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Long-term monitoring of a pilot-scale AGS-SBR treating real citrus-processing wastewater: effects of seasonal operation on granulation and process performance

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Research Article

Keywords: Aerobic granular sludge, citrus-processing wastewater, granular sludge stability, sequencing batch reactor, variable OLR

Posted Date: November 18th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11515920/v1

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Additional Declarations: No competing interests reported.

Long-term monitoring of a pilot-scale AGS-SBR treating real citrus-processing wastewater: effects of seasonal operation on granulation and process performance

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Abstract

This study investigated the long-term performance of a pilot-scale sequencing batch reactor (SBR) with aerobic granular sludge (AGS) treating real citrus-processing wastewater over two consecutive production campaigns. The reactor was subjected to wide fluctuations in organic loading rates (OLR) due to the strongly seasonal nature of the wastewater, and prolonged summer shutdown periods without feeding. The AGS-SBR achieved high removal efficiencies for chemical oxygen demand (COD) and biochemical oxygen demand (BOD_5), showing high potential for water reclamation. The AGS-SBR biomass maintained a hybrid granular structure throughout the monitoring period. Complete granulation and fully mature granules were not achieved, likely because of repeated OLR shifts in OLR and short steady-state periods. In addition, prolonged substrate limitation during summer shutdowns caused structural deterioration and partial degranulation; the severity of deterioration depended on the structural state of the biomass before shutdown.

Overall, the findings of this study suggested that AGS can effectively handle seasonal agro-industrial wastewaters, providing useful insights into the resilience and operational limits of granular systems treating highly variable industrial wastewater.

Keywords: Aerobic granular sludge; citrus-processing wastewater; granular sludge stability; sequencing batch reactor; variable OLR.

1. Introduction

Citrus-processing industry is an important component of the agro-food sector in many countries of Europe, North Africa and the Americas. Production of juices, concentrates, jams, and essential oils generates large wastewater volumes (Lucia et al. 2022). This wastewater mainly derives from fruit washing, juice extraction, pulp processing and equipment cleaning. Citrus processing wastewater typically contains high concentrations of organic matter, suspended solids and easily biodegradable compounds, including sugars, pectins and organic acids (Zema et al. 2016).). A major challenge for biological treatment is the pronounced seasonal variability of both flow and organic load. During full production, wastewater flow and organic load increase, whereas both decline during start-up and final stages of processing, until stopping during the summer shutdown period (Di Trapani et al. 2019). In addition, the organic content, as chemical oxygen demand (COD) concentrations, can vary widely from approximately 1 to 10 g/L, depending on the production activities (Calabrò et al. 2018). Conventional activated sludge (CAS) systems are widely applied to treat agro-industrial wastewater. However, sudden changes in organic load and flow can affect sludge settling, causing bulking, biomass washout and poor effluent quality (Andiloro et al. 2013; Corsino et al. 2018). In this context, aerobic granular sludge (AGS) is a promising alternative because it can provide excellent settling properties and high biomass retention, allowing facing extreme operating conditions (Derlon et al. 2016; Campo et al. 2020).

AGS consists of dense microbial aggregates allowing high biomass retention and the removal of organic matter and nutrients in compact reactors, while showing excellent settling properties (de Kreuk et al. 2005; Haaksman et al. 2020). Aerobic granulation is mainly observed in biological reactor operating in sequencing batch mode (SBR - sequencing batch reactor). In this reactor, the alternation of feast-famine conditions and the application of high hydraulic selection pressure (or short settling time) are considered crucial for transition of flocculent activated sludge to stable AGS (Campos et al. 2009).

Despite these advantages, maintaining stable granules can be difficult when treating seasonal industrial wastewater (Zheng et al. 2006). Most AGS studies have been carried out under controlled conditions, using synthetic wastewater or relatively stable organic loads (Corsino et al. 2017; Iorhemen and Liu 2021). In real pilot and full-scale industrial applications, changes in organic loading, wastewater composition and substrate availability can affect granule formation and stability. High organic loads may promote biomass growth, whereas prolonged substrate limitation can driveo biomass to endogenous decay, aggregate fragmentation and reversion toward flocculent sludge (Nancharaiah and Kiran Kumar Reddy 2018).

Unlike previous studies mainly conducted with synthetic wastewater or under relatively stable operating conditions, this study investigated the long-term performance of a pilot-scale AGS-SBR treating real citrus-processing wastewater under highly variable seasonal conditions. Its novelty lies in assessing aerobic granulation dynamics and granule stability across two consecutive industrial processing campaigns, including periods of fluctuating organic loading and substrate limitation. Specific attention was given to: (i) characterize the effects of seasonal OLR variation on granulation, biomass concentration, and settling; (ii) assess granule deterioration and recovery across shutdown and restart; (iii) quantify organic-matter removal and anaerobic substrate uptake; and (iv) examine effluent-quality indicators relevant to potential water reuse. The findings were aimed to provide new insights into the feasibility of AGS technology for treating seasonal agro-industrial wastewater, while also exploring opportunities for effluent valorization

2. Materials and methods

2.1 Site and wastewater description

The pilot plant was installed at a citrus-processing facility located in Sicily (Italy), produce juices and essential oils from citrus fruits. The industrial facility operates seasonally, with fruit processing occurring during the citrus production campaign (November-June) and a complete shutdown during summer, typically from August to the end of September. Wastewater generation and its organic strength vary significantly during the year in accordance with the factory production. Before the beginning of fruit processing, wastewater derives mainly from equipment washing, juice storage tank rinsing, and residual stock processing from the previous season. These operations produce a relatively low organic load, with total COD (tCOD) in the range 300 - 700 mg/L). As fruit processing begins (October-December), both wastewater volume and organic content increase, with COD progressively rising to 2000-3000 mg/L. Peak production occurs between January and May, when maximum wastewater flows are generated and COD concentrations reach up to 4000-5000 mg/L. Toward the end of spring and beginning of summer, organic loading gradually decreases below 1000 mg/L as operations shift to equipment cleaning and final stock processing, until production ceases during August and September.

Raw citrus wastewater was characterized by acidic pH values (3.0-4.5) and a severe nutrient deficiency, exhibiting a carbon-to-nitrogen-to-phosphorus (C:N:P) mass ratio exceeding 2000:5:1. To provide a suitable influent for biological treatment, the pilot plant was located downstream of an equalization tank, where pH was automatically adjusted to 7.0±0.2 with sodium hydroxide and nutrients (urea and dipotassium phosphate) were added to achieve an operational C:N:P mass ratio of 100:5:1.

2.2 Pilot-scale SBR

The pilot system was part of an international project involving academic and industrial partners of the mediterranean area (SWRIPS - *Sustainable Water Re-use with Innovative Purification and Sensing system* – funded by the European Program PRIMA - Partnership for Research and Innovation in the Mediterranean Area). The plant consisted of a cylindrical reactor operating as a SBR, with an internal diameter of 1.0 m, a total height of 1.80 m, and a working volume of 1.0 m³ (Figure 1).

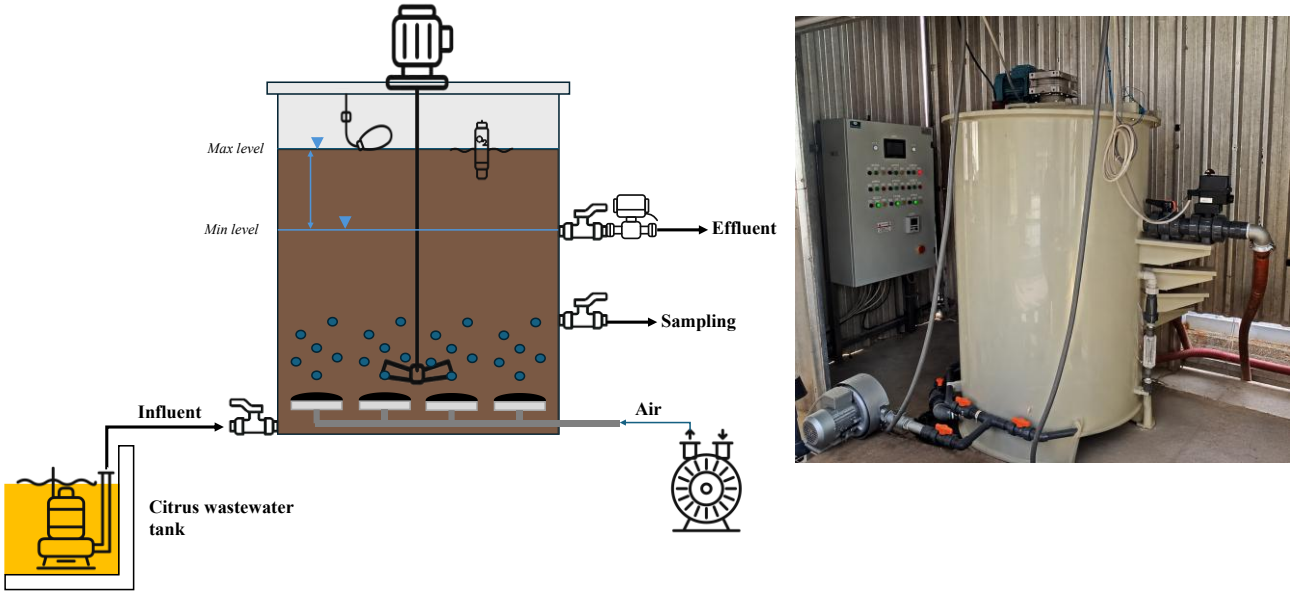


Figure 1: Layout and picture of the SBR pilot plant

Aeration was supplied through four fine-bubble disk diffusers placed at the reactor bottom and connected to two blowers operated in alternating duty-standby mode. The reactor operated on a 4-h cycle (6 cycles per day) comprising 1 min of influent feeding, 60 min of mixed anaerobic conditions, 130-157 min of aeration with dissolved oxygen (DO) controlled at 4.0 mg/L, 14 min of mixed deoxygenation, 3-30 min of settling, and 5 min for effluent discharge and idle time. At the beginning of each campaign, hydraulic selection pressure (HSP) was progressively increased by reducing the settling time stepwise from 30 min down to 25, 20, 15, 10, 8, 6, 5, 4, and 3 min (one step per week). This corresponded to an HSP increase from 0.4 to 7.0 m/h. Conversely, at the end of the processing campaign (August-September), the reactor was maintained under continuous aeration without any feed and discharge of wastewater.

The volumetric exchange ratio (VER) of the reactor was maintained at 20% (0.2 m^3 per cycle), corresponding to a daily treated flow rate of 1.2 m^3/d and a hydraulic retention time (HRT) of 20 h. The solids retention time (SRT) was controlled between 15 and 20 days through periodic wasting of mixed liquor during the aerated phase. DO concentration was measured by an on-line sensor (FDO WTW IQ Sensor Net). Complete mixing during the anaerobic and deoxygenation phases was ensured by a vertical-axis mechanical stirrer.

Influent wastewater was pumped from the post-equalization unit of the parent wastewater treatment plant (WWTP) until the nominal working volume of 1.0 m^3 was reached. Treated effluent was discharged by gravity through a motorized ball valve positioned at the minimum water level. The SBR was inoculated with conventional activated sludge (TSS = 13.4 g/L; VSS = 9.9 g/L) collected from the full-scale WWTP operating at the same industrial site. The full-scale plant operated with an anaerobic/oxic (A/O) layout, including a high-load anaerobic reactor, followed by a low-load aerobic stage. Thus, the inoculum was already acclimated to alternating high-load anaerobic and low-load aerobic conditions.

2.3 Operational periods

Pilot-plant operation lasted 549 days covering two consecutive citrus-processing campaigns (named C1 and C2) and an intermediate summer shutdown (C1-SD and C2-SD). Operational phases were categorized according to factory production cycles and the corresponding organic loading rates (Table 1).

A complete processing campaign comprised four sequential periods: a low-load period (LL), when plant operation restarted before fruit processing (e.g., cleaning operations); an intermittent-load period (IL), when fruit transformation occurred on alternate days; a full-load period (FL), with all production lines operating at maximum capacity; and a decreasing-load period (DL), corresponding to the end of fresh-fruit processing. Each campaign was followed by a summer shutdown (SD), during which the industrial plant was not operated, and the reactor was aerated without wastewater feeding. Campaign 1 (C1) included only the FL and DL periods, as the pilot reactor was started at the end of March 2025, whereas Campaign 2 (C2) covered the full operational sequence from LL to DL. The operational periods during the citrus-processing campaigns are summarized in Table 1.

Table 1: Operational periods of the experimental period

Period	Dates	Days	Industrial status	Typical influent COD
C1-FL	31 Mar-13 Jun 2025	0-74	Full citrus production	3000-5000 mg/L
C1-DL	14 Jun-1 Aug 2025	75-123	Decreasing production	3000-1000 mg/L
C1-SD	2 Aug-30 Sep 2025	124-183	Summer shutdown	No wastewater
C2-LL	1 Oct-13 Nov 2025	184-227	Pre-season with low organic load	500-1000 mg/L
C2-IL	14 Nov-31 Dec 2025	228-275	Alternate-day processing	1000-2000 mg/L

Period	Dates	Days	Industrial status	Typical influent COD
C2-FL	1 Jan-7 Jun 2026	276-433	Full citrus production	3000-5000 mg/L
C2-DL	8 Jun-28 Jul 2026	434-484	Decreasing production	3000-1000 mg/L
C2-SD	29 Jul-30 Sep 2026	485-549	Summer shutdown	No wastewater

2.4 Analytical methods

Total chemical oxygen demand (tCOD), soluble COD (sCOD; samples filtered through a 0.45 µm membrane), biochemical oxygen demand (BOD5), total suspended solids (TSS), volatile suspended solids (VSS), and sludge volume indices after 30 and 5 min (SVI_{30} and SVI_5, respectively) were determined in triplicate according to Standard Methods (APHA 2012). Dissolved oxygen was continuously measured by an online optical probe (WTW, FDO type). Soluble COD was measured at the end of the anaerobic mixing phase to assess the amount of organic substrate consumed during the anaerobic feast phase.

The particle size distribution (PSD) of sludge aggregates was evaluated by dynamic image analysis using an optical granulometer (QICPIC, Sympatec). Biomass aggregates were classified into flocs (< 100 µm), protogranules (100-200 µm), and granules (> 200 µm), defining the granulation rate as the volumetric percentage of particles larger than 200 µm (Van Dijk et al. 2022).

Microscopic observations were carried out using a phase-contrast optical microscope (Olympus. Gram, Neisser, and Sudan Black staining were performed according to Jenkins et al. (2003) (Jenkins et al. 2003), to qualitatively evaluate sludge flocs morphology and the phenotypes of specific microorganisms.

3. Results and discussion

3.1 Influent wastewater characteristics and organic loading dynamics

The temporal profiles of influent tCOD and sCOD are shown in Figure 2a. Both the parameters showed a clear relationship with the seasonality of the citrus processing operation. Indeed, during full production phases, corresponding to the periods (C1-FL and C2-FL), the tCOD concentration reached its highest values, with averages of 3110±476 mg/L and 3401±413 mg/L, and maximum peaks exceeding 4000 mg/L. During these periods, the sCOD was the main component of the organic matter, remaining stable between 78% and 80%. Conversely, during the transition phases, specifically the decreasing-load periods (C1-DL and C2-DL), and pre-season restart (C2-LL), the tCOD concentration dropped sharply, from 1360-2351 mg/L to an average of 696±26 mg/L. The soluble fraction simultaneously decreased from approximately 75-80% under full-load conditions to 56-67%, reaching minimum values close to 35-40% during the summer period before the plant shutdown. During the summer shutdown period no data were available since the absence of wastewater.

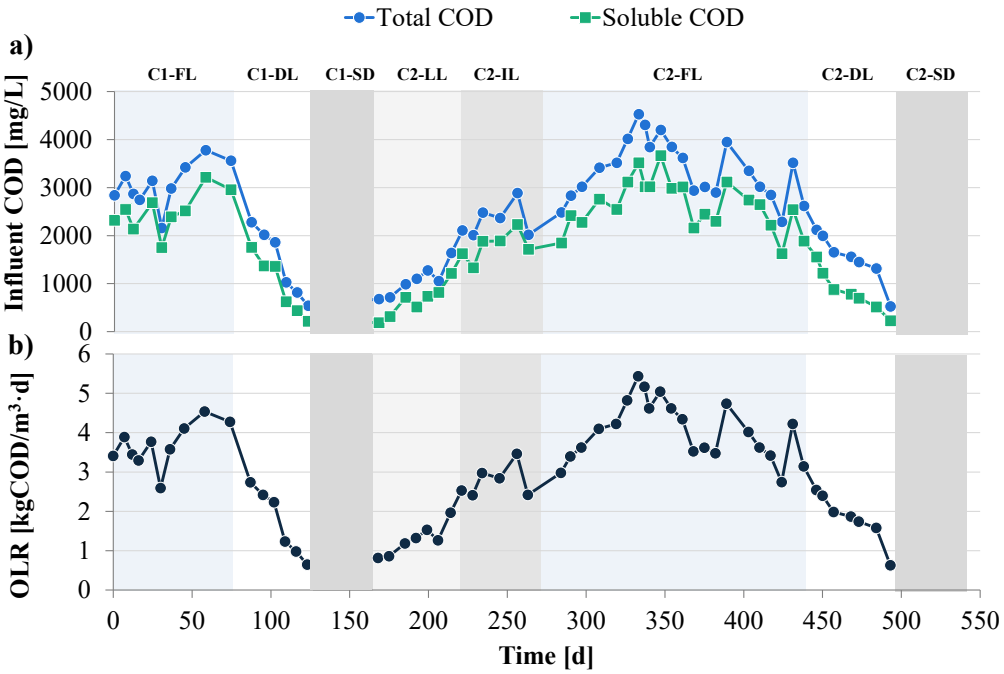


Figure 2: Time profile of (a) influent total COD and soluble COD, and (b) applied OLR across the monitoring period.

The trend of the applied organic loading rate (OLR) (Fig. 2b) closely reflected the variations in COD wastewater concentration and factory production volumes. Since the pilot SBR was fed at constant influent flow rate, the variation in OLR was related to the changes in the tCOD concentration only. Overall, the process was characterised by high OLR values, particularly during the full-production phases, although with significant fluctuations over time. Specifically, the OLR increased mostly at the beginning of citrus processing activities, reaching values above 3 kgCOD/m^3·d and, during the full production periods, exceeding 5 kgCOD/m^3·d. Conversely, during the transition periods, the organic loading decreased substantially, reaching values below 1 kgCOD/m^3·d. The corresponding OLR in the full-scale plant ranged between 0.65 and 2.86 kgCOD/m^3·d.

3.2 Granulation dynamics

Figure 3 summarises the evolution of the biomass over the two processing campaigns.

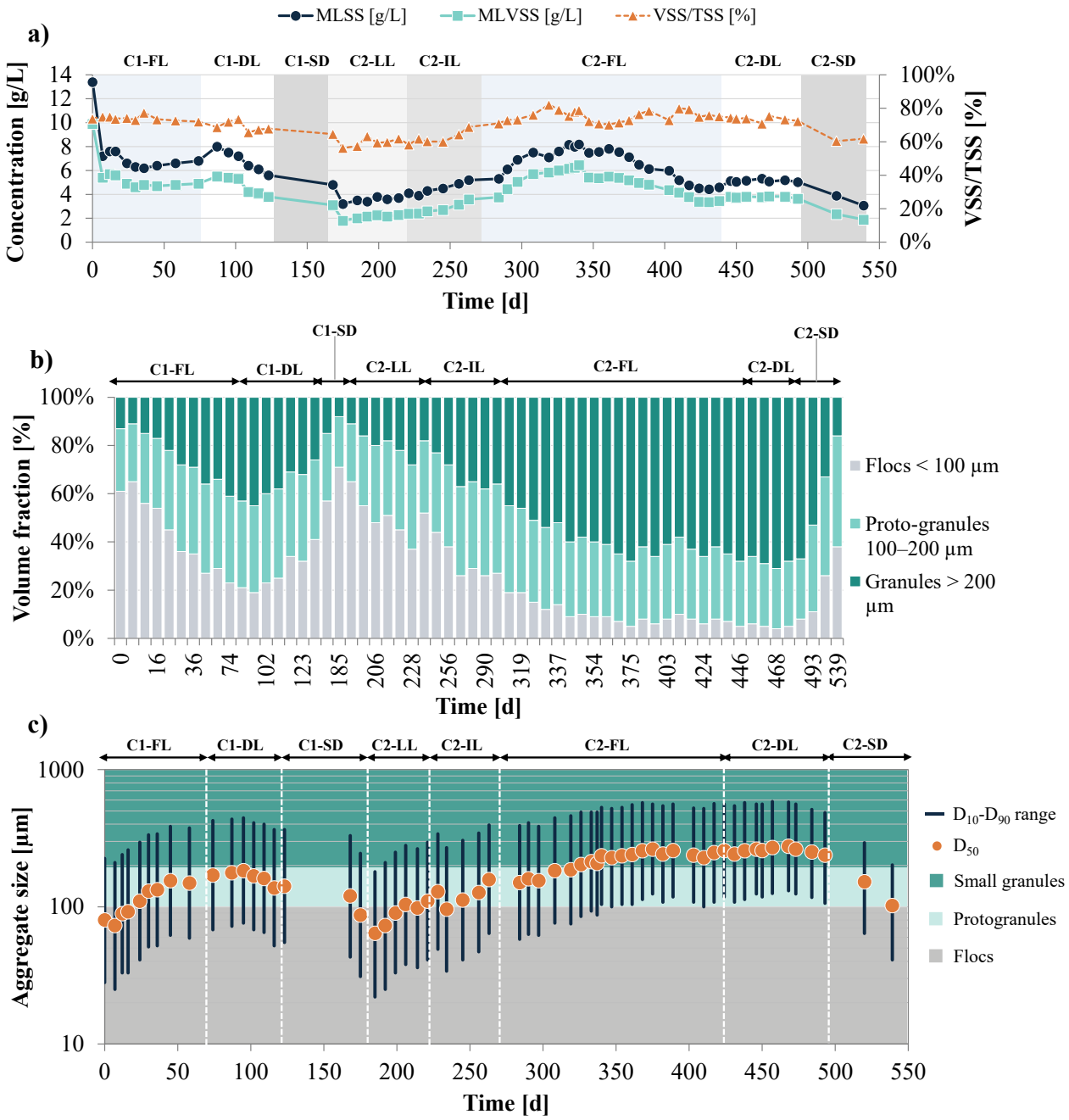


Figure 3: Time evolution of biomass characteristics and granulation during the experiment: (a) MLTSS, MLVSS and VSS/TSS; (b) particle-size distribution of the biomass; and (c) D10, D50 and D90 particle diameters.

The SBR startup was carried out during the full-load period of the first campaign (C1-FL). The initial MLTSS concentration was close to 13.4 gTSS/L. Within the first week, MLTSS decreased to approximately 7.0 gTSS/L due to the washout of the poorly settling flocs of the inoculum under the shorter settling time applied in the pilot SBR (30 min) than the parent full-scale plant (Fig. 3a). The biomass concentration stabilised between 6.2 and 6.8 g/L, whereas the VSS/TSS ratio was between 0.72 and 0.77. During this period, the granulation rate increased almost linearly from 13% to 41% over 74 days (Fig. 3a). This increase was accompanied by a progressive decrease in the floc fraction and corresponding increases in protogranules and small granules (Fig. 3b). Consistently, the median

particle diameter (D_{50}) increased over time, although the D_{10}-D_{90} range still indicated a broad particle-size distribution dominated by relatively small aggregates (Fig. 3c).

When the influent OLR began to decrease during C1-DL, MLTSS increased slightly, reaching a maximum of 8.0 gTSS/L, mainly because of a temporary reduction in the sludge wasting rate. The granulation rate continued to increase for approximately three weeks, reaching 45% on day 95 (Fig. 3b). During this period, the fraction of protogranules increased, whereas the contribution of flocs progressively decreased (Fig. 3b). The increase in D_{50} observed in Fig. 3c further suggested that the biomass was still undergoing aggregation despite the reduction in substrate availability, although the relatively wide D_{10}-D_{90} range indicated the coexistence of small flocs and larger aggregates.

These results suggested that granules formed under high-load conditions could maintain their growth for some time even after substrate availability started to decrease. However, when influent COD dropped below approximately 1000 mg/L, the granulation rate decreased to 31–32% and the VSS/TSS ratio fell to 0.66-0.68, suggesting the onset of extended famine conditions and likely endogenous decay. This deterioration was also reflected in the particle-size distribution, with a reduction in the contribution of larger aggregates and a decrease in D_{50}.

During the first summer shutdown (C1-SD), the MLTSS decreased from 4.8 to 3.2 g/L, while MLVSS reached a minimum of 1.8 g/L, resulting in a VSS/TSS ratio of 0.56, the lowest value observed during the study. At the same time, the granulation rate decreased from 26% to only 8% (Fig. 3a). The loss of granular biomass was accompanied by a marked change in particle size. Indeed, flocs accounted for approximately 50-71% of the biomass volume, while D_{50} remained below 100 μm and the D_{10}-D_{90} range was approximately 20-260 μm (Fig. 3b, c). The low D_{50}, together with the relatively wide D_{10}-D_{90} range, indicates that the sludge was predominantly composed of small flocs with only a limited fraction of larger aggregates. Thus, the prolonged period of very low substrate availability resulted not only in a substantial loss of total and active biomass, but also in the breakdown or loss of most of the larger aggregates. Overall, approximately two months of near-starvation conditions caused the loss of most of the granular sludge and more than half of the active biomass, highlighting the critical role of seasonal shutdowns for AGS stability.

After the citrus processing operation restarted, the influent COD gradually increased during the low-load period of the second campaign (C2-LL). MLTSS remained between 3.4 and 4.1 gTSS/L, whereas the VSS/TSS ratio remained low, between 0.57 and 0.63. Nevertheless, the granulation rate progressively increased from 8% to 22% over approximately six weeks. However, the increase in granulation rate was initially not accompanied by a correspondent increase in particle size and the biomass was dominated by small aggregates, with D_{50} still below 100 μm. This recovery was slower than the initial granulation observed during C1-FL, despite a similar F/M ratio of approximately 0.7 kgCOD/kgVSS·d. Therefore, substrate availability alone did not explain the low structural recovery.

During the alternate-day processing period (C2-IL), the reactor received high-strength wastewater on processing days and weaker wastewater on intervening days. The granulation rate fluctuated between 18% and 37% in parallel with the irregular loading pattern. High-load days may have promoted formation and consolidation of denser aggregates, whereas lower-load intervals may have favoured partial breakup. Particle-size data showed corresponding fluctuations in D_{50} and a broad D_{10}-D_{90} range, indicating coexistence of small flocs and developing aggregates (Fig. 3c). Despite these fluctuations, MLTSS increased from 3.9 to 5.2 g/L and the VSS/TSS ratio recovered to 0.69, indicating renewed biomass growth. The PSD also showed progressive development of protogranules in the 100-200 μm range.

During the C2-FL period, the granulation process accelerated markedly. The granulation rate increased from approximately 35% in early January to 60% in early March and reached 68% in April, exceeding the maximum value of 45% obtained during the first campaign. The granulation rate

increase was accompanied by a progressive shift from flocculent biomass towards larger aggregates. The fraction of protogranules (100-200 µm) increased initially, reaching up to approximately 37% at intermediate granulation rates of 30-45%, and subsequently decreased as the fraction of granules larger than 200 µm increased. As shown in Fig. 3c, this transition was accompanied by a clear increase in D_{50}, which exceeded 200 µm once the granulation rate was over 50%. The D_{10}-D_{90} range also progressively shifted towards larger particle sizes, indicating the development of a more heterogeneous sludge containing an increasing fraction of large aggregates. At the same time, MLTSS reached a maximum of approximately 8.2 gTSS/L and the VSS/TSS ratio increased to 0.82, indicating substantial biomass growth and a high organic fraction of the sludge.

After approximately day 340, MLTSS progressively decreased from about 8.2 to 4.4 g/L by day 430 despite the high OLR and continued increase in granulation rate. In contrast, particle size continued to increase, with D_{50} reaching approximately 270-280 µm and D_{90} approaching 600 µm. The concurrent decrease in MLTSS and increase in particle size and granulation rate are consistent with preferential washout of poorly settling flocculent biomass under the short settling time while larger aggregates were retained. Consequently, the relative contribution of granular biomass increased despite the decline in total biomass concentration. By the end of C2-FL, the flocculent fraction was below 10% of total biomass volume, while D_{50} and D_{90} reached approximately 270-280 and 600 µm, respectively. Thus, the high-load period promoted selective enrichment of larger aggregates, although their size remained below the 0.5-1 mm range commonly reported for mature granular sludge in full-scale SBRs (Pronk et al., 2015).

In contrast to the first campaign, the decrease in influent COD during C2-DL did not result in a significant deterioration of the granular biomass. Although influent COD decreased from approximately 3500 to 1300 mg/L, the granulation rate remained between 65% and 71%, while MLTSS stabilised at 4.6–5.3 g/L and the VSS/TSS ratio remained between 0.71 and 0.75. The particle-size distribution was also relatively stable, with D_{50} remaining in the range of approximately 250–280 µm and a persistent fraction of large aggregates (Fig. 3b, c). The persistence of the granular structure under decreasing substrate availability was likely related to the more effective removal of the flocculent fraction achieved during the preceding C2-FL period. The lower proportion of flocculent biomass may have reduced competition for the available substrate and favoured its uptake and storage by the granular biomass. Consequently, the granules remained relatively stable and did not undergo the marked deflocculation observed during C1-DL.

During the second shutdown period (C2-SD), the reduction in substrate availability again resulted in a deterioration of the granular structure. Both MLTSS and the granulation rate decreased, while the particle-size distribution progressively shifted towards smaller aggregates (Fig. 3b, c). In particular, the fraction of small granules decreased markedly, from approximately 60% at the beginning of the shutdown to less than 20% at its end, while the fractions of protogranules and flocs increased. This shift indicates that the larger granular aggregates underwent progressive disintegration and were converted into smaller aggregates and flocs under prolonged substrate limitation. However, the extent of this deterioration was considerably lower than during C1-SD. In C1-SD, the granulation rate decreased to approximately 8%, with D_{50} remaining below 100 µm and flocs accounting for about 50-71% of the biomass volume. In contrast, during C2-SD a substantial fraction of aggregates larger than 200 µm was still present at the end of the shutdown, and the particle-size distribution remained shifted towards larger sizes compared with C1-SD. Therefore, C2-SD did not preserve the granular structure, but rather resulted in a partial degranulation of a more mature and selectively enriched granular biomass. The lower severity of degranulation compared with C1-SD suggested that the structural state of the biomass at the onset of the shutdown influenced its resistance to prolonged substrate limitation.

Overall, the second campaign achieved higher granulation rates than the first under comparable loading conditions, reaching 56±10% versus 25±11% at full load and 68±2% versus 38±6% during decreasing-load conditions. Two factors may have contributed to this difference. First, the second campaign included a much longer full-load period, lasting approximately five months compared with about two and a half months in the first campaign, thereby providing more time for granule development and hydraulic selection. Second, the lower flocculent fraction during the second campaign may have reduced competition for readily available substrate and favoured persistence of the granular fraction.

The comparison between campaigns also showed that recovery after the prolonged shutdown was not faster than initial granulation from activated sludge. A granulation rate of 45% was reached within 95 days during the first campaign, whereas approximately 120 days were required during the second campaign. The slower structural recovery coincided with the marked reduction in anaerobic COD uptake after C1-SD (Section 3.4), suggesting that restoration of granulation required recovery of substrate-uptake and storage functions. However, intracellular storage polymers were not directly quantified; therefore, this mechanistic interpretation should be regarded as a hypothesis.

Overall, the comparison between the two shutdown periods suggested that the seasonal substrate limitation did not simply determine whether granules were lost. Rather, it affected the extent of structural deterioration of the biomass. The pronounced degranulation during C1-SD contrasted with the less severe, although still evident, degranulation during C2-SD, when the decrease in granules was accompanied by increases in protogranules and flocs. A more developed granular structure before shutdown was therefore associated with greater retention of large aggregates under prolonged substrate limitation, but it did not prevent degranulation. Thus, the degree of granulation achieved before the shutdown seemed to influence the resilience of the granular sludge and its ability to retain larger aggregates under prolonged low-substrate conditions.

3.3 Sludge morphology and settling properties

Figure 4 depicts microscopic images of the sludge during the two campaigns.

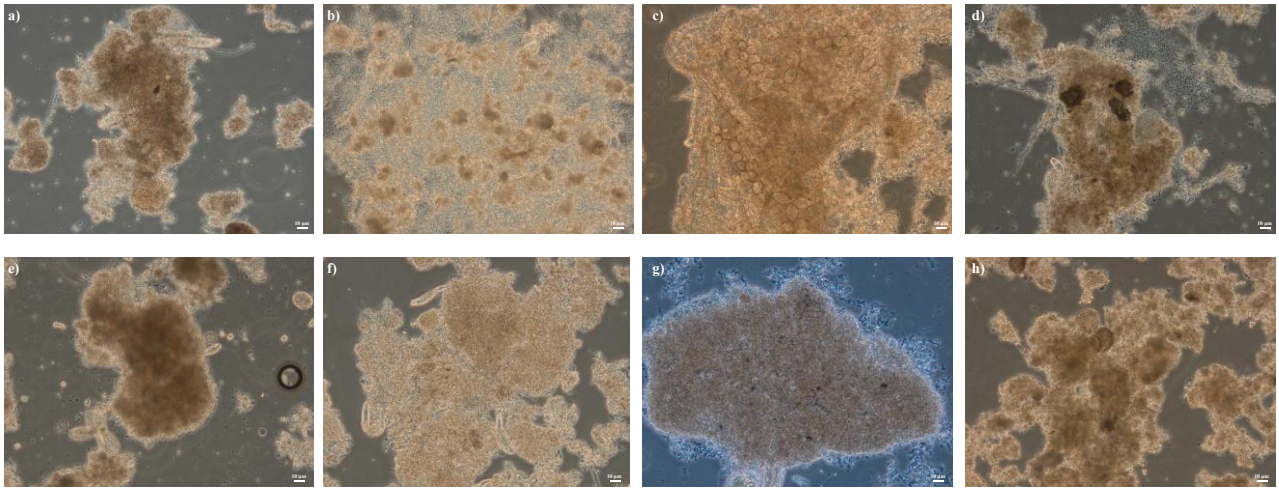


Figure 4: Microscopic images of the AGS-SBR sludge during the experiment (top row: first campaign; bottom row: second campaign). Proto-granules (a, e), small granule during densification (b, f) at the beginning of FF periods, small granule during FF periods (c, g), and granule during the decreasing-load period (d, h).

Protogranules were identified according to aggregate size and morphology. They were characterized by a dense, compact core and relatively defined boundaries, whereas conventional flocs were irregular, lacked a clear core, and showed a more open structure. The protogranules observed during

the first campaign (Fig. 4a) had a dark compact core, a size of approximately 130-150 µm, and an irregular outer boundary. Several smaller dense clusters and filamentous bacteria were attached to its surface. The protogranules observed during the second campaign (Fig. 4e) was more compact and rounded, of similar size, and had a darker, more homogeneous core with a clearer boundary.

Images collected during increasing-load periods (Fig. 4b, f) showed the formation of small granules. In the first campaign (Fig. 4b), several small dense nuclei were visible within a loose aggregate structure. In the second campaign (Fig. 4f), the nuclei were larger (approximately 100 µm) and more clearly defined, while the surrounding floc matrix was less abundant. These observations are consistent with granule formation through development of dense nuclei within activated-sludge flocs followed by growth and coalescence. The larger nuclei observed during the second campaign also suggest that biomass surviving the shutdown may have contributed to subsequent aggregate development.

At full load, the small granules shown in Fig. 4c and g were compact and clearly separated from the surrounding liquid, with sizes of approximately 300 µm. The granule observed during the first campaign (Fig. 4c) had a well-defined boundary and contained several clusters of large coccoid cells. The granule from the second campaign (Fig. 4g) was similar in size (approximately 290 µm) and showed a dense, homogeneous structure with a smooth boundary. Rod-shaped bacteria and short filaments were mainly observed at the surface, with little loose biomass attached.

Neisser staining provided further information on the coccoid clusters observed in the granules (Fig. 5).

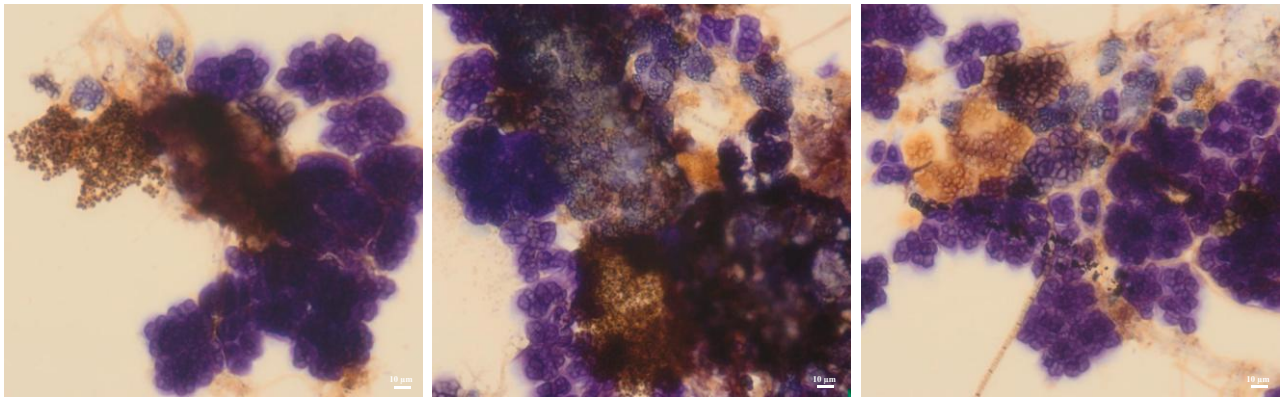


Figure 5: Clusters of Neisser-positive cells observed in the AGS-SBR biomass

The aggregates were mainly composed of large clusters of coccoid cells and tetrads forming organisms. These clusters showed a strong Neisser-positive reaction (purple colour). They were closely packed and accounted for most of the granule volume.

Tetrad-forming organisms were commonly observed in granular sludge operated under anaerobic feast and aerobic famine conditions and may include polyphosphate accumulating organisms (PAO) and glycogen accumulating organisms (GAO)-like organisms (Weissbrodt et al. 2013). Nevertheless, PAO-like activity was not detected, since no polyphosphate release was measured at the end of the anaerobic phase. Besides, phosphorus was added only to meet the biomass growth requirement, thus enhanced biological phosphorus removal was stimulated. Therefore, the observed Neisser-positive cluster were assumed as GAO-like phenotypes. Indeed, sugar rich wastewater is reported to stimulate the enrichment of such microorganisms in activated flocculent and granular sludge (Winkler et al. 2011).

Overall, these dense cell clusters formed compact structures, while the surrounding floc matrix and filaments provided additional connections among them. This combination resulted in compact but morphologically irregular aggregates.

During the decreasing-load periods (Fig. 4d, h), the granules size reduced to about 200-250 μm and showed different changes in their structure. In the first campaign (Fig. 4d), the granule maintained a dense core, but its surface became more irregular. Filamentous bacteria overgrowth was observed, and dispersed cells were visible around the aggregate. These features were consistent with the onset of granule disintegration, in agreement with the decrease in granulation rate observed at the end of the first campaign. In the second campaign (Fig. 4h), the granule showed a different pathway of deterioration. The dense core was still visible, but the aggregate was only partly broken and surrounded by smaller compact structures and loose biomass. This suggested that granule break-up generated smaller aggregates, including protogranules-like structures, rather than resulting mainly in a transition towards a flocculent structure. This observation was consistent with the changes in particle-size distribution and granulation rate reported in Section 3.2. In contrast, during the first campaign (Fig. 4d), granule deterioration was more advanced, with the aggregate progressively losing its compact structure and developing into a predominantly flocculent biomass.

Overall, the microscopic analysis confirmed that the reactor contained a mixture of granules, protogranules and flocs throughout the study. The granules remained relatively small, generally below 0.5 mm, and were often irregular in shape. Large, smooth and spherical granules typical of fully developed AGS were not observed (de Kreuk et al. 2005; Giesen et al. 2013). Therefore, granulation mainly evolved through the densification and subsequent growth of dense nuclei within the floc matrix, rather than through the direct formation of large granules. The continuous fluctuation in OLR observed in full-scale application with such type of industrial wastewater, together with the short duration of pseudo-steady-state periods, likely limited the maturation of the granules and prevented the development of the larger, more compact aggregates typically observed under more stable feeding conditions.

The sludge morphology had a significant impact on settling properties. Figure 6 shows the trend of SVI_5, SVI_{30} and their ratio throughout the experiment.

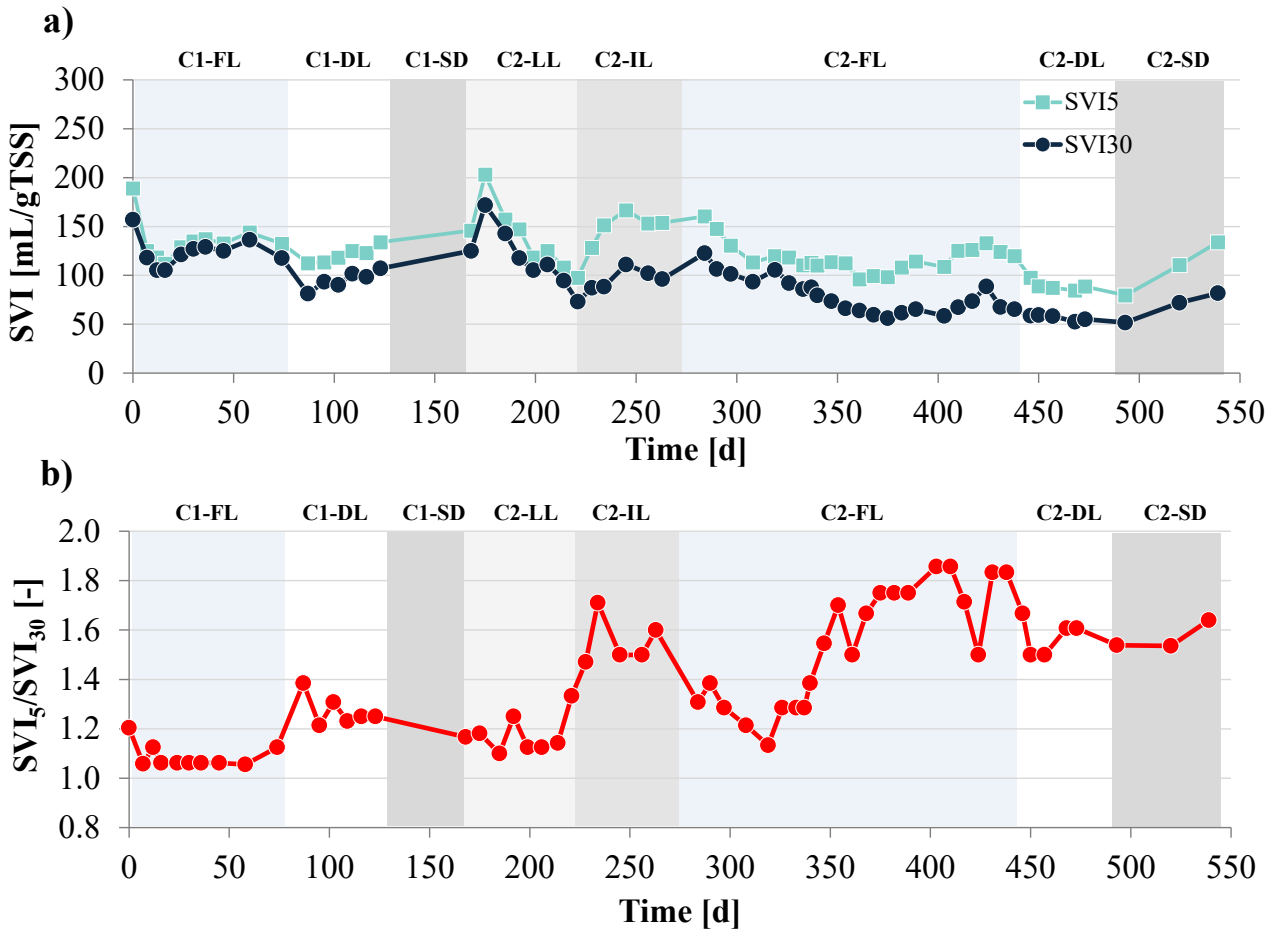


Figure 6: Settling properties of the sludge: (a) SVI_5 and SVI_{30}; (b) SVI_5/SVI_{30} ratio

At the beginning of the first campaign, both SVI_5 and SVI_{30} decreased from approximately 185 mL/gTSS and 150 mL/gTSS, respectively to about 120 mL/gTSS and 105 mL/gTSS in about 4 weeks, indicating a rapid improvement in settling properties after the SBR start-up. However, as the granulation proceeded, both SVI_5 and SVI_{30} showed a temporary increase, despite the continued increase in the fraction of particles larger than 200 µm. This suggested that the increase in granulation rate did not immediately involve the improvement settling properties. In this phase, the sludge still contained a mixture of flocs, protogranules and small granules, and the newly formed aggregates were not yet sufficiently compact to dominate the settling behaviour.

After this initial phase, SVI_5 and SVI_{30} progressively decreased, particularly during the period in which the granulation rate increased to its highest values. SVI_5 decreased to approximately 60-65 mL/gTSS, while SVI_{30} reached values close to 45-50 mL/gTSS close day 90. The decrease in both indices, together with the increase in granulation rate to around 40%, indicates a progressive improvement in settling as the aggregates became larger and more compact. After the end of the FL period in C1, both SVI_5 and SVI_{30} increased, according to the increase of the flocculent fraction in the sludge.

SVI_5 remained consistently higher than SVI_{30}, showing that the sludge continued to undergo significant compaction after the first 5 min of settling. The microscopic observations showed the simultaneous presence of small granules, proto-granules and flocs, with no dominance of the large, compact granules typical of mature AGS systems (Pronk et al. 2015; Hamza et al. 2022). Therefore, the sludge behaved as a hybrid granular sludge, in which settling was still largely governed by the

"zone settling" rather than by the rapid individual settling of large and dense granules. This mixed structure can also explain the persistent difference between SVI_5 and SVI_{30}.

The behaviour was also observed during the second campaign. Indeed, both the SVIs showed different trends that did not always follow that of granulation rate. This further confirmed that the increase of aggregates size, including flocs and protogranules, did not necessarily result in an improvement of settling properties particularly if this was not accompanied by a sludge densification. Moreover, despite the higher granulation rate, which reached approximately 70%, the SVI_5/SVI_{30} did not approach the behaviour expected for a fully mature granular sludge.

However, a clear difference than the previous campaign was noted during the decreasing-load period. The SVI values remained relatively stable even as the granulation rate slightly decreased. This was consistent with the partial degranulation observed microscopically. Although some granules broke down into smaller aggregates and floc-like structures, a fraction of the larger aggregates remained and continued to contribute to good overall settling.

The opposite trends observed between the granulation rate and SVIs were contrast with the results generally reported in the literature (de Bruin et al. 2004; Dobbeleers et al. 2025), where the development of granular sludge is typically accompanied by a progressive improvement in settling properties. It should be reminded that granulation rate only indicates the fraction of aggregates larger than 200 µm and does not provide information on their structure. The formation of larger aggregates can occur before the development of dense and well-settling granules. In the present study, this distinction was important because granulation remained a gradual process, with the sludge retaining a mix of flocculent and granules-like aggregates throughout the experiment. The relatively low SVI values thus indicate good overall settleability, but not the complete transition to the predominantly granular settling behaviour characteristic of mature AGS systems.

3.4 Organic matter removal and anaerobic substrate uptake

The SBR achieved high organic matter removal throughout the study (Fig. 7).

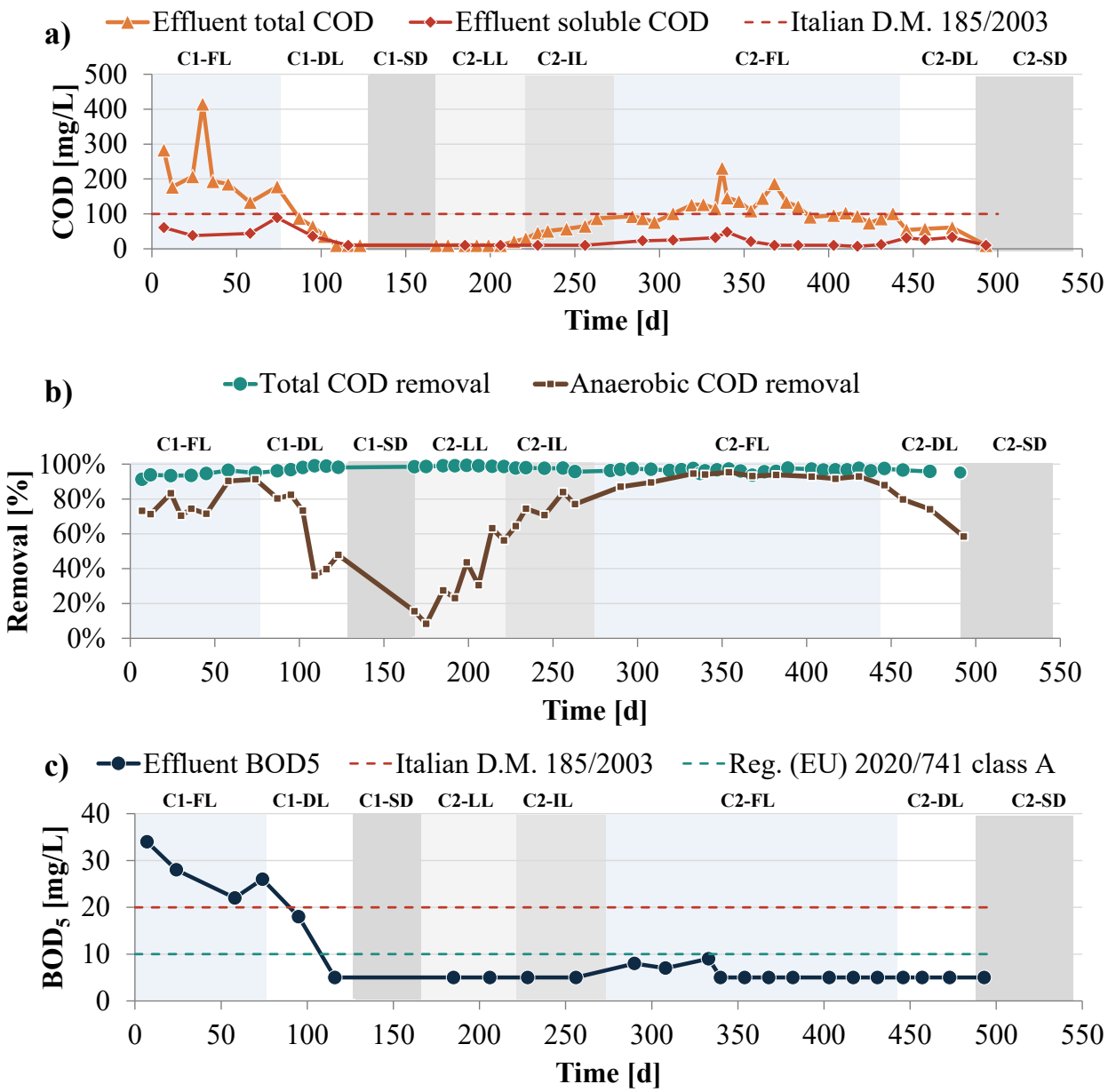


Figure 7: Organic matter removal performance of the SBR: (a) effluent total COD and soluble COD concentrations; (b) soluble COD removal during the anaerobic phase and overall tCOD removal; (c) effluent BOD_5 concentration compared with the Class A limit established by Regulation (EU) 2020/741.

During the SBR start-up phase (C1-FL) the tCOD removal was 94.0±1.6% and increased to approximately 98% in the following periods, indicating consistently high organic matter removal despite variations in influent loading.

The effluent concentration of tCOD showed a relevant variability (Fig. 7a). During the C1-FL period, it averaged 224±94 mg/L, when the granular sludge was developing and the hydraulic selection pressure was increasing. In the following periods, the effluent tCOD concentration decreased to below 40 mg/L towards the end of the first campaign, indicating a high stability of the SBR in organic matter removal. During the C2-IL period, the effluent tCOD increased again reaching about 100 mg/L. This trend was confirmed during the C2-FL period, during which the effluent tCOD concentration averaged 118±38 mg/L and reached a maximum of 231 mg/L between days 330 and 370. Because

the soluble COD concentration remained low during this period, these increases were associated with particulate matter, which accounted for approximately 72% of effluent total COD on average. This coincided with the decrease in MLTSS from 8.2 to 4.4 g/L observed in the same period, while the granulation rate continued to increase (see section 3.2). Therefore, it was speculated that the short settling time might have favoured the washout of poorly settling flocs, increasing the particulate COD in the effluent. Nevertheless, after this phase was stable at approximately 100 mg/L until the end of C2-FL period, while showing a decreasing trend in the following C2-DL because of the decreasing influent OLR.

The effluent soluble COD remained low, generally between 10 and 30 mg/L from C1-DL onward, with values up to 48 mg/L during the second campaign. These results indicated that soluble organic matter was efficiently removed under both full-load and reduced-load conditions, despite the large variations in influent COD.

The removal efficiency of soluble COD during the anaerobic phase and the overall are reported in Fig. 7b. During the full-load operation periods (C1-FL, C2-FL), the anaerobic uptake was on average 76±7% and 83±5% in in C1-FL and C2-FL, respectively. A decreasing trend was observed during both the decreasing-load periods (C1-DL, C2-DL), reaching about 41% in C1 and 60 in C2. This suggested a gradual loss in the substrate uptake capacity of the SBR biomass under anaerobic conditions when the influent OLR decreased. After the first shutdown (C1-SD), the substrate anaerobic uptake capacity fell to 10%. However, it was partially recovered during the C2-IL period, reaching about 61%. The recovery of anaerobic COD uptake was accompanied by the reprise of granulation. This relationship suggested that the capacity to take up readily biodegradable substrates under anaerobic conditions was associated with the recovery of the granular sludge (Carrera et al. 2019; Iorhemen and Liu 2021). Indeed, anaerobic uptake can favour granulation by directing substrate towards intracellular storage rather than allowing it to support growth during aeration (Rollemberg et al. 2021). This may limit the competitive advantage of fast-growing floc-forming organisms and favour the development of a more compact sludge structure (Carrera et al. 2019).

The concentration of the effluent BOD_5 followed a similar trend to sCOD (Fig. 7c). Overall, the average concentration resulted close to 30 mg/L during C1-FL, and it decreased below 10 mg/L towards the end of the first campaign, while between 5 and 8 mg/L during the entire second campaign. These values indicated an effective removal of biodegradable organic matter.

From a water reuse perspective, the effluent quality was particularly relevant, especially in relation to the Italian Ministerial Decree No. 185/2003 (D.M. 185/2003), which regulates wastewater reuse, including industrial applications. Although effluent tCOD occasionally exceeded the reference value of 100 mg/L, the consistently low sCOD concentrations indicated that these exceedances were primarily associated with suspended particulate matter rather than residual soluble organic compounds. Therefore, a final filtration step could be sufficient to reduce effluent tCOD below 100 mg/L, without requiring further biological treatment for organic matter removal.

The effluent BOD_5 values were also below the threshold of 10 mg/L established for Class A reclaimed water under Regulation (EU) 2020/741, which defines minimum water quality requirements for agricultural irrigation. They were also below the reference limit of 20 mg/L established by D.M. 185/2003 reuse. Overall, these results highlighted the potential of the SBR for producing effluents suitable for subsequent reclamation for agricultural and industrial applications. In particular, the low concentrations of soluble COD and BOD_5 suggested that the main limitation in effluent quality was associated with suspended solids rather than insufficient biological treatment. Accordingly, a final filtration step could provide the additional solid-liquid separation required to improve effluent quality and meet the relevant physicochemical requirements. Nevertheless, compliance with the applicable

reuse standards would also require verification of the remaining quality parameters, particularly microbiological indicators.

4. Conclusions

This study investigated the AGS technology for the treatment of strongly seasonal agro-industrial wastewaters. The results indicated that although the wide OLR fluctuations and short pseudo-steady-state periods, the biomass successfully maintained a hybrid granular structure. This allowed to obtain high removal efficiencies for COD and BOD_5 alongside a strong potential for water reclamation and low SVI. Furthermore, while prolonged substrate limitation during summer shutdowns caused structural deterioration of the aerobic granules, the extent of degranulation was closely dependent on the prior structural state and maturity of the biomass. Overall, the study provides long-term pilot-scale evidence on both the robustness and operational limitations of AGS for highly seasonal agro-industrial wastewater and identifies shutdown management as a key target for future optimization.

Acknowledgments

The authors would like to thank Agrumaria Corleone for the fundamental support provided for pilot plant installation and monitoring.

Declarations

Funding: This study was funded by SWRIPS (Sustainable Wastewater Re-use with Innovative Purification and Sensing system for the agrifood supply chain) project - PRIMA Section 2 -2022, CUP B73C22001900008.

Author contributions:

Corsino Santo Fabio: writing - original draft; methodology; data curation¸ conceptualization; *Riccardo Campo*: writing - original draft; methodology; data curation; review and editing; *Marco Capodici*: writing - original draft; methodology; review and editing; *Maria Castiglione*: writing - original draft; formal analysis; data curation; *Alida Cosenza*: review and editing; *Federica De Marines*: writing - original draft; formal analysis; data curation; *Daniele Di Trapani*: review and editing; *Gaetano Di Bella*: writing - review and editing; *Michele Torregrossa*: methodology; conceptualization; review and editing.

Competing interests: 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.

Ethics approval: Ethics declaration: not applicable

Consent to participate: The manuscript is based solely on experimental activity carried out with activated sludge and does not involve any experiments with human participants requiring consent.

Consent to publish: All authors have read and approved the manuscript, and consent to its publication in Environmental Processes

Data availability: Data supporting the findings of this study are available from the corresponding author upon reasonable request.

Code availability: Not applicable

Clinical trial study: This study is not a clinical trial. Therefore, no trial registration number or registry information is applicable.

References

Andiloro S, Bombino G, Tamburino V, et al (2013) Aerated lagooning of agro-industrial wastewater: depuration performance and energy requirements. J Agric Eng 44:0–5. https://doi.org/10.4081/jae.2013.s2.e166

APHA (2012) Standard Methods for the Examination of Water and Wastewater

Calabrò PS, Pontoni L, Porqueddu I, et al (2018) Agro-industrialwastewater treatment with decentralized biological treatment methods. Waste Manag 33:8993–8999. https://doi.org/10.1016/j.biortech.2016.04.106

Campo R, Sguanci S, Caffaz S, et al (2020) Efficient carbon, nitrogen and phosphorus removal from low C/N real domestic wastewater with aerobic granular sludge. Bioresour Technol 305:122961. https://doi.org/10.1016/j.biortech.2020.122961

Campos JL, Figueroa M, Corral AM, Mendez R (2009) Aerobic sludge granulation: state-of-the-art. Int J Environ Eng 1:136. https://doi.org/10.1504/IJEE.2009.027311

Carrera P, Campo R, Méndez R, et al (2019) Does the feeding strategy enhance the aerobic granular sludge stability treating saline effluents? Chemosphere 226:865–873. https://doi.org/10.1016/j.chemosphere.2019.03.127

Corsino SF, di Biase A, Devlin TR, et al (2017) Effect of extended famine conditions on aerobic granular sludge stability in the treatment of brewery wastewater. Bioresour Technol 226:150–157. https://doi.org/10.1016/j.biortech.2016.12.026

Corsino SF, Di Trapani D, Torregrossa M, Viviani G (2018) Aerobic granular sludge treating high strength citrus wastewater: Analysis of pH and organic loading rate effect on kinetics, performance and stability. J Environ Manage 214:23–35. https://doi.org/10.1016/j.jenvman.2018.02.087

de Bruin LMM, de Kreuk MK, van der Roest HFR, et al (2004) Aerobic granular sludge technology: An alternative to activated sludge? Water Sci Technol 49:1–7

de Kreuk MK, Pronk M, van Loosdrecht MCM (2005) Formation of aerobic granules and conversion processes in an aerobic granular sludge reactor at moderate and low temperatures. Water Res 39:4476–4484. https://doi.org/10.1016/j.watres.2005.08.031

Derlon N, Wagner J, da Costa RHR, Morgenroth E (2016) Formation of aerobic granules for the treatment of real and low-strength municipal wastewater using a sequencing batch reactor operated at constant volume. Water Res 105:341–350. https://doi.org/10.1016/j.watres.2016.09.007

Di Trapani D, Corsino SF, Torregrossa M, Viviani G (2019) Treatment of high strength industrial wastewater with membrane bioreactors for water reuse: Effect of pre-treatment with aerobic granular sludge on system performance and fouling tendency. J Water Process Eng 100859:. https://doi.org/10.1016/j.jwpe.2019.100859

Dobbeleers T, Feyaerts M, Dries J (2025) Impact of the Anaerobic Feeding Strategy on the Formation and Stability of Aerobic Granular Sludge Treating Dairy Wastewater. Water (Switzerland) 17:1–15. https://doi.org/10.3390/w17111648

Giesen A, van Loosdrecht M, de Bruin B, et al (2013) Full-scale Experiences with Aerobic Granular Biomass Technology for Treatment of Urban and Industrial Wastewater. In: International Water Week

Haaksman VA, Mirghorayshi M, Loosdrecht MCM Van, Pronk M (2020) Impact of aerobic availability of readily biodegradable COD on morphological stability of aerobic granular sludge. Water Res 187:116402. https://doi.org/10.1016/j.watres.2020.116402

Hamza R, Rabii A, Ezzahraoui F zahra, et al (2022) A review of the state of development of aerobic granular sludge technology over the last 20 years: Full-scale applications and resource recovery.

Case Stud Chem Environ Eng 5:100173. https://doi.org/10.1016/j.cscee.2021.100173
Iorhemen OT, Liu Y (2021) Effect of feeding strategy and organic loading rate on the formation and stability of aerobic granular sludge. J Water Process Eng 39:101709. https://doi.org/10.1016/j.jwpe.2020.101709
Jenkins D, Richard MG, Daigger GT (2003) Manual on the Causes and Control of Activated Sludge Bulking, Foaming and Other Solids Separation Problems. IWA, London ISBN 1566706475
Lucia C, Laudicina VA, Badalucco L, et al (2022) Challenges and opportunities for citrus wastewater management and valorisation: A review. J Environ Manage 321:115924. https://doi.org/10.1016/j.jenvman.2022.115924
Nancharaiah Y V., Kiran Kumar Reddy G (2018) Aerobic granular sludge technology: Mechanisms of granulation and biotechnological applications. Bioresour Technol 247:1128–1143. https://doi.org/10.1016/j.biortech.2017.09.131
Pronk M, de Kreuk MK, de Bruin B, et al (2015) Full scale performance of the aerobic granular sludge process for sewage treatment. Water Res 84:207–217. https://doi.org/10.1016/j.watres.2015.07.011
Rollemberg SL de S, Ferreira dos Santos A, Tavares Ferreira TJ, et al (2021) Evaluation of the production of alginate - like exopolysaccharides (ALE) and tryptophan in aerobic granular sludge systems. Bioprocess Biosyst Eng 44:259–270. https://doi.org/10.1007/s00449-020-02439-w
Van Dijk EJH, Haaksman VA, Loosdrecht MCM Van, Pronk M (2022) On the mechanisms for aerobic granulation - model based evaluation. Water Res 216:118365. https://doi.org/10.1016/j.watres.2022.118365
Weissbrodt DG, Schneiter GS, Fürbringer JM, Holliger C (2013) Identification of trigger factors selecting for polyphosphate- and glycogen-accumulating organisms in aerobic granular sludge sequencing batch reactors. Water Res 47:7006–7018. https://doi.org/10.1016/j.watres.2013.08.043
Winkler M-KH, Bassin JP, Kleerebezem R, et al (2011) Selective sludge removal in a segregated aerobic granular biomass system as a strategy to control PAO–GAO competition at high temperatures. Water Res 45:3291–3299. https://doi.org/10.1016/j.watres.2011.03.024
Zema DA, Andiloro S, Bombino G, et al (2016) Comparing Different Schemes of Agricultural Wastewater Lagooning: Depuration Performance and Microbiological Characteristics. Water Air Soil Pollut 227:. https://doi.org/10.1007/s11270-016-3132-4
Zheng YM, Yu HQ, Liu SJ, Liu XZ (2006) Formation and instability of aerobic granules under high organic loading conditions. Chemosphere 63:1791–1800. https://doi.org/10.1016/j.chemosphere.2005.08.055