Aerobic granular sludge treating anaerobically pretreated brewery wastewater at different loading rates
Alessandro di Biase, Fabio Santo Corsino, Tanner Ryan Devlin, Michele Torregrossa, Giulio Munz and Jan A. Oleszkiewicz

ABSTRACT
In this study, three different aerobic granular sludge (AGS) reactors fed with anaerobically pre-treated brewery wastewater were studied. The AGS reactors were operated under different conditions including organic loading rates (OLR) between 0.8 and 4.1 kg COD m\(^{-3}\) d\(^{-1}\), C:N:P ratios (100:10:1 and 100:6:1) and food to microorganism ratios (F/M) between 0.8 \pm 0.6 and 1.2 \pm 0.5 and 0.9 \pm 0.3 kg-TCOD kg-VSS\(^{-1}\)d\(^{-1}\). Stable granulation was achieved within two weeks and the size of the granules increased according to the OLR applied. The results indicated that low C:N:P and F/M ratios were favorable to achieve stable aerobic granules in the long term. The carbon removal rate was load-independent in the range examined (TCOD removal >80%), whereas TN removals were inversely proportional to the OLRs. Overall, a longer aeration reaction time with a lower OLR was beneficial to granular structure, which exhibited a compact and defined architecture. Performance results within the other conditions studied further indicated that the microbial community and its complex functionality in nutrient removal was efficient at operational parameters of OLR at 0.8 \pm 0.2 kg-TCOD m\(^{-3}\) d\(^{-1}\) and F/M ratio at 0.5 \pm 0.2 kg-TCOD VSS\(^{-1}\)d\(^{-1}\). Moreover, the protein to polysaccharide ratio increased as OLR decreased, leading to a stable granular structure.

Key words | aerobic granular sludge, brewery wastewater, food to microorganism ratio, nutrient removal, organic loading rates

HIGHLIGHTS

- AGS fed with anaerobically pre-treated brewery wastewater was studied.
- Rapid granulation occurred and granule size increased with OLR.
- High organic carbon removal performance was observed independently from the OLR.
- Low C:N:P ratio and F/M were favorable for granular sludge stability in the long term.
- Longer cycle duration improved granular sludge stability.

INTRODUCTION
Food and beverage industries are increasing worldwide, which inevitably has an impact on the environment. These industries are increasing consumption of resources such as water and energy, which leads to more production of wastes and wastewater (Olajire 2012). In the brewery industry, the amount of water consumption depends on the production and cleaning practices, which lead to different beer to wastewater ratios. For instance, in Europe it has been reported that 1 L of beer generates on average 2.7 L of wastewater, whereas in North America the typical reported value is 3.5 L L\(^{-1}\) (Chastain et al. 2011). Brewery wastewater is mainly constituted by sugars, soluble starches, ethanol, and fatty acids while very low concentrations of heavy metals are present (Simate et al. 2011). The organic
load of brewery wastewater is easily biodegradable with a biochemical oxygen demand (BOD₅) to total chemical oxygen demand (TCOD) ratio of 0.6–0.7 (Brito et al. 2007). The typical composition of brewery wastewater is: 2,000–6,000 mg-COD L⁻¹, 25–80 mg-TN L⁻¹, 10–50 mg-TP L⁻¹, with pH between 4.5 and 12 (Valta et al. 2014). Therefore, brewery wastewaters often lack sufficient nitrogen and phosphorus levels for microbial communities to achieve the biological activity level required for biological processes. Even though nitrogen and phosphorus fractions over carbon increase after anaerobic treatment, it is still necessary to supply these nutrients from external resources to ensure proper conditions for biological processes.

It has been widely demonstrated that anaerobic processes like up-flow anaerobic sludge blanket reactors (UASB), anaerobic membrane bioreactors and anaerobic moving bed biofilm reactors (AnMBBR) are reliable and robust technologies that achieve high organic carbon removal performance while recovering energy through biogas production (di Biase et al. 2017). However, anaerobic bioprocesses generate effluents with residual pollution that require further treatments before discharge into the environment (Chastain et al. 2011). Among the aerobic post-treatments, aerobic granular sludge (AGS) is considered a promising technology that is feasible and competitive towards the treatment of several industrial wastewaters due to its small footprint and high nutrient removal performance (Hamza et al. 2018).

The organic loading rate (OLR) and food to microorganism ratio (F/M) are two of the parameters that most affect the AGS formation and long-term stability of granular structure (Hamza et al. 2018). Several studies have demonstrated that AGS operates with an OLR higher than conventional technologies. However, there were discrepancies in the literature regarding the stability of AGS under high OLRs, especially in the treatment of industrial wastewater (Adav et al. 2010). Furthermore, a lack of mechanisms is presented to support AGS stability under increasing OLR. In addition, the application of design or management criteria illustrated in the literature refers to municipal wastewater. Therefore, these resulted in unsatisfactory outcomes when applied to a specific industrial wastewater due to its different composition. Consequently, in order to comply with the discharge limits imposed by regulators, it is necessary to establish specific design and management criteria for the specific kind of industrial wastewater.

Aerobic granular sludge has been used in the treatment of brewery wastewater. For instance, Stes et al. (2018) investigated the treatment of brewery wastewater with AGS operating at different OLRs between 1.2 and 1.5 kg-TCOD m⁻³ d⁻¹. Wang et al. (2007) demonstrated the capability of high-rate degradation as well as simultaneous nitrogen and phosphorus removal on raw brewery wastewater. However, these studies lacked clarification on long-term operating strategies to improve granular sludge stability, preserving their integrity, and nutrient removal performances. Moreover, post-treatment of anaerobic pre-treated brewery wastewater with AGS technology has never been assessed.

Hence, the aim of this study was to determine the feasibility of using AGS as a polishing process immediately following the anaerobic pre-treatment of brewery wastewater. This work evaluated the effects of operating parameters such as OLR, F/M and COD:N:P ratios on granular sludge development and stability, as well as nutrient removal performance.

MATERIAL AND METHODS

Reactor configuration and operation

Three 4 L sequencing batch reactors (SBR) were seeded with conventional activated sludge from the ‘West End Water Pollution Control Centre’ (WEWPC; Winnipeg, Manitoba, Canada) with total suspended solids of 3.4 ± 0.2 g-TSS L⁻¹ and volatile suspend solids of 2.7 ± 0.2 g-VSS L⁻¹ concentrations. Brewery wastewater collected from Fort Garry Brewery, a local brewery in Winnipeg, was previously anaerobically treated with three 4 L anaerobic moving bed biofilm reactors (AnMBBR) using AC920 media manufactured by Headworks BIO (Victoria, BC, Canada) with 40% media filling at an 8 h hydraulic retention time (HRT) in a mesophilic environment (35 ± 1 °C). Studies conducted by di Biase et al. (2017) suggested that at this operational condition, the AnMBBR was capable of achieving soluble COD (sCOD) removal greater than 80% without compromising methanogenesis and buffering the pH to neutral levels. The raw brewery wastewater was stored in a cold chamber (4 °C) to prevent pre-fermentation of the substrate before anaerobic digestion pre-treatment. The wastewater was prepared by diluting the fermenter underflow (140 ± 10 kg-sCOD m⁻³) to approximately 1:40 ± 10 times the initial concentration. This resulted in an AnMBBR influent concentration of 3.7 ± 0.3 kg-sCOD m⁻³ and 4.5 ± 0.5 kg-TCOD m⁻³ over the 120 days studied; influent concentrations were comparable to the average values of typical North American
brewery wastewater (Chastain et al. 2011). The measured \( \text{BOD}_5 \) was 3.2 ± 0.2 kg \( \text{BOD}_5 \) m\(^{-3}\), resulting in a BOD/ COD ratio of the raw brewery wastewater of on average 0.70 ± 0.05, which is within the typical ratio of 0.6–0.7 reported in literature (Brito et al. 2007). After AnMBBR pre-treatment, the effluent wastewater was sieved through a screen with 0.6 mm pore diameter and subsequently used as the influent for three aerobic granular SBR reactors.

The SBRs were three identical column-type reactors (100 cm height) operated at room temperature (i.e. 19 ± 1 °C). Air was supplied at a flow rate of 2.5 L min\(^{-1}\) via three circular ceramic stones laid on the bottom of the reactor at 120° angle. This resulted in a superficial up-flow air velocity of 0.5 cm s\(^{-1}\) and a dissolved oxygen (DO) concentration of 7 mg L\(^{-1}\). Two COD:N:P ratios were investigated in two independent periods. Table 1 reports typical ranges of brewery wastewater characterization, and discharge into sewer bylaw of three North American areas (i.e. Winnipeg, Manitoba, Canada; Ottawa, Quebec, Canada; and Minnesota, USA bylaws). Table 1 also shows the influent brewery wastewater characterization to AGS after AnMBBR treatment. It has been shown by Wang et al. (2007) that 100COD:10N:1P by weight is required to avoid aerobic heterotrophic growth limitations and the appearance of filamentous bacteria. To maintain this ratio, typical brewery wastewater reported in Table 1 has a deficit in nitrogen and phosphorus. For these reasons, in the first period studied, nitrogen and phosphorus concentrations were adjusted to have the desired ratio of 100COD:10N:1P. During the second period, however, the nutrients in the influent wastewater were not added, resulting in a measured COD:N:P ratio of 100:6:1. Following anaerobic digestion pre-treatment, the BOD/COD ratio was measured at between 0.4 and 0.5 while pH was 7.1 ± 0.2. The reaction cycle was set with 40 minutes of up-flow anaerobic feeding followed by an aerobic phase which varied in the two periods, and at the end was 2 minutes of discharge. The number of cycles differed between the two periods (i.e. 100COD:10N:1P and 100COD:6N:1P) to increase the HRT and reduce the OLR while maintaining unvaried volume exchange ratio (VER) set at 75, 50, and 25% in the three AGSs, allowing therefore direct comparison – Table 2. Thus, the first period had 4 cycles per day (6 hours each) while the second period had 2 cycles per day (12 hours each). Settling times were also varied to maintain similar hydraulic selective pressure within the two periods.

Influent characteristics reported in Table 1 above and operational parameters in Table 2 (i.e. VER and number of cycles) were used to calculated flow (Q; Equation (1)), OLR (Equation (2)), and F/M ratio (Equation (3)).

\[ Q \left( \frac{L}{d} \right) = \text{VER} \times V_R \times N_{cycles} \left( \frac{#}{d} \right) \]  

(1)

Table 1 | Typical brewery wastewater characterization, discharge into sewer bylaw and influent wastewater to AGS (after anaerobic AnMBBR) in the two periods operated at different C:N:P ratios

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Typical brewery wastewater</th>
<th>Discharge into sewer bylaw*</th>
<th>100COD:10N:1P Value</th>
<th>100COD:6N:1P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCOD (mg L(^{-1}))</td>
<td>1,000–8,000</td>
<td>500–600</td>
<td>1,500</td>
<td>1,800</td>
</tr>
<tr>
<td>( \text{BOD}_5 ) (mg L(^{-1}))</td>
<td>600–5,000</td>
<td>250–300</td>
<td>800</td>
<td>720</td>
</tr>
<tr>
<td>TN (mg L(^{-1}))</td>
<td>10–30</td>
<td>50–100</td>
<td>138</td>
<td>98</td>
</tr>
<tr>
<td>TP (mg L(^{-1}))</td>
<td>10–30</td>
<td>10</td>
<td>19</td>
<td>17</td>
</tr>
<tr>
<td>pH</td>
<td>4.5–12</td>
<td>5.5–11</td>
<td>7.1</td>
<td>7.1</td>
</tr>
</tbody>
</table>

TCOD, total chemical oxygen demand; \( \text{BOD}_5 \), biochemical oxygen demand; TP, total phosphorus; TN, total nitrogen.

*City of Winnipeg (Manitoba, Canada) SEWER BY-LAW NO 92/2010; City of Ottawa BY-LAW NO. 2003-514; Minnesota bylaw (www.metrocouncil.org); D. Lgs. 152/06 (Parle Terza, Allegato 5, Tabella 3) Veneto Province.

Table 2 | Operational parameters in the two periods (100COD:10N:1P and 100COD:6N:1P)

<table>
<thead>
<tr>
<th>Operational parameter</th>
<th>100COD:10N:1P</th>
<th>100COD:6N:1P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycle length (h)</td>
<td>( R_{0,1} )</td>
<td>( R_{0,1} )</td>
</tr>
<tr>
<td>OLR (kg-TCOD m(^{-3}) d(^{-1}))</td>
<td>4.1 ± 0.6</td>
<td>2.8 ± 0.4</td>
</tr>
<tr>
<td>HRT (h)</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>Q (L/d)</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>VER (%)</td>
<td>75</td>
<td>50</td>
</tr>
<tr>
<td>Settling time (min)</td>
<td>4.5</td>
<td>3</td>
</tr>
</tbody>
</table>

\( R_0 \) X represent the organic loading rate (OLR) of the reactor; VER, volume exchange ratio; HRT, hydraulic retention time.
where $Q$ is expressed in liters per day (L d$^{-1}$), VER is expressed as a percentage, and $n'_c$ cycles is the number of cycles per day (# d$^{-1}$).

$$\text{OLR} \left( \frac{\text{kg TCOD}}{\text{m}^3 \text{d}} \right) = \frac{\text{TCOD}_{\text{in}} \left( \frac{\text{kg TCOD}}{\text{m}^3} \right) \times Q \left( \frac{\text{m}^3}{\text{d}} \right)}{\text{VR}}$$

(2)

where OLR is reported in kg-TCOD m$^{-3}$ d$^{-1}$, TCOD$_{\text{in}}$ is the influent concentration of TCOD in kg-TCOD m$^{-3}$, $Q$ is calculated using Equation (1) but expressed in m$^3$ d$^{-1}$, and $VR$ is the reactor volume in m$^3$ (i.e. 0.004 m$^3$).

$$\frac{F}{M} \text{ ratio} \left( \frac{\text{kg TCOD}}{\text{kg VSS d}} \right) = \frac{\text{TCOD}_{\text{in}} \left( \frac{\text{kg TCOD}}{\text{m}^3} \right) \times Q \left( \frac{\text{m}^3}{\text{d}} \right)}{\text{biomass} \left( \frac{\text{kg VSS}}{\text{m}^3} \right) \times \text{VR} (\text{m}^3)}$$

(3)

where the $F/M$ ratio is calculated as kg-TCOD kg-VSS$^{-1}$d$^{-1}$, TCOD$_{\text{in}}$ is the influent concentration in kg-TCOD m$^{-3}$, $Q$ is the flow calculated using Equation (1) expressed in m$^3$d$^{-1}$, biomass is the concentration of VSS in the reactor reported in kg-VSS m$^{-3}$, and $VR$ is the reactor volume in m$^3$ (i.e., 0.004 m$^3$).

**Analytical methods**

Physical-chemical analyses of influent and effluent (COD, total nitrogen – TN, total phosphorus – TP, total and volatile suspended solids – TSS and VSS) were performed in accordance with standard methods (APHA 2005). Biochemical oxygen demand was measured using the OxiTop Control BOD measurement system (WTW, Ohio, USA). The nitrogen fraction as total ammonia nitrogen (TAN-N), nitrite nitrogen (NO$_2$-N), nitrate nitrogen (NO$_3$-N), and orthophosphate phosphorus (PO$_4^{3-}$-P) were determined with a QuickChem 8500 Flow Injection Analyzer (FIA) system. Samples for effluent TSS and VSS were measured three times per week, while total and volatile mixed liquor suspended solids were measured twice a week. Extracellular polymeric substances (EPS) were quantified according to the two-step heating extraction method described by Corsino et al. (2016). A 50 mL sample of mixed liquor was immediately filtered through a 0.22 μm pore-size membrane to extract the unbound EPS. The precipitate was then suspended again with deionized water to its original volume (50 mL) and placed in a thermal batch at 80 ± 1 °C for 10 minutes. Afterwards, the sample was centrifuged at 7,000 rpm for 10 minutes at 4 ± 1 °C and the supernatant was filtered through a 0.22 μm pore-size membrane allowing the determination of tightly-bound EPS. This research focused on the characterization of tightly-bound EPS in terms of protein and polysaccharide concentrations. The carbohydrates and protein concentrations, therefore, were determined according to the phenol-sulphuric acid methods with glucose as the standard and by the Folin method with bovine albumin serum as the standard (Corsino et al. 2016). Morphological characteristics of the granular sludge were evaluated through stereomicroscope observations (Zeiss, Toronto, ON, CA), while their size distribution was determined by measuring 100 granules through IM50-Leika image analyser software.

**Solids retention time estimation**

The VER applied (Table 2) and the geometry of the reactors (i.e. discharge valve located at 1 L reactor height) allowed the smallest volume for biomass retention in R$_{4.1}$. The sludge retention time (SRT) was therefore not directly controlled but managed by discharging the excess solids settled above the valve at an operational C:N:P ratio of 100:10:1. In R$_{2.8}$ and R$_{1.4}$, however, the SRT was manually maintained as close as possible to R$_{4.1}$. At a nutrient ratio of 100C:6N:1P, the SRT was not controlled in any of the reactors. At steady state, the SRT was estimated according to the following equation – Equation (4):

$$\text{SRT} = \frac{X_{\text{TSS}} (\text{g TSS L}^{-1}) \cdot V_R (\text{L})}{X_{\text{TSS steady state}} (\text{g TSS L}^{-1}) \cdot V_S (\text{L d}^{-1}) + X_{\text{TSS}} (\text{g TSS L}^{-1}) \cdot V_D (\text{L d}^{-1})}$$

(4)

where $X_{\text{TSS}}$ is the average TSS concentration over 20 days of steady state in g-TSS L$^{-1}$, $V_R$ is the reactor volume in liter (i.e., 4 L), $V_S$ is the volume of samples withdraw per day for analytical measurements, $X_{\text{TSS steady state}}$ is the concentration of solids in the effluent in g-TSS L$^{-1}$, and $V_D$ is the volume discharged in L d$^{-1}$. Granular sludge settles faster than the flocculant and suspended biomass, therefore, the mass of solids wasted per day was assumed as floccular, suspended, and biomass detached from granular sludge not contributing to the granular biomass. Equation (4) allowed estimation of the total SRT of the system. The granular sludge retention time was also estimated with Equation (4) by disregarding the volume discharge, $V_D$, in the denominator.
RESULTS AND DISCUSSION

Granular sludge development

Mixed liquor and effluent solids

The total mixed liquor and effluent solids profile at different OLRs for both nutrient ratios studied are shown in Figure 1. Figure 1(a) reports the early stages of operation in which granulation took place at 100C:10N:1P. Under these conditions, R4.1 achieved an average maximum mixed liquor solids concentration of 3.6 ± 0.3 g-TSS L⁻¹ two weeks after seeding. In R4.1, the solids concentration was maintained by effluent discharge from a valve located at 1 L reactor height. In R2.8 and R1.4, TSS in mixed liquor concentrations were of 3.5 ± 0.2 and 2.0 ± 0.2 g L⁻¹, respectively. This favored granulation over free-floating suspended and flocculant biomass development. At steady state conditions (i.e. from day 30 to 35), mixed liquor TSS concentrations were measured to be 3.6 ± 0.7, 3.7 ± 0.6, and 2.0 ± 0.2 g-TSS L⁻¹ in R4.1, R2.8, and R1.4 respectively. Concentration of mixed liquor solids were found statistically similar (p-value <0.05) in R4.1 and R2.8, suggesting that manual discharge of settled solids was capable towards maintaining mixed liquor solids at comparable values at F/M ratios of 1.8 ± 0.6 and 1.2 ± 0.5 kg-TCOD kg-VSS⁻¹d⁻¹. However, lower OLR in R1.4 did not achieve similar mixed liquor solids concentration, with a F/M ratio of 0.9 ± 0.3 kg-TCOD kg-VSS⁻¹d⁻¹. At a nutrient ratio of 100COD:6N:1P and a lower OLR (Figure 1(b)), no manual selective solids waste was provided to any reactor. The mixed liquor solids concentrations at steady state were 3.1 ± 0.8, 3.0 ± 1.0, 2.0 ± 0.2 g TSS L⁻¹ in R2.1, R1.5, and R0.8, resulting in F/M ratios of 0.75 ± 0.25 kg-TCOD kg-VSS⁻¹d⁻¹. Effluent solids reported in Figure 1(c) (i.e., 100C:10N:1P) and Figure 1(d) (i.e., 100C:6N:1P) resulted as comparable and therefore independent from OLR. Effluent solids concentrations at steady state were consistently below 350 mg-TSS L⁻¹ but a few measurements. Scaling up the AGS process as a polishing step following anaerobic digestion may require solids separation technologies after treatment to further reduce effluent TSS concentrations to effluent discharge levels imposed by regulators (e.g. 350 mg-TSS L⁻¹ in the city of Winnipeg, Canada, sewer bylaw). Alternatively, an independent selective wasting system could be employed to ensure that lighter mixed liquor fractions of biomass do not contribute to TSS effluent solids concentration. Selectively wasting the lighter fractions

Figure 1 | Mixed liquor suspended solids in the three reactors operated at different OLR (X of the RX) and COD:N:P ratio of 100:10:1 in A and 100:6:1 in B; effluent TSS at different OLR and COD:N:P ratio of 100:10:1 in C and 100:6:1 in D.
of TSS have also been shown to improve granular structural morphology and its stability (Wilén et al. 2018).

**Granular sludge morphology**

Figure 2 shows the profile of granular sludge development and the difference in morphological structure during experimental periods as a function of the F/M ratio calculated according to Equation (3) presented in Reactors configuration and operation. Stereomicroscopic imaging from weeks 2, 4, and 8 are reported.

After one week from seeding, the first granular structure precursors were observed under stereomicroscopy. Full granulation was observed after 2 weeks of operation (Figure 2(a)). Aerobic granules dominated after 4 weeks, when their average sizes reached $1.6 \pm 0.2$, $1.9 \pm 0.3$, and $1.2 \pm 0.1$ mm at F/M ratios of $1.8 \pm 0.6$, $1.2 \pm 0.5$, and $0.9 \pm 0.3$ kg-TCOD kg-VSS$^{-1}$d$^{-1}$, respectively. The F/M ratio resulting from varying OLRs had an impact in granular sludge development. Results shown that the lower the OLR and F/M ratio, the smaller is the diameter of the granules which is in accordance with literature (Hamza et al. 2018). However, the reactor operated at an OLR of $2.8 \pm 0.4$ kg-TCOD m$^{-3}$d$^{-1}$ resulting in a F/M ratio of $1.2 \pm 0.5$ kg-TCOD kg-VSS$^{-1}$d$^{-1}$ did not follow this trend of decreasing granular size with respect to OLR due to different organic loads and settling time. After seven weeks, the reactors accumulated solids on granules surfaces and in the bulk mixed liquor (Figure 1(a) and 1(b)) which is presented in Figure 2(a) at 8 weeks. This was caused by changes in operational parameters (i.e. previously, 7d SRT control with 4 cycles a day 6 h long) which did not affect granular sludge stability, settling characteristics, or TSS effluent concentrations (Stes et al. 2018). At the beginning of week 8, the SRT was not controlled and the number of cycles were decreased to two per day to further reduce OLR while maintaining the first period’s operational parameters (Table 2).

At 100COD:6N:1P ratio (Figure 2(b)), the reactors received approximately half the OLR compared to the previous stage. Longer cycle lengths and no SRT contributed to extending the aerobic oxidation of organics further allowing the redistribution and recovery of granular structure into a discrete morphology. This is evident when comparing the stereomicroscopic image at 4 weeks to 8 weeks in Figure 2(b). Hence, one month was required to re-establish the granular morphology into a more defined architecture. The granular sludge became dominant, presenting an average diameter of $1.5 \pm 0.3$, $1.2 \pm 0.3$, and $1.0 \pm 0.1$ mm at OLRs of $2.1 \pm 0.6$, $1.5 \pm 0.4$, and $0.8 \pm 0.2$ kg-TCOD m$^{-3}$d$^{-1}$ corresponding to F/M ratios of $1.0 \pm 0.3$, $0.7 \pm 0.3$, and $0.5 \pm 0.2$ kg-TCOD kg-VSS$^{-1}$d$^{-1}$, respectively.

During the second period (100COD:6N:1P), the difference in OLRs and F/M ratios emphasized the impact on granular structure and morphology. The trend observed in the first period became more evident as the OLR decreased. The resulting size distribution was directly dependent on the OLR, F/M ratio, and longer cycle length, leading to a longer famine period, produced discrete granular architecture.
The granular structure presented more irregularities at higher F/M ratios, suggesting that the applied loads affected the appearance and biomass structural aggregation of the granules. Hamza et al. (2018) concluded that high F/M ratios adversely affect granular stability, showing overgrown biomass and poor settleability at F/M ratios above 1.5 kg-TCOD kg-TSSm⁻³d⁻¹, and leading to the breakdown and washout of biomass at values above 2.5. Hamza et al. (2018) indicated an F/M ratio ranging from 0.5–1.4 kg-TCOD kg-TSSm⁻³d⁻¹ as optimal for long-term granular stability. Stes et al. (2018) found that granular structure treating brewery wastewater is resistant to operational parameter fluctuation at lower F/M ratios (i.e., 0.01–0.3 kg-TCOD kg-TSS⁻¹d⁻¹) and pH. During one-step AGS brewery wastewater treatment, Stes et al. (2018) observed long-term granular stability, and preserved both performance and settling properties. A change in granular morphology was observed, in the present study, due to the microbiology of different microorganisms forming granules, EPS production/accumulation, and cycle length. Ordinary heterotrophs (OHO) are fast growing microorganisms that reside on the surface of the granules and drive its structural appearance (Devlin et al. 2017). At high OLR and F/M ratio, the OHO had more substrate available for growth and more EPS are stored both within and on the granular surface, which led to an amorphous superficial architecture. This phenomenon is also driven by the aerobic cycle length and superficial air velocity supplied to the reactor (Devlin et al. 2017). Longer aeration cycles led to the loss of loosely bound EPS and increased the consumption of tightly bound EPS, which promoted the appearance of discrete granules (Corsino et al. 2016). The stereoscopic imaging of granular sludge in Figure 2 suggested that the lower OLR and lower F/M ratio presented the most discrete and compact granules, thereby agreeing with literature (Hamza et al. 2018).

**Aerobic granular sludge performance**

**Carbon, nitrogen, and phosphorus removal**

Performance of organic carbon, nitrogen, and phosphorus removal rates for both 100C:10N:1P and 100C:6N:1P periods as a function of loading rates are presented in Figure 3. At different OLR, a strong correlation between organic removal rates was found with an overall percent removal of 82.5% with R² of 0.99 during the two periods considered – Figure 3(a). Specifically, at a nutrient ratio of 100C:10N:1P, the reactors operated at different OLR, resulting in comparable organic removal rates of around 80% TCOD. Effluent concentrations were measured between 0.2 and 0.8 g-TCOD L⁻¹ at OLR from 1.4 to 4.1 kg-TCOD m⁻³d⁻¹. In the second test period, by applying a nutrient ratio of 100COD:6N:1P and lower OLR from 4 down to 2 cycles per day, the TCOD removal improved by 5% to 85%, producing effluent concentrations lower than 0.2 g-TCOD L⁻¹. The bylaws in brewery industry reported in Table 1 legislate a maximum dischargeable concentration of organic carbon into the sewer system of 0.3 g-BOD₅ L⁻¹. The effluent BOD₅ for 100C:10N:1P ranged from 0.06 to 0.24 g-BOD₅ L⁻¹ at 1.4–4.1 kg-TCOD

![Figure 3](image-url)
m⁻³ d⁻¹ OLR with BOD₅/COD ratio of 0.2–0.3. In the second period, 100C:10N:1P with OLR at 0.8–2.1 kg-TCOD m⁻³ d⁻¹, BOD₅ concentrations were found below 0.2 g-BOD₅ L⁻¹. Hence, all the reactors complied with the organic carbon bylaws reported in Table 1 at the operational conditions applied.

Nitrogen removal rates as a function of loading rates are plotted in Figure 3(b), and resulted in a high correlation (i.e. R² of 0.90) with an overall nitrogen removal of approximately 60% when considering both trial periods. Similar overall removals were found when plotting phosphorus results as a function of loading rate, with a weaker correlation of 0.635 R² (Figure 3(c)).

In the first period at 100C:10N:1P, nitrogen removal ranged between 35 to 40% of the influent nitrogen, which was adjusted by dosing ammonia to maintain the nutrient ratio. An increasing trend was observed (Figure 3(a) and 3(b)) demonstrating a higher organic loading rate correlating to higher nitrogen removal rates. This suggested a higher nitrogen demand supported microbial growth and activity without nutrient limitation at the operational conditions imposed. Zhao et al. (2015) demonstrated that heterotrophic removal through assimilation contributed more towards nitrogen removal than autotrophic nitrification at high COD and nitrogen loading rates (NLR). Total nitrogen effluent concentration was found to be higher than 80 mg-L⁻¹, not complying with bylaws for discharge into the sewer system reported in Table 1. Similarly, phosphorus effluent concentration limits of 10 mg-TP L⁻¹ were not achieved; phosphorus removals were observed to be 30% of the TP loaded.

In the second period (i.e. 100C:6N:1P) with no addition of nitrogen, nitrogen removal increased from 40 to 60% while TP removal (i.e. 30%) showed no improvement over the previous period. The effluent BOD₅ ranged from 50 to 75 mg L⁻¹, effluent TN concentrations were measured below 40 mg-TN L⁻¹ and in compliance with the bylaws reported in Table 1. However, effluent TP concentrations were above 14 mg-TP L⁻¹, not meeting the required limit of less than 10 mg-TP L⁻¹.

Carbon removal rates were load-independent in the range examined (i.e. 0.8–4.1 kg-TCOD m⁻³ d⁻¹). The higher removal (~5%) during the second period of experimentation was linked to the doubled aerobic reaction time, which allowed more time to hydrolyze the residual particulate COD for further degradation. Agreeing independent correlation between loading rates from 10 to 61 g-TP m⁻³ d⁻¹ and removal rates from 5 to 40 g-TP m⁻³ d⁻¹ were observed in the effluent TP – Figure 2(c). The TN removal rate was greater during lower loading rates of 50–140 g-TN m⁻³ d⁻¹ (i.e. 100COD:6N:1P ratio) versus the higher loading rates ranging from 140 to 400 g-TN m⁻³ d⁻¹ (i.e. 100COD:10N:1P ratio). Total nitrogen removal rates were determined to be inversely proportional to the OLRs.

**Nitrification and free ammonia inhibition**

Soluble nitrogen fractionation in effluent as total ammonia nitrogen (TAN), nitrite, and nitrate allows for the understanding of the nitrification process in the two operational periods. Figure 4 shows nitrogen speciation in effluents from both periods.

In the first period, 100COD:10N:1P (Figure 4(a)), the influent TAN was converted to nitrite by ammonia-oxidizing bacteria (AOB) with no presence of nitrite-oxidizing bacteria (NOB) metabolism observed. Results showed that the nitritation process is inversely proportional to the organic loading rate. Full nitrification was never reached at 100C:10N:1P ratio with a 6 h cycle regime. In the second period, 100COD:6N:1P (Figure 4(b)), the influent TAN was converted to nitrite by ammonia-oxidizing bacteria (AOB) with no presence of nitrite-oxidizing bacteria (NOB) metabolism observed. Results showed that the nitritation process is inversely proportional to the organic loading rate. Full nitrification was never reached at 100C:6N:1P ratio with a 6 h cycle regime. In the second...
operational period, 100COD:6N:1P presented in Figure 4(b), when the aeration contact time was doubled and external nitrogen was no longer provided, thereby reducing the nitrogen loading rate, full nitritation was observed only for the higher OLR of 2.1 ± 0.6 kg-TCOD m⁻³d⁻¹. However, nitrification increased when OLR decreased to full nitrification observed at OLR of 0.8 ± 0.2 kg-TCOD m⁻³d⁻¹.

Two factors explain the observed phenomenon: free ammonia (FA; NH₃-N) inhibition and OHO interference with AOB and NOB metabolism. Total ammonia nitrogen concentrations in influent wastewater were measured at 120 ± 10 and 80 ± 7 mg-TAN L⁻¹ resulting in FA concentrations of 34 ± 4 and 23 ± 2 mg-NH₃-N L⁻¹ at C:N:P nutrient ratios of 100:10:1 and 100:6:1, respectively. Nitrogen loading rates were 0.41 ± 0.05, 0.27 ± 0.02, and 0.14 ± 0.04 g-TAN L⁻¹d⁻¹ in COD100:10N:1P and 0.28 ± 0.05, 0.18 ± 0.02, and 0.04 ± 0.01 g-TAN L⁻¹d⁻¹ in 100COD:6N:1P. The reactors' pH was not controlled over the study, leading to an alkaline environment after aeration time (i.e. pH > 9), which inhibited AOB and NOB metabolism and their establishment through FA inhibition (Wei et al. 2012). A study conducted by Claros et al. (2013) suggested that the optimal pH for AOB is in the range of 7.4–7.8 and the inhibitory effect increase at higher pH values in which FA concentration increases. Research from Anthonisen et al. (1976) has demonstrated that AOB are more resistant to FA than nitrous acid-sensitive NOB. Anthonisen et al. (1976) defined acute FA inhibitory concentrations ranging from 10 to 150 mg-NH₃-N L⁻¹ for AOB and concentrations ranging from 0.1 to 1 mg-NH₃-N L⁻¹ for NOB, with initial effects occurring at concentrations as low as 0.2–3.0 mg-NH₃-N L⁻¹. Acclimation of nitrifiers to FA is, however, possible over long-term exposure depending on biomass characteristics (e.g. suspended, granular) and NLR (Liu et al. 2008). Researchers have shown that nitrifying granular sludge can tolerate fluctuation of NLR from 0.7 to 1.8 kg-TAN m⁻³d⁻¹ at pH 8 and an aerated length between 2 to 5 hours at DO saturation (Liu et al. 2008). Other researchers have shown in nitrifying AGS designed for complete nitrification to nitrate at a NLR of 0.4 g-TAN L⁻¹d⁻¹ while 80% of nitrification was achieved at twice the NLR with DO concentration of ~8 mg-O₂ L⁻¹ and pH ranging from 7.0 to 8.5 (Vázquez-Padín et al. 2010). It has been shown that in AGS systems also designed for carbon removal, the OLR plays an important role in enabling nitrification. Figueroa et al. (2011) reported that FA inhibition and oxygen limitation could cause a decrease in nitrogen removal when NLR reached values of ~1.3 kg-TN m⁻³d⁻¹. Increasing OLR will decrease granular nitrifying activity caused by the reduction of oxygen penetration into the granules’ depth and its availability for AOB (Chen et al. 2008).

The differences in OLR presented in this presented study agree with the reported literature. Microorganisms in the granules (i.e. OHO, AOB and NOB) developed in an efficient architecture and fully performed at the lowest OLR and NLR of 0.04 ± 0.01 g-TAN L⁻¹ (Figure 4(b)). Compared to nitrifiers, OHO have a higher DO affinity; their accelerated growth and EPS production/storage may have hindered AOB establishment within the granular structure. In the first test period with 100C:10N:1P, the measured specific tightly-bound EPS on granules ranged between 160 and 200 mg-TB-EPS VSS⁻¹ at OLR from 1.4 to 4.1 kg-TCOD m⁻³d⁻¹ with 1.2 to 2 mm granular size. Liu et al. (2015) concluded in their research that particle size between 0.6 and 1.8 mm provide an ideal environment for AOB and NOB establishment with optimal specific oxygen utilization rates. The second period 100C:6N:1P exhibited a smaller granular size of 1 to 1.5 mm and lower EPS production/storage of 75 to 90 mg-TB-EPS VSS⁻¹ at OLR from 0.8 to 2.1 kg-TCOD m⁻³d⁻¹. For these reasons, the lower OLR of 0.8 ± 0.2 kg-TCOD m⁻³d⁻¹ resulted in greater stability in granular architecture (see Figure 2(b)) promoting nitrification (Figure 4(b)).

Extracellular polymeric substances

Differences in EPS composition have been reported to be important toward granular structure stability. Liu et al. (2015) reported how the abundance of protein and carbohydrates significantly affects granular stability. In particular, the sludge hydrophobicity is enhanced by protein content while a high protein to polysaccharides ratio promoted cellular aggregation by improving cell adhesion.
capability. In Figure 5, the protein to polysaccharides ratio at different OLR in steady state condition for both C:N:P nutrient ratios of 100:101 and 100:6:1 is shown. The protein to polysaccharides ratio decreased as the OLR and therefore F/M ratio increased, suggesting that, for higher OLR, granular structure could be compromised and reduced granular stability could be observed as also described by Hamza et al. (Hamza et al. 2018).

The abundance of protein in contrast with carbohydrates resulted in a protein to polysaccharide ratio ranging between 8 to 15. The highest protein to polysaccharide ratio was achieved when the OLR was decreased by increasing the cycle length from 6 to 12 h, and decreasing the number of cycles from 4 to 2 per day at a nutrient ratio of 100COD:6N:1P, therefore increasing the organic carbon oxidation potential. Research has shown that an extended aeration period leads to a granular famine stage that increases granular stability (Corsino et al. 2016). Adav et al. (2010) studied a one-step AGS reactor treating high strength synthetic wastewater at OLR between 9 to 21 kg-TCOD m⁻³·d⁻¹. This research concluded that EPS protein content is an indicator of granular stability. Protein production decreased as the OLR increased, leading to a weakened granular structure and their eventual breakdown. Longer aeration reaction times with lower OLR are qualitatively beneficial to granular structure, which exhibited a more compact and defined architecture compared to the other operational conditions evaluated (Figure 2). Performance results also indicated that, within the conditions studied, the microbial community and its complex functionality in nutrient removal is more efficient at operational parameters of OLR at 0.8 ± 0.2 kg·TCOD m⁻³·d⁻¹ yielding an F/M ratio of 0.5 ± 0.2 kg·TCOD VSS⁻¹·d⁻¹.

**Design considerations**

The solids retention time was determined according to Equation (4). Total SRT of 7d comprehensive of granular and suspended solids was estimated for the 100C:10N:1P nutrient ratio while the granules’ SRT was calculated as 11d (Figure 1). In the second test period with a C:N:P ratio of 100:6:1, the total SRT was estimated to be 13d while the granular SRT was calculated as triple the total SRT (i.e. 36d). At ambient room temperature (20°C), the minimum SRT for OHO, phosphorus accumulating organisms (PAO), AOB, and NOB are 0.4d, 1.2, 1.4, and 1.9 d, respectively (BioWin 6.0, Environsim, 2020). The reactors had a sufficient SRT to retain and favor the growth of the microbial population, leading to carbon, nitrogen, and phosphorus removal. Full nitrification was inhibited in all conditions studied excluding at NLR of 0.04 ± 0.01 g·TAN L⁻¹, OLR of 0.8 ± 0.2 kg·TCOD m⁻³·d⁻¹, which resulted in an F/M ratio of 0.5 ± 0.2 kg·TCOD VSS⁻¹·d⁻¹ (Figure 4(b)). However, simultaneous nitrification-denitrification and biological phosphorus removal (Bio-P) did not occur during any of the applied conditions. Kinetic tests showed that the readily biodegradable COD (rb-COD) was removed within 2 hours after aeration by OHO. The scarcity of rb-COD, together with the lack of anoxic conditions, did not provide a suitable environment for simultaneous nitrification-denitrification. The presence of oxygen-bound compounds (nitrite and nitrate; Figure 4) at the end of the cycle had a critical effect on the anaerobic feeding period, thereby becoming anoxic, which negatively affected PAO establishment. Hence feeding time impacted both Bio-P and denitrification processes. Hydrolysis of the brewery substrate and further fermentation to consistent production of volatile fatty acids (VFA) likely required more than the imposed 40 minutes-long feeding time. Following the anaerobic pre-treatment, most of the rb-COD was depleted by digestion resulting in more time required to ferment the recalcitrant particulate COD. Thus, without rb-COD (i.e. with VFA as acetate), the PAO were incapable of growing and integrating to the microbial granular structure. A longer feeding time would have enabled denitrification and an increase in acetate production via fermentation, ensuring anaerobic feeding conditions for Bio-P (Stes et al. 2019). Also, selectively removing microorganisms by wasting the lighter solids fraction via a shorter settling time, SKT control, or implementing a dedicated selective waste system, has been shown to enhance Bio-P and reduce effluent solids concentration (Wilén et al. 2018).

**CONCLUSIONS**

Granulation was observed after one week of operations. Following two weeks, a defined granular structure developed and granulation completed in all reactors independent from the OLR and F/M ratios. Aerobic granular sludge stability was significantly affected by the operating conditions. At a high C:N:P ratio and OLRs (or F/M ratio), irregular and weak granules developed. In contrast, a low C:N:P ratio and OLRs (or F/M ratio) were favorable toward achieving a long-term stable aerobic granular architecture. Carbon removal rate was higher than 80% in all reactors and was load-independent. However, TN removal was inversely proportional to the OLRs. Longer aeration reaction times with
lower OLR were beneficial to the granular structure, which exhibited a clear prevalence of proteins in the EPS matrix. The ideal operating conditions within the range studied were: an OLR below $0.8 \pm 0.2$ kg-TCOD m$^{-3}$d$^{-1}$, an F/M ratio below $0.5 \pm 0.2$ kg-TCOD VSS$^{-1}$d$^{-1}$, and a C:N:P ratio of 100:6:1.

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DATA AVAILABILITY STATEMENT

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

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