DOC removal capacity of the sludge from the full-scale installation. To this end, endogenous sludge samples from the full-scale SBR were transported to the lab for an ex-situ batch experiment. 400 mL of the sampled sludge was washed (3 times) with a wash buffer (0.1M $NaHCO_3$ + 0.05M KCl) and transferred to a beaker on a magnetic stirring plate. In two parallel experiments, respectively 40 and 100 mL of wastewater was then added to the suspended sludge. Samples for DOC analysis were then taken at the start and at the end of the test (after 180 min) to calculate the DOC removal.

Microbial community composition analysis

Biomass samples were collected, centrifuged, and pellets were then stored at −80 °C until DNA extraction. DNA was isolated from triplicate samples using FastDNA® SPIN kit. This kit was used because of its high cell-breaking capability (Guo & Zhang 2013) needed for compact flocs or granular sludge following the adapted extraction protocol for AS samples (van Loosdrecht *et al.* 2016). The DNA concentration was then measured using a Qubit dsDNA Assay kit (Invitrogen, USA) following manufacturers protocol.

The bacterial 16S rRNA gen hypervariable region, targeting V1–3, was amplified by polymerase chain reaction (PCR) in a CFX96 Thermocycler (Bio-Rad). The V1–3 region was chosen since it is been described as the most suitable region for AS samples (Albertsen *et al.* 2015; Dueholm *et al.* 2021). Barcoded primers were used 27F (5′-AGAGTTTGATCCTGGCTCAG-3′), 534R (5′-ATTACCGCGGCTGCTGG-3′) and KAPA HiFi HotStart PCR kit (Boston, MA, USA). The sequencing of the amplified libraries was carried out on an Illumina MiSeq platform at the Center for Medical Genetics (Antwerp, Belgium), using a MiSeq Reagent kit v (Illuminia) following standard guidelines.

The reads were then processed using USEARCH v.11.0.667. Processed reads were clustered into Operational Taxonomy Units (OTUs) based on 97% sequence similarity threshold (Edgar 2013). Taxonomy was assigned using the MiDAS4 data base (Dueholm *et al.* 2021), and the data analysis was performed in R v. 3.2.3 using the ampvis2 package v. 1.24.0 (Andersen *et al.* 2018).

RESULTS AND DISCUSSION

Present study investigates the implementation of an anaerobic feeding strategy to improve the sludge settling properties and sludge morphology in a SBR treating industrial wastewater, first on lab-scale, then on full-scale. The results of the lab-scale test are reported first, followed by the results of the full-scale implementation. The impact of the anaerobic feeding strategy is discussed in the third section. The last section discusses the degree of granulation achieved in the lab-scale and full-scale SBRs.

Lab-scale testing

The seeding sludge for the lab-scale test, collected form the full-scale installation, had a MLSS and MLVSS concentration of 3.71 $g{\cdot}L^{-1}$ and 3.26 $g{\cdot}L^{-1}$ respectively. The SVI after 30 min was about 330 $mL{\cdot}g^{-1}$, indicative of very poor settling sludge, which was confirmed by the filamentous nature of the sludge morphology (Figures 1 and 2).

After seeding the lab-scale SBR, an anaerobic/aerobic process operation was implemented. During the first week of operation (P1 in Table 1), a mixed unaerated feeding pattern was applied, which was subsequently changed to a static feeding regime (P2 to P4 in Table 1). The latter was done to create a build-up of substrate in the SBR, resulting in a feast–famine regime (Stes *et al.* 2020).

The first 25 days of operation were characterized by a significant loss of total suspended solids (TSS) from the reactor, bad settling behaviour (SVI values above 250 $mL{\cdot}g^{-1}$) and a predominant filamentous floc morphology (Figure 1). After 50 days of operation, a clear effluent was obtained, and sludge settling improved, reaching 100 $mL{\cdot}g^{1}$ and lower after approximately 75 days. Filaments disappeared almost completely, and more compact sludge aggregates were formed. In the same period, between 25 and 50 days after start-up, a rapid increase in the DOC removal, up to approximately 80%, during the anaerobic mixing phase was observed (Figure 3). Although intracellular storage polymers, such as PHA, were not measured in this study, it is very likely that a large part of the DOC, especially the VFA fraction, was taken up and stored anaerobically. This was confirmed by the enrichment of carbon-storing organisms, mostly GAO, in the sludge (see below).

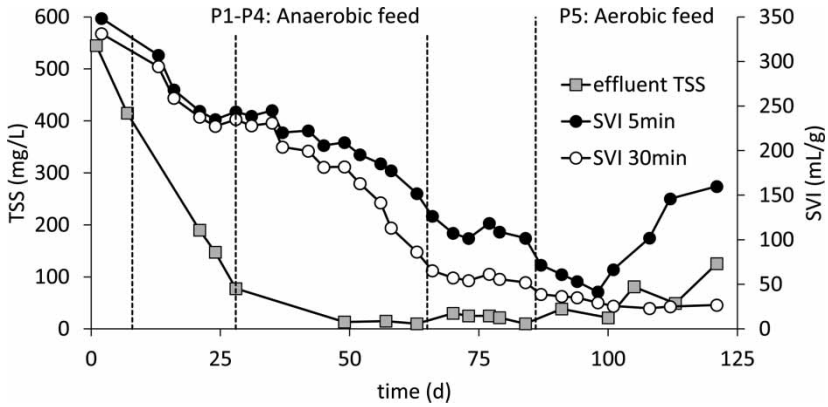


Figure 1 | Evolution of effluent TSS, and SVI after 5 and 30 min during the lab-scale experiment. The vertical dotted lines delineate the periods (P1 to P5) with the different operational conditions described in Table 1. After 86 days, the feeding regime was switched from an anaerobic (P1 to P4) to an aerobic feed (P5).

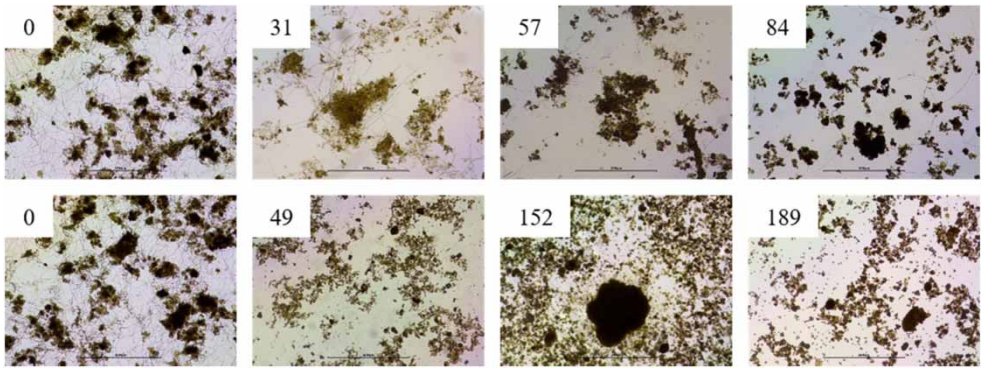


Figure 2 | Evolution of the sludge morphology at different time points during the lab-scale test (top) and full-scale FS-SBR2 test (bottom) (scale bar = 1,000 μm; boxed numbers refer to the number of days after start-up).

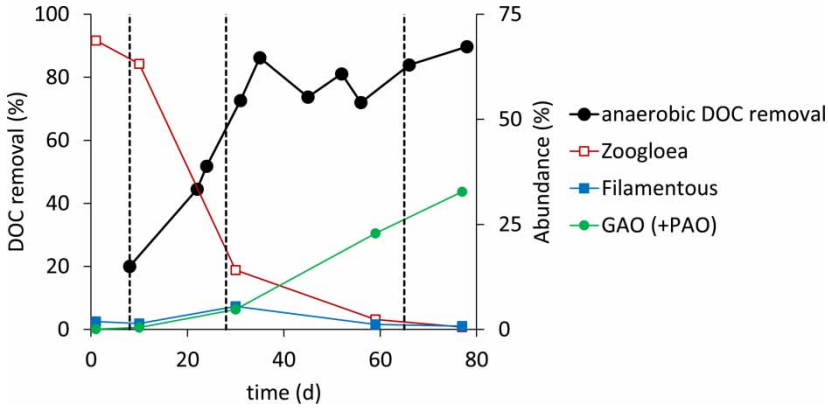


Figure 3 | Evolution of the DOC removal during the anaerobic phase of the lab-scale SBR cycle, and changes in the relative abundance, based on 16S rRNA gene sequencing, of *Zoogloea*, filamentous organisms and GAO, throughout periods P1 to P4 when the SBR was operated with an unaerated feeding step (the vertical dotted lines delineate the periods (P1 to P4) with the different operational conditions described in Table 1).

Although the sludge morphology of the seed sludge was dominated by filamentous organisms (Figure 2), filamentous bacteria were only present in low numbers: read abundances of known filamentous genera were below 5% (Figure 3). The main filamentous genera found were *Thiothrix* and *Kouleothrix*. Both organisms have been associated with serious bulking problems in (industrial) activated sludge plants (Nielsen *et al.* 2000; Nittami *et al.* 2020).

Non-filamentous bulking organisms, i.e. *Zoogloea*, dominated the seed sludge composition. *Zoogloea* bulking is caused by nutrient deficiency, most probably due to a suboptimal nutrient dosing in the full-scale installation (Jenkins *et al.* 2003). During the lab test, nutrients were added to fulfil the requirements for growth. A COD/N/P ratio of 100/3.0/0.5 was adopted.

The implementation of an unaerated feeding step resulted in a gradual increase in the degree of anaerobic DOC removal, coinciding with the enrichment of carbon-storing GAO at the expense of the bulking organisms (Figure 3). The abundance of PAO remained low throughout the entire study, although there was a slight increase in the abundance of *Candidatus* Accumulibacter, up to 1%. Two known genera of GAO were mainly enriched, i.e. *Defluviicoccus* and *Candidatus* Competibacter, from less than 1% abundance at the start to respectively 7% and 25% after 77 days. Both GAO can store short-chain VFA, such as acetate and propionate, and convert them to PHA under anaerobic conditions (Burow *et al.* 2007). VFAs represent an important COD fraction (about 35% on COD basis) in the influent. Storage by GAO prevents 'leakage' of this rbCOD fraction into the aerobic phase which could otherwise lead to stimulation of filamentous organisms, specially under oxygen-limited conditions (Pronk *et al.* 2015b; Haaksman *et al.* 2020).

After 86 days of operation, the feeding regime of the lab-scale SBR was switched to a fully aerobic feeding. Although the SVI30 remained low until the end of the experiment, a gradual increase in SVI5 and effluent TSS was recorded (Figure 1), pointing towards a deterioration of the reactor performance. The fraction of GAO dropped to less than 4% abundance after 100 days (data not shown), although neither *Zoogloea* nor other known filamentous organisms significantly increased in abundance. The lab-scale experiment was terminated after 125 days.

Full-scale implementation

The promising results obtained in the short lab-scale experiment prompted the TTC company to implement the new feeding strategy in one of the two parallel full-scale SBRs. FS-SBR2 was operated using an anaerobic/aerobic sequence, while FS-SBR1 acted as a fully aerobic reference.

The start sludge in both reactors had an MLSS of about 4.5 $g{\cdot}L^{-1}$ and a SVI of 200 $mL{\cdot}g^{-1}$, indicating the poor settling properties of the sludge. The morphology of the sludge was dominated by filamentous bacteria (Figure 2). In the first 50 days of operation, the SVI remained high in both reactors with average values between 200 and 270 $mL{\cdot}g^{-1}$. Seventy-five days after the implementation of the new feeding strategy, a clear difference between the SVI values in both reactors was observed: SVI values dropped consistently below 100 $mL{\cdot}g^{-1}$ in FS-SBR2, with an average of 75 $mL{\cdot}g^{-1}$ between days 75 and 150, while the average SVI in FS-SBR1 was 220 $mL{\cdot}g^{-1}$. The improved SVI values in FS-SBR2 were associated with a significant increase in the anaerobic DOC removal capacity of the sludge, measured in ex-situ batch test (Figure 4). In this batch test, the sludge from FS-SBR2 was exposed to a pulse feed of wastewater at a volume exchange rate (VER) of 20%, which is higher than the VER in the full-scale plant, i.e. 10%. Hence, the results of the test indicate the maximal removal

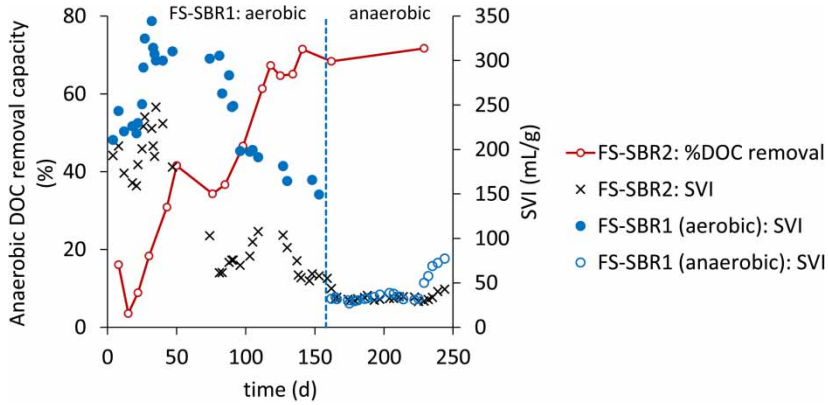


Figure 4 | Evolution of the DOC removal capacity during the anaerobic phase in the full-scale FS-SBR2, and the SVI (after 30 min) in both full-scale SBRs (FS-SBR2 was operated using an anaerobic/aerobic sequence, while FS-SBR1 was operated under fully aerobic conditions, until day 158, when the cycle was switched to an anaerobic/aerobic sequence as well).

capacity of the sludge. The anaerobic DOC removal capacity increased from less than 20% initially to almost 70% after 100 days of operation, suggesting sludge adaptation to the new feeding regime. The in-situ COD removal was always above 80% (data not shown). The evolution of the sludge morphology in FS-SBR2 was in line with changes in the settling properties. Filaments disappeared after about 50 days, and the sludge evolved into floccular sludge with an open structure (Figure 2). In the last 100 days, the sludge morphology consisted of both small flocs and larger, denser, granular-like structures.

In the last 100 days of the full-scale test, the anaerobic feeding strategy was applied to both SBRs. FS-SBR1 was emptied and restarted with sludge from FS-SBR2. This resulted in stable and low SVI values in both reactors, with an average of 37 $mL \cdot g^{-1}$.

At the start of the full-scale test, the sludge from both SBRs was high in abundance in *Zoogloea*, and the filamentous genera *Thiothrix* and *Kouleothrix*, much like the sludge in the lab-scale test (see above). In FS-SBR2, operated with an extended anaerobic feeding step, a decline in *Zoogloea* abundance, and a rise followed by a decline in filamentous genera, was observed during the first 100 days of operation (Figure 5(a)). The abundance of known bulking organisms was consistently well below 1% during the last 100 days of the test. The abundance of carbon-storing organisms, GAO and PAO, rapidly increased to average values of approximately 7%. As in the lab-scale test, the enriched GAO genera were *Defluviicoccus* and *Candidatus* Competibacter. The average abundance of PAO remained below 1% (data not shown).

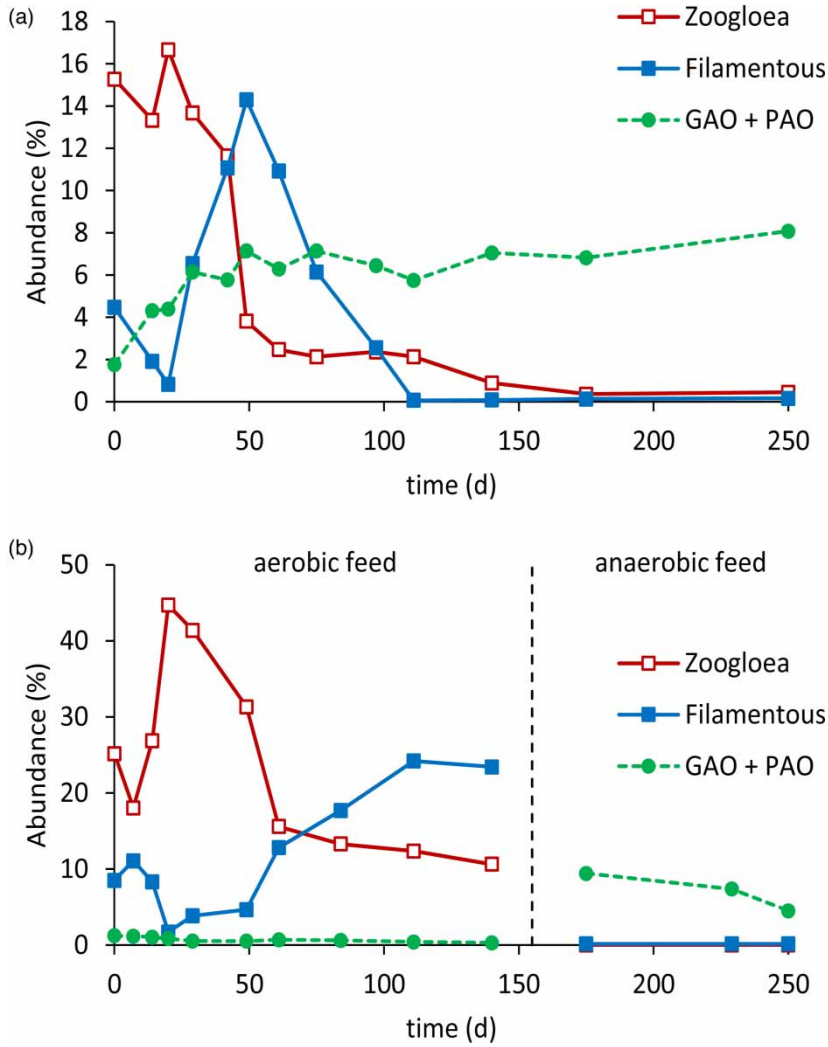


Figure 5 | Changes in relative abundances of *Zoogloea*, filamentous organisms, GAO and PAO, based on 16S rRNA gene sequencing, in (a) FS-SBR2, operated with an anaerobic feed, and (b) FS-SBR1, operated with an aerobic feed until day 158. After day 158, FS-SBR1 was seeded with sludge from FS-SBR2, and operated with an anaerobic feed.

In FS-SBR1 the abundance of bulking organisms remained high as long as the reactor was operated using an aerobic feeding strategy, although the abundance of *Zoogloea* and filamentous organisms did vary over time (Figure 5(b)). After 158 days, when the reactor was seeded with sludge from FS-SBR2 and operated using an anaerobic feeding regime for the remainder of the study, the population of GAO and PAO was maintained at similar levels (approximately 7% abundance).

Impact of the anaerobic feeding strategy

The implementation of an unaerated feeding regime in the SBR process is equivalent to the introduction of an anaerobic selector zone. The average sludge loading rate during the anaerobic phase was 0.6 ± 0.1 kg COD·(kg MLSS·d)$^{-1}$ in the full-scale SBR and 0.8 ± 0.2 kg COD·(kg MLSS·d)$^{-1}$ in the lab-scale SBR, values which are at the low end of typical design criteria for anaerobic selectors (Jenkins *et al.* 2003). The metabolic selection of carbon-storing GAO, both in lab and full-scale, is effective in combating filamentous bulking in the presented industrial case (Figure 4). The main advantage of the anaerobic selector strategy is that influent can be fed at a slow rate into the AS system. The anaerobic fill time ratio (FTR), describing the ratio of the anaerobic feeding time to (active) cycle time was 13.6% in the lab-scale test, and 20% in the full-scale installation. These FTRs are comparable to values reported earlier: Martins *et al.* (2004a) applied an anaerobic FTR of >35% and achieved good settling sludge fed with acetate-based synthetic wastewater, while Stes *et al.* (2020) and Meunier *et al.* (2016) applied FTRs of respectively 25.6% and 17% yielding AGS fed with real industrial wastewaters. On a full-scale dairy wastewater treatment plant, Henriet *et al.* (2017) applied an anaerobic FTR of 31% and obtained well-settling densified sludge. In contrast, under fully aerobic conditions, an aerobic FTR > 6.2% led to low substrate concentrations and bad sludge settleability (Martins *et al.* 2003).

In the lab-scale test, a static feeding was employed in order to create a substrate build up, and ensure full penetration of the substrate into the whole sludge volume during the subsequent mixing phase (Iorhemen & Liu 2021). The low influent pH (4.3 ± 0.3), however, precluded the application of such a static feeding step in the full-scale installation as it would require a significant amount of base to neutralize the wastewater before feeding, depending on the variable influent alkalinity (data not shown). Therefore, a slow mixed feeding was implemented in the full-scale installation. The results indicate that there was no build-up of substrate in the full-scale (not shown), but the fed COD was removed, and probably stored by GAO, almost completely while being fed in the SBR. As Martins *et al.* (2004a) showed for acetate-based synthetic wastewater, this metabolic selection was sufficient to yield well settling sludge (Figures 4 and 5).

Incomplete granulation

An additional aim of the present study was the achievement of AGS by implementation of the metabolic selection strategy as the main driver for granulation (de Kreuk & van Loosdrecht 2004). According to de Kreuk *et al.* (2007), the quantitative definition of AGS includes the SVI5/SVI30 or SVI10/SVI30 ratios, which should be close to 1, to characterize the sludge settleability, and the minimum granule size of 0.2 mm. The average SVI5/SVI30 ratio of the full-scale sludge was 1.85. The lowest average SVI5/SVI30 ratio at the end of the lab-scale test was 1.41. Since particle size determination was not performed, the average sludge size could not be determined. Microscopy analysis shows that the average size was below 0.2 mm (Figure 2). Based on both criteria, it is clear that full granulation was not achieved in the timeframe of the study.

Microscopy analysis showed a difference in morphology between the lab and full-scale sludge (Figure 2). The lab-scale sludge showed the clear formation of dense sludge aggregates consisting of both small and larger particles. The authors hypothesize that the duration of the lab test was too short to result in full granulation. Other works, only applying a metabolic selection for granulation in systems fed with real industrial wastewater, report granule formation times of 30–150 days (e.g. Meunier *et al.* 2016; Caluwé *et al.* 2017; Stes *et al.* 2020).

The full-scale morphology showed more diversity with the presence of larger granular-like structures embedded in a matrix of loose sludge particles. Besides the short time of the study, other factors may have played a role. Most importantly, the full-scale installation suffered periodical disturbances, both in the wastewater treatment plant, especially the DAF, as in the truck cleaning operation. Without going into too much detail, these disturbances lead (1) to significant overloading of the SBR, (2) insufficient COD storage in the anaerobic phase, and (3) extended low DO periods during the aerobic phase. Previous research shows that the presence of suspended solids, e.g. due to a failing DAF, could prevent or be detrimental for (full) granulation (de Kreuk *et al.* 2010; Martins *et al.* 2011; De Vleeschauwer *et al.* 2021). Also leaking of soluble, potentially rapidly degradable, COD into the aerobic phase likewise hinders granulation, especially if aeration is insufficient (Pronk *et al.* 2015b; Haaksman *et al.* 2020). In addition, the absence of a substrate build up, due to the slow mixed feeding, may represent an

insufficient driver for granulation (Franca *et al.* 2018; Stes *et al.* 2019). As discussed in the introduction, most literature references on AGS formation in industrial settings applied a pulse feeding, to mimic the plug flow regime applied in the full-scale Nereda plants (Pronk *et al.* 2015a; Franca *et al.* 2018). In the presented full-scale study, the sludge loading rate during the feeding phase, and the associated substrate build-up, may be increased by shortening the feeding time, within the practical limitations of the existing pumps, and taking the impact of feeding acidic wastewater into account. Finally, the absence of a hydraulic selection pressure prevents the selective washout of the floccular sludge fraction, resulting in a hybrid sludge consisting of both floccular and granular structures (Stes *et al.* 2020; De Vleeschauwer *et al.* 2021). Further research should explore these hypotheses in more detail, to optimize the granulation in the full-scale treatment plant.

CONCLUSIONS

The introduction of an anaerobic (feeding) step in the SBR operation alleviated the filamentous sludge bulking problem in the industrial wastewater treatment plant. Slow, unaerated feeding of the wastewater led to the metabolic selection of substrate-storing GAO, the elimination of filamentous genera, and resulted in the formation of well-settling dense sludge, although full granulation was not achieved in the time frame of the study. The implementation of the densification strategy is easy and straightforward: the main investment to be done is the installation of a submerged mixer to keep the sludge in suspension during the unaerated phase, while an anaerobic (feeding) step can easily be implemented in the PLC system of the plant. These results are of great importance for the applicability of the proposed strategy in full-scale industrial SBR systems.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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