Monitoring the stability of aerobic granular sludge using fractal dimension analysis†

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Cyclic episodes of granules formation and disintegration took place in two lab-scale aerobic granular sludge sequencing batch reactors, one fed with synthetic wastewater (COD: 0.6 g L$^{-1}$ and NH$_4^+$-N: 0.06 g L$^{-1}$) and operated at a constant organic loading rate (2.5 g COD per L d), and the other fed with real wastewater (soluble COD: 0.27–1.37 and NH$_4^+$-N: 0.02–0.16 g L$^{-1}$) and with a variable loading rate (between 1.1 and 5.5 g COD$_{\text{soluble}}$ per L d). The sludge volume index, density and diameter (mean value and relative standard deviation) of the granular biomass showed great fluctuations, without any clear tendency during the operational period. However, changes in granules fractal dimension values (both mean and relative standard deviation) matched with the formation and disintegration dynamics of the granular biomass. Statistical data analysis showed that the relative standard deviation of the granules fractal dimension could be a useful parameter for monitoring the granules status. Indeed, an increase of its value during the maturation or steady-state granulation stages is an early warning of disintegration episodes. A control strategy to maintain granules integrity based on this parameter is proposed.

1. Introduction

Aerobic granulation is currently one of the most promising techniques for wastewater treatment due to its advantages over conventional treatment systems.$^1$ Aerobic granular sludge systems are a feasible option in terms of operating and capital costs to replace existing activated sludge systems, since they can achieve reductions in sludge production and capital costs to replace existing activated sludge systems,9,10

However, the long start-up periods required for the development of suitable granules from flocculent sludge, especially for nutrient removal, and the instability of granular biomass during prolonged operational periods,7,8 still hinder the optimization and large-scale application of aerobic granular sludge systems.$^9,10$

In this context, Yuan et al. (2017)$^{11}$ observed that the particle size is one of the parameters influencing the stability of aerobic granules due to mass transfer limitation and shifts in microbial communities. Granule breakdown due to critical size is determined by the respective rates of growth and size reduction processes influenced by the wastewater characteristics, aeration rate, reactor geometry, mixing, and solids concentration.$^{12}$ Thus, in the case of fluctuating operating conditions, predicting the critical size value of a granule is not a simple task and, therefore, it may be discarded as a control parameter to assess granular biomass disintegration episodes. It is hard to give a suitable qualitative or quantitative delimitation to establish when a granular sludge system enters into a phase of deterioration that could cause an unstable operation.

To get a better understanding of granulation stability is necessary to get information about the microstructure of granular sludge. In this sense, physical characterization has provided insights into some intrinsic architectural properties
of aggregated biomass such as activated and granular sludge, and may be used to evaluate granule disintegration. For instance, from granular sludge fractal analysis, it is possible to determine properties such as permeability, density and porosity, which have important implications for the kinetics of aggregation, and granules break-up and settling velocities. The fractal dimension \((D_f)\) of an object is a measure of its space filling capacity, and also a measure of aggregation; open and branching structures from clustering of particles due to diffusion-limited aggregation have lower values of \(D_f\) compared to more compact and denser clusters. For instance, large flocs and filamentous biomass in activated sludge, corresponding to lower values of fractal dimension, have demonstrated poor compressibility and settleability. Fractal dimension has also been used to evaluate the biological activity and physical structure of aggregated biomass. In activated sludge, high values of \(D_f\) correlate to low bioactivity due to non-porous compact flocs. In aerobic granular sludge systems this is particularly important, because high bioactivity and compactness are simultaneously required in the reactor. Fractal properties may also provide information about the strength of granules. Physical analysis of granular sludge through quantitative image analysis, has been pointed out as a promising tool to monitor the operation of aerobic granular systems. However, little information is available in the literature focusing on the structural integrity of granular sludge and fractal dimension.

The main objective of this work was to determine if fractal dimension of aerobic granular sludge can be used to predict granule disintegration episodes. To achieve this goal, cycles of formation and disintegration of aerobic granular sludge were assessed under long-term operation in conventional lab-scale sequencing batch reactors, using synthetic and real wastewater. Furthermore, the relationship between fractal characteristics and biomass granulation was established.

2. Materials and methods

2.1. Aerobic granular sludge reactors

Two aerobic granular sludge sequencing batch reactors (SBR) were operated, one fed with a synthetic medium (SBR1) and another fed with a real wastewater effluent from a seafood processing plant (SBR2). Both reactors had a total volume of 2.7 L and a working volume of 1.8 L, characterized by a height 480 mm and an inner diameter 85 mm. SBRs were operated at room temperature (15–20 °C). In both reactors, agitation was achieved by aeration through an air diffuser, and peristaltic pumps were used to feed and discharge the system. The exchange volume was set at 50%. The SBRs were operated daily in 8 cycles of 3 h, each distributed as follows: 3 min feeding, 171 min aeration, 1 min settling and 5 min effluent withdrawal. A programmable logic controller (PLC) controlled the different cycle phases of operation. The synthetic wastewater used to feed the SBR1 had a chemical oxygen demand (COD) of 0.6 g L\(^{-1}\) and its composition was, in g L\(^{-1}\): CH\(_3\)COONa·3H\(_2\)O 1.7, NH\(_4\)Cl 0.23, K\(_2\)HPO\(_4\) 0.12, MgSO\(_4\) 0.04, CaCl\(_2\)·2H\(_2\)O 0.07, KCl 0.02, and 1 mL L\(^{-1}\) of a trace solution. The effluent from the seafood facility used to fed the SBR2 had the following characteristics, in g L\(^{-1}\): total COD 0.32–1.64; soluble COD 0.27–1.37; NH\(_4\)\(^+\)N 0.02–0.16; PO\(_4\)\(^3-\)P 0.02–0.05; total suspended solids (TSS) 0.01–0.37; conductivity 1.8–4.6 mS cm\(^{-1}\); pH 6.7–6.9.

SBR1 and SBR2 were inoculated with 2.5 g volatile suspended solids (VSS) and 1.6 g VSS, respectively, of activated sludge from an urban wastewater treatment plant whose sludge volume index (SVI) were 200 and 125 mL g\(^{-1}\) TSS, respectively. SBR1 was operated at a constant organic loading rate (OLR) of approximately 2.5 g COD per L d, while the OLR applied to SBR2 ranged between 1.1 and 5.5 g COD\(_{\text{soluble}}\) per L d. SBR1 and SBR2 were operated for 340 and 220 days, respectively, to ensure a sufficient operational period to study the granular biomass dynamics. In both cases, no purge of biomass was done during the operating period (except for biomass sampling).

2.2. Analytical methods

Concentrations of total and soluble COD, ammonia, nitrate, nitrate, VSS, TSS and SVI and pH were determined according to standard methods. The size distribution of the granules was regularly measured using an image analysis procedure with a stereomicroscope (Stemi 2000-C, Zeiss) according to Tijhuis et al. (1994). Biomass density in terms of g VSS per L\(_{\text{granules}}\) was determined with the dextran blue method.

2.3. Fractal dimension analysis

The fractal dimension value \((D_f)\) was determined by means of image analysis using the ImageJ software package (National Institute of Health, USA). In brief, granular biomass images were transformed to grey-level and automatically segmented, resulting in binary images. Later, images were processed using the distance map function that generates a Euclidian distance map (EDM), where each foreground pixel in the binary image was replaced with a grey value equal to that to that pixel's distance from the nearest background pixel. Pixel size calibration was done with a stage micrometer. For imaging purposes, granular sludge samples from both SBRs were obtained along the experimental period. In order to calculate the \(D_f\) three images were taken per sampled day and four individual granules were randomly selected per image (i.e., twelve granules per day).

2.4. Statistical analyses

Experimental data of the biomass physical properties obtained from SBR1 and SBR2; SVI, density, diameter (mean and relative standard deviation (SD) values) and fractal dimension (mean and relative SD values), were statistically
analyzed together. First, the Shapiro-Wilk normality test was applied to the data of each physical property in order to evaluate normality. Then, the data were grouped according to the observed granular biomass evolution stage (i.e., formation, maturation, steady-state and disintegration). For normally distributed data sets, one-way ANOVA and Tukey’s HSD tests at a confidence level of 95% were performed. For the remaining data sets (not normally distributed) nonparametric tests were applied and the Kruskal-Wallis test with Dunn’s post-test (with Bonferroni correction using a critical value of 0.0083) were used. All statistical calculations were carried out using XLstat version 2014 (Addinsoft).

3. Results and discussion

3.1. Granular biomass development

The biomass concentration of both reactors was monitored over time (Fig. 1). In the case of SBR1, two cyclic episodes related to the formation and disintegration of granules were observed. Initially, biomass concentration inside the reactor increased due to the formation of granular biomass (Fig. 1a). After 120 experimental days, floccular biomass was not observed and the biomass concentration remained at 8 g VSS per L, indicating steady-state operation. However, on day 190, this concentration sharply decreased to 3.5 g VSS per L due to the disintegration of most of the granular aggregates. A significant fraction of biomass was then washed out in the effluent withdrawal, due to the flocculent character of disintegrated granules. After this drastic loss, the biomass concentration was recovered and increased until achieving a steady concentration (day 304) to disintegrate again at day 325.

Other authors previously observed a similar profile of biomass concentration overtime during the long-term operation of aerobic granular reactors, where no control actions about the biomass stability were established, and generally divide the development of aerobic granules into four phases according to their physicochemical properties such as shape, suspended solids concentration and settleability.11,26 Corsino et al. (2016)26 identified these four different stages following the cycle of formation and breakage, and granules extracellular polymeric substances (EPS) production: 1) formation stage where biomass concentration is almost constant and the formation of the first aggregates takes place; 2) maturation stage characterized by an exponential biomass growth and a high EPS production; 3) steady-state stage where biomass concentration remains constant and; 4) disintegration stage where granules break and biomass wash-out occurs.

Despite the operating conditions of SBR1 were maintained constant along the whole operation period, biomass concentration observed during both steady-state stages was different (around 8 and 10 g VSS per L for first and second steady-states, respectively, Fig. 1a). In SBR 1, different particle diameter was also obtained for the first and second steady-states of approximately 2.4 and 0.9 mm, respectively (Fig. 2a). Previous studies have been shown that the solids retention time (SRT) affects the achieved biomass concentration and the granule size values.27 Nevertheless, similar SRTs were obtained during both steady-state stages (around 30 d). Therefore, these results could indicate that steady-state conditions achieved in the system depend not only on the operating conditions but on the initial conditions of the granulation process too. Verawaty et al. (2013)12 also observed that steady-state conditions achieved were affected by the initial size and biomass concentration values.

In the case of SBR2, the first aggregates appeared on day 21, but it was from day 130 when a clear improvement on the properties of the aerobic granules was observed. From this day onwards, biomass concentration exponentially increased from around 2.0 g VSS per L up to 11.8 g VSS per L (day 199). Then, granules breakage took place, causing partial biomass wash-out until a final concentration of 6.6 g VSS per L by the end of the reactor operation (Fig. 1b). Contrary to what happened during the SBR1 operation, a steady-state stage with stable biomass concentration was not achieved after granules maturation, which could be attributed to the fluctuating operational conditions due to the varying characteristics of the real wastewater influent.

The observed COD removal efficiencies were 95 ± 2% and 84 ± 9% for SBR1 and SBR2, respectively, and no decrease of COD removal efficiency was observed during the granular biomass disintegration episodes in both cases (Fig. S1 and S2, of the ESI†). Regarding nitrogen removal, its efficiency depended on the performance of the nitrification process, which was mainly affected by the system biomass retention capacity, that is, by
the SRT of the system. During the first formation stage of SBR1, nitrification efficiency was 49 ± 23% (Fig. S3†), limited mainly by the low and fluctuating SRT values achieved in this stage (3.1 ± 1.5 d). Later, during the first maturation stage, the SRT increased up to values of around 30 d due to biomass accumulation inside the system and a nitrification efficiency of 94% was reached. This efficiency remained during the rest of the operational period despite the disintegration episodes and the SRT was always higher than 9 days. For SBR2, during the formation stage, no nitrification was observed due to the low SRT with average values of 1.2 ± 0.5 d (Fig. S4†). Then, during the maturation stage, the SRT value gradually increased up to 14.3 d and the nitrification process started-up reaching efficiencies of around 65%. Then, the granules breakage caused the total loss of the nitrification process.

Generally, literature reports that the formation of stable granules is associated with the decrease of the biomass SVI, the increase of its density and the homogenization of the particle size.26,28,29 However, SVI, density, and diameter mean and relative standard deviation (SD) values of the biomass, monitored for both reactors along the operational time, did not show a cyclic behavior that could be correlated to the granules’ formation stages (Fig. 2). The statistical analysis of the data confirmed this observation (Tables S1–S4†).

3.2. Fractal dimension
The $D_f$ mean values of the biomass showed cyclic changes that correlated with the different stages of granular sludge development (Fig. 2c and f). In the first granulation cycle of
SBR1, during the formation stage, the mean value of the biomass fractal dimension increased from 2.65 to 2.79 (Fig. 2c). This value remained relatively constant (2.81–2.83) during the maturation and steady-state stages (from day 70 to 190), until the biomass suffered disintegration episodes, where the mean value of $D_f$ decreased to 2.74. A similar trend was observed during the second granulation episode of SBR1, and during the operating period of SBR2 (Fig. 2c and f).

The image-based approach used to calculate $D_f$ describes the difference in density of biomass, which could indicate changes in strength and compactness. The granulation process typically increase the fractal dimension mean value of the granular aggregates, indicating an increase in their compactness. This may result in more dense and less porous granules, which could be easily retained inside the system. However, this could also increase mass transfer limitations, decreasing the specific biomass activity, which may provoke the breakage of granular biomass. Thus, high $D_f$ values may be undesired despite the better settleability of compact granules. Therefore, it would be convenient to maintain the $D_f$ at values allowing to obtain a suitable tradeoff between settleability and physical integrity of the aerobic granules.

The fractal dimension can also be considered as an indicator of the granules shape irregularity (Fig. 3). The increase of $D_f$ mean values during the steady-state stage could be a signal of initial filamentous microbial growth, or the occurrence of cracks on the surface of the granules before their breakage. Nevertheless, the mean values of the fractal dimension usually vary in a small range, hindering the use of this parameter to assess the disintegration of granules. In fact, the statistical data analysis showed that there were significant differences between the mean values of the fractal dimension during maturation/steady-state and formation stages (p-values ≤0.005), but not between those of maturation/steady-state and disintegration stages (p-values ≥0.045) (Table S5†).

Image analyses of biomass samples showed that $D_f$ values followed a normal distribution, where the relative SD values also had a cyclic behavior along the granulation process time frame. As shown in Fig. 2c and f, the SD of the $D_f$ values sharply changed during the formation and disintegration stages, while they remained at values lower than 1.0% during the maturation and steady-state stages. In the case of SBR1, the relative SD reached a minimum value during both observed steady-state stages (0.60%). Later, previous to disintegration episodes, the relative SD increased steadily. A similar trend for the relative SD of $D_f$ values was observed during the maturation stage of SBR2 (in this case steady-state stage was not observed). Statistically significant differences were found between these relative SD values during maturation/steady-state and disintegration stages (Fig. 4 and Table S6†). Thus, the increase of this parameter during the maturation or steady-state stages could be used as an early warning of granular sludge breakage.

Although there are previous studies where the fractal dimension of granular biomass was determined, this parameter has been generally used, as an indirect measurement of properties such as sedimentation settling velocity, porosity, or biomass activity, and, on the other hand, as an index to differentiate between flocculent and granular biomass. In these works, specific samples of biomass from one or more reactors were studied, but there was no continuous monitoring of the fractal dimension throughout the formation–disintegration cycles.

Fig. 3 Biomass appearance and image processing in the different granulation stages for SBR1: (a) formation (day 4), (b) maturation (day 94), (c) steady-state (day 172), and (d) disintegration (day 207).
of granular biomass. Therefore, the novelty of the present work is the use of this parameter to define the change of the granular biomass state in order to carry out an early detection of the disintegration episodes. Li et al. (2020)\textsuperscript{16} proposed the fractal dimension as a tool to simultaneously control the bioactivity and sedimentability of an active sludge system and, for this, they performed calibration curves of the fractal dimension values of various types of active sludge against their SVI and oxygen consumption rate values. However, similar to the present work, the range of values observed for $D_f$ was relatively narrow (1.55–1.67), which makes the calibration curves obtained very sensitive to the uncertainty in determining this value. Thus, a relative value of the fractal dimension, such as that of its standard deviation, could be a more suitable parameter to determine a possible change of state of the granular biomass.

Image analysis is increasingly being applied to characterize granular biomass.\textsuperscript{17,37} In fact, the mean value of its diameter is a parameter conventionally used to monitor the evolution of the granules, but it is not an adequate parameter to characterize the state of the biomass since its value will depend on the operating conditions.\textsuperscript{12} Since an improvement in the physical properties of the granules is associated with a more homogeneous size distribution,\textsuperscript{38} it may be expected that the standard deviation of the size distribution would be a useful parameter to define the state of the granular biomass, but the results found in the present work showed the opposite. Our results suggest that the fractal dimension is a suitable parameter to monitor the stability of the granular biomass, however, its measurement could be demanding and the development of an appropriate methodology for its online determination is still a challenge.

Possible causes of granule disintegration reported in literature are the accumulation of EPS in the granule’s pores,\textsuperscript{26} substrate limitation and changes in microbial populations.\textsuperscript{11,39} Then, other alternatives to monitor the granules stability would be the measurement of EPS inside the biomass, the analysis of the microbial community or the determination of substrates profiles along the granules, approaches that are likely more complex than the image analysis used to determine the fractal dimension.

3.3. Control of granules stability

In order to improve the long-term stability of aerobic granules, several operating strategies are proposed in the literature. Likewise selective wash-outs of floccular biomass during the granulation start-up period enhance granular sludge selection, a selective purge of granules depending on their age or size is suggested to prevent disintegration episodes. Granules of higher age and/or size, and with lower porosity are more prone to become unstable as a consequence of substrate diffusion limitation, and the subsequent promotion of biological endogenous activity.\textsuperscript{29,38,40} Other measures to maintain granule stability are based on readjusting the configuration of the operating cycle periods. For instance, longer famine periods can favor the hydrolysis of EPS that may be clogging granules pores, counteracting their overproduction.\textsuperscript{26} A controlled aerobic phase to maintain a suitable dissolved oxygen level, or an initial anaerobic phase where the readily biodegradable COD is mostly consumed; are recommended to avoid the growth of filamentous microorganisms which could cause granules to disintegrate.\textsuperscript{41–43} Controlling the food to microorganisms ratio, after the maturation stage, between 0.5 and 1.4 g COD per g TSS d has also been proposed to maintain the stability of the granules.\textsuperscript{39}

The strategies discussed above have been applied in systems operating with synthetic media and under highly controlled operational conditions. In the case of effluents whose characteristics are continually changing, optimizing the value of granule size and age, or the distribution of the operating cycle, is not feasible since these would require changes for each different condition.

Results obtained in this work showed that values of the fractal dimension relative standard deviation between 0.6 and 1.0% (Fig. 4), are well suited in order to get a good balance between settleability and stability of aerobic granular sludge. However, if the measurement of the fractal dimension is time-consuming, it may not be a practical approach to monitor the granular biomass status. In this sense, an important finding is that the value of the fractal dimension relative standard deviation is directly associated with the different stages of granular biomass evolution. Therefore, the own biomass concentration profile could directly provide a rough idea about the status of the granules. To avoid the risk of disintegration episodes, control measures could be applied during the maturation phase, once the maximum nitrogen removal efficiency is achieved, according to the applied volume exchange ratio. In this way, a compromise may be obtained between organic matter and nitrogen removal efficiency and the settling properties and stability of granules.

The proposed strategy to control granular sludge stability consists of a periodic purge of biomass, without any complex scheme of selection, to maintain the minimum biomass...
concentration securing the required removal efficiencies (point A, Fig. 5). If a higher biomass concentration is desired in the system to provide a buffer treatment capacity for overloading events, the biomass purge rate should be decreased. Then, periodical and controlled breakage of a fraction of granular biomass should be applied (e.g. using abrasion, mechanical breakdown), in order to maintain the fractal dimension relative standard deviation at a constant value (point A’, Fig. 5). This may prevent the development of unstable granules. Moreover, the resulting granules’ fragments may serve as nuclei for the formation of new granule. For instance, if this measure is applied during the maturation stage, and not during the steady-state stage, these granule precursors would be porous and not diffusion-limited, and thus may favour mass-transfer.

4. Conclusions

Parameters such as SVI, density and diameter of granules cannot be used to characterize the different stages of aerobic granular sludge development. The mean value of the fractal dimension $D_f$ and its relative standard deviation shows a cyclic behavior that can be well correlated to biomass granulation and breakage episodes. Statistical analyses of the experimental data, showed that only the relative standard deviation of $D_f$ could be used as a parameter to assess the stability granules. As a consequence, biomass control should be applied during the granular sludge maturation stage in order to maintain the integrity of the granules and reduce the risk of granule disintegration.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This work was funded by the Chilean Government through projects FONDECYT 1180650, FONDECYT 11181107, ANID/FONDAP/15130015 and ANID PIA/BASAL FB0002, and by the Spanish Government through TREASURE [CTQ2017-83225-C2-1-R] and GRANDSEA [CTM2014-55397-JIN] projects. The authors from Universidade de Santiago de Compostela belong to CRETUS Strategic Partnership [ED431E 2018/01] and to the Galician Competitive Research Group [GRC ED431C 2017/29]. All the Spanish programs are co-funded by FEDER (EU).

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