Image analysis as a tool to recognize anaerobic granulation time

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ABSTRACT

Image analysis techniques were used to monitor the development of anaerobic granules into an EGSB reactor fed with a synthetic substrate based on glucose (30-40% COD) and volatile fatty acids (70-60% COD) during 400 days. Two types of objects were recognized and quantified: aggregates and free filaments. The aggregates were classified into five ranges based on the equivalent diameter: smaller than 11.3 mm (residual), between 11.3 and 35.7 mm, between 35.7 and 112.8 mm, between 112.8 and 356.8 mm and larger than 356.8 mm. For each size range the following parameters were measured: projected area, number of aggregates, width, length, perimeter, and convex envelop. For filaments, the measurements were: number of free filaments and total free filament length. Other parameters were calculated based upon this data: average equivalent diameter, percentage in number of aggregates, percentage in total area, fractal dimensions and a shape factor (P_2A), based on the perimeter and on the projected area. Besides, a new parameter was defined in terms of the ratio of filament length to total floc area (L_fA). It showed to be very sensitive to the changes in the morphology of the developing granules, and, based upon our granule definition, it was used to determine the granulation time (θg) in our system (120 days). The P_2A factor allowed the quantification of aggregates roughness and was much more sensitive than fractal dimensions. The study was complemented with measurements of specific methanogenic activity (SMA) of the granules in presence of acetate, propionate, butyrate, ethanol and a gas mixture of hydrogen/carbon dioxide.

KEYWORDS

granulation time, EGSB, methanogenic activity, image analysis

INTRODUCTION

The upflow–type reactor is one of the most used systems in anaerobic wastewater treatment. The UASB (Upflow Anaerobic Sludge Blanket) were developed 20 years ago, having naturally converged to the EGSB (Expanded Granular Sludge Blanket) reactor. Since both types of reactors are based upon the differentiation between the hydraulic retention time and the solids retention time, the use of granulated biomass leads to a better performance. Granules can be considered as spherical biofilms, formed by auto-immobilisation. The phenomenon of granulation has been studied from different viewpoints (Hulshoff Pol, 1989, Schmidt and Ahring, 1996). The intrinsic process remains to be unveiled. Although there is a large set of data based upon physico-chemical measurements, this is not enough to allow the proposal of a unique wide accepted granulation theory. Regarding the factors that are considered of major importance to the granulation process there are a lot of different opinions based on observed experiments. Fang (2000) reported that granular structure is strongly dependent on the nature of the substrates, while Batstone (2001) supports that the role of the operating parameters (for example the upflow velocity and the reactor design) prevails over the one of substrate characteristics. Sure is that both beliefs have to be part of a bigger and unified theory about granulation, which was not yet enunciated. The wide range of techniques used to monitor the process may, in part, be responsible for the different opinions about it. There is an urgent need on harmonization of physical, microbial and metabolic methodologies, besides the access to integrated, unified and organized knowledge.

The recent advances in molecular and image acquisition and processing techniques will allow new insights on granulation process. The quantification of granular morphology and particle size distribution are important indicators of sludge bed stability and image analysis techniques can potentially perform it on-line. Furthermore it allows the monitoring of the dynamic changes along the process and the identification of critical moments, like the granulation time (Singh et al., 1998). In this scope, we use the image analysis tools to obtain valuable information about the formation, disintegration and other changes suffered by aggregates along their growth into granules. The aim of the present study was to identify critical moments of the

anaerobic granulation process, by determining morphological parameters involving filament length and total floc area and by following the changes of aggregates within different defined size ranges.

METHODS

EGSB reactor

An EGSB reactor was used for the granulation assay. It was made of Plexiglas with a volume of 11.47 L, 2.22 m high and a height to diameter ratio of 27, in order to achieve high superficial velocities and at the same time a wide range of hydraulic retention times. Figure 1 presents a scheme of the experimental set-up. The inoculum obtained from a local municipal sludge digester, was screened by a 0.7 mm sieve and the final volatile suspended solids content was 13.5 gSSV/L.

Analytical methods

The chemical oxygen demand (COD) and volatile suspended solids (VSS) were determined according to Standard Methods (1989). The specific methanogenic activity (SMA) assays were performed using a pressure transducer technique (Colleran et al., 1992) and was measured against different individual substrates: acetate, propionate, butyrate, ethanol and H_2/CO_2. No calcium or trace-nutrients were added. Methane was measured by gas chromatography with Helium as the carrier gas and a TCD detector. The electronic microscopy observations were carried out using a Leica-Cambridge S360 SEM microscope equipped with an Oxford Instruments energy dispersion spectrograph (EDS). The samples were prepared according to Harper and Pohland (1997).

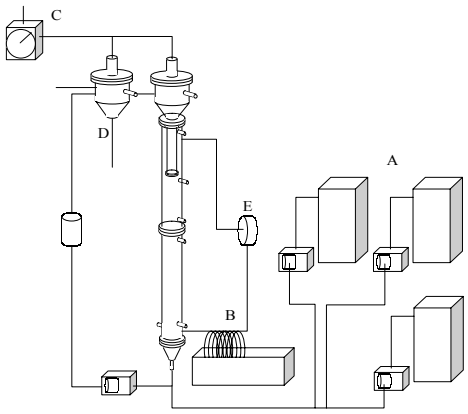

Figure 1. Experimental set-up. A. Feed containers. B. Water. C. Biogas flow-meter. D. External settler. E. Water recirculation pump.

Image Analysis

The aggregates were divided into five size ranges defined as follow: residual aggregates with an equivalent diameter below 11.3 μm, aggregates with equivalent diameters between 11.3 and 35.7 μm, aggregates with equivalent diameters between 35.7 and 112.8 μm, aggregates with equivalent diameter between 112.8 and 356.8 μm and aggregates larger than 356.8 μm. Residual aggregates were not considered in the calculations. For each size range, image analysis allowed the quantification of average equivalent diameter, percentage in number of aggregates, percentage in total area and a shape factor that quantifies the roughness and is defined through the perimeter (P) and the projected area (S) (Noesis, 1998) (Eq. 1)

$$FF = \frac{P^2}{4\pi S} \qquad \text{(Eq. 1)}$$

Alongside this parameter, other morphological parameters were determined such as fractal dimensions, convexity, compactness, roundness, solidity, extent, eccentricity, width and length (data not shown). Although some of these morphological descriptors were already used for microbial aggregates (Bellouti et al., 1997, Alves et al., 2001), in the present work, the shape factor (P_2A) was chosen due to its higher sensitivity. The filaments were characterized in terms of total free filament length and ratio of filament length to total floc area (L_fA). Free filaments are not only the dispersed bulk filaments, but also those that are attached to a floc and still have one free extremity (Figure 2).

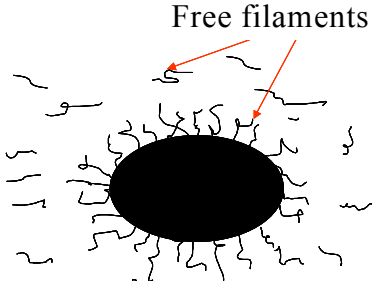

Figure 2. Schematic representation of an aggregate and free filaments.

From each sample 35 μL were put on slides and covered with a cover slip and 2.8 mL of sample were poured in Petri dishes. The slides were visualized on a Diaphot 300 Nikon microscope (Nikon Corporation, Tokyo) with a 100x magnification (phase contrast), and a total of 60 images per sample and on a Zeiss Axioscop microscope (Zeiss, Oberkochen) with a 100x magnification, and a total of 60

images per sample. The petri dishes were visualized on an Olympus SZ 40 stereo microscope (Olympus, Tokyo) with a 40x magnification, and a total of 60 images per sample. The acquired images from the Nikon microscope were used to quantify filament properties (total filament length and number). The images acquired in the Zeiss microscope were used to quantify the size and the morphological properties of aggregates smaller than 112.8 μm (equivalent diameter) which were defined as microflocs. Images acquired from the Olympus stereo microscope were used to quantify the size and the morphological properties of aggregates larger than 112.8 μm (equivalent diameter) which were defined as macroflocs. All the images were digitized with a CCD AVC D5CE Sony camera (Sony, Tokyo) a DT 3155 Data Translation frame grabber (Data Translation, Marlboro), with a 768 x 576 pixel size and 256 grey levels and with the Image Pro Plus (Media Cybernetics, Silver Spring) software package.

Three programs were created in Matlab 6.1 for determining morphological descriptors for the microflocs, macroflocs and filaments:

Microflocs. First, the original image is divided by the background image (background elimination), followed by a histogram equalisation and Wiener filtering. The image is then segmented in black (background) and white (objects). This segmentation is achieved by the simultaneous use of a boundary's image and a region image. Next, all the objects smaller than 3x3 pixels were removed and small gaps (6x6 pixels) were filled on the remaining objects. Subsequently, in order to remove debris (mainly filaments) all the objects smaller than 2000 pixels that presented a gyration radius above 1.2 were deleted. Finally, the objects cut off by the image boundaries were removed, and the morphological characterization of the microflocs was performed.

Macroflocs. First, the original image is divided by the background image (background elimination), followed by a Wiener filtering. The image is then segmented in black (background) and white (objects), by a given threshold. This threshold value can be given directly by the user or automatically determined by the image histogram minima, maximum slope or minimum slope. Next, small gaps (6x6 pixels) were filled on the objects, and small debris (3x3 pixels or less) was subsequently removed. Finally, the objects cut off by the image boundaries were removed, and the morphological characterization of the macroflocs was performed.

Filaments. First, the original image is divided by the background image (background elimination). A bottom hat filter is then applied to the image in order to retrieve the filaments from the image and a comparison of the remaining image to the background one is performed. The flocs are seemingly identified and removed from the image. The filaments are further enhanced and finally segmented by a two-step procedure using a chosen threshold and percentile. Finally, in order to remove debris, all the objects smaller than 32 pixels or with a gyration radius bellow 1.2 were deleted and, the morphological characterization of the filaments was performed. Biomass samples must be diluted for image analysis, but an optimal value should be found. Excessive dilution increases the number of objects detected and if the dilution is insufficient the object will be overlaid. Experiments were done in order to determine the optimal dilution value for filaments, micro and macroflocs. The optimal dilution value was determined as the lowest dilution that enabled the maximum % of recognition. The % of recognition is the ratio between the area of objects that are completely inside the image and the total area of objects in the image, including those that are in the boundaries and are not completely recognized.

EGSB operation

The reactor was operated continuously for a 400 days period. The operation of the EGSB reactor was divided into three operating stages (ST1-day 0 to79, ST2-day 110 to 207 and ST3-day 237 to 400. During the whole continuous operation period the reactor was fed with a concentrated synthetic feed, which was diluted online with tap water in order to maintain the feed COD at a constant value of 1500 mgCOD/L. In order to ensure the growth of acidogenic populations, during the first stages (ST1 and ST2), the feed was composed of 60% COD as glucose and 40% COD as volatile fatty acids (acetic and propionic acid in a COD ratio of 70:30). At day 326 the glucose was lowered to 30% COD. The 70% remaining VFA-COD was kept as before. Sodium bicarbonate and calcium carbonate were used as alkalinity source. Micro and macronutrients were added as described elsewhere (Alves et al, 2001). A low acetate concentration was chosen, in order to favour the selection of Methanosaeta-like organisms (Wiegant and de Man, 1985). In these first two stages, the superficial up-flow velocity was kept below 4m/h to prevent the washout of growing granulation nuclei, but also high enough to maintain a mixing pattern that promoted the contact

between the aggregates. During stage ST3, the reactor was operated at organic loading rates (OLR) up to 6.5 kg COD/m^3.d) and higher upflow liquid velocities (up to 6 m/h) in order to prevent possible growth limitation of the biomass and to maintain a strong hydraulic selection pressure over the biomass, respectively.

RESULTS AND DISCUSSION

EGSB operation

During the whole operation period, the EGSB reactor has showed regular stability in the removal efficiency, about 95% COD (Figure 3a). The increase in the upflow liquid velocity at day 250 did not affect significantly the SMA of the biomass (Figure 3b and 3c). This could point out that the solely reduction in the external diffusion limitations was not enough to improve the SMA of the sludge. At day 295 the OLR value was increased to 4 kgCOD/m^3.d and this induced a considerably increase in all the SMA measured at day 326. As OLR was the sole parameter changed after day 250 and before that day, this increase in the specific activity can be attributed to the higher load of substrate supply. The SMA on acetate, propionate and butyrate increased steadily between days 287 and 400. The hydrogenophilic activity decreased significantly between days 358 and 400.

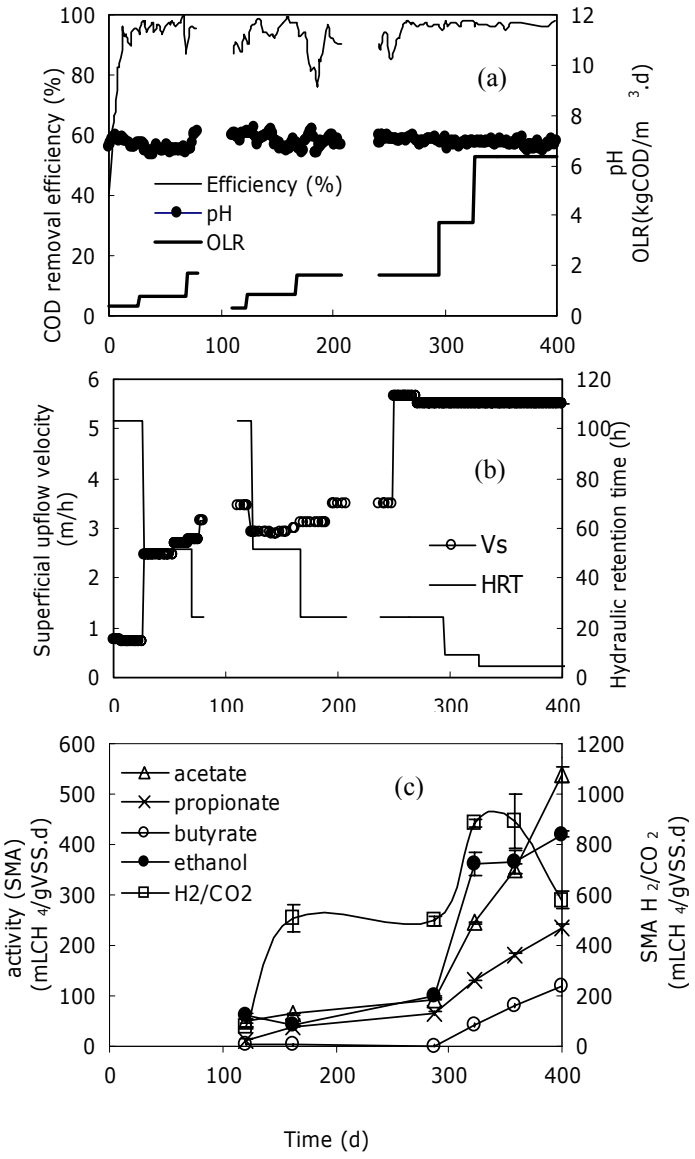

Figure 3. Time course of COD removal efficiency, pH and OLR (a), Vs and HRT (b), and SMA (c).

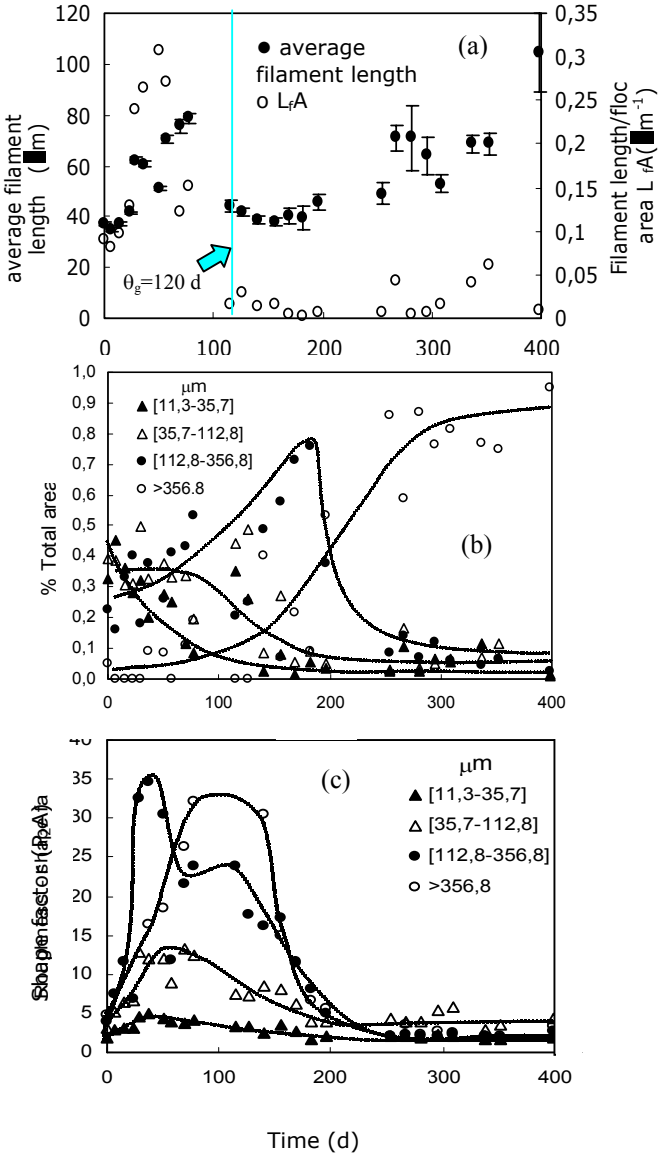

Figure 4. Time course of ratio of filament length to total floc area (L_fA) and average filament length (a), % of total area of different size ranges (b), and shape factor P_2A (c).

Image analysis

Among all the morphological parameters calculated by image analysis, some of them gave good information on dynamic changes on aggregates morphology and size, but others were less sensitive since their values remained almost constant during all the operation. This was the case of the several fractal dimensions determined. In fact, although this morphological parameter was already used to differentiate between flocs and granules (Bellouti *et al.,* 1997), in this work it did not allow the monitoring of granulation dynamics, probably because the parameters involved in fractal dimension definitions changed in a proportional way. Instead of using fractal dimensions, the new defined L_fA factor and the P_2A shape factor gave more accurate insights of the morphological changes along the process (Figure 4a and c). One important parameter of the granulation process is the granulation time (θ_g), which is totally dependent on the granule definition. We understand it as a structure achieved after nucleation and aggregation stages that should maintain a stable morphology, but that also evolves in time due to the dynamics between growth, erosion and fragmentation. The image analysis data revealed an evolution in the morphology of the aggregates that could be used to identify θ_g in terms of the length of filament to total floc area ratio (L_fA) (Figure 4a). This Figure presents also the average filaments length along the process. The changes in the L_fA value are related to the balance between the free filaments (as defined in Figure 2) and the total floc area, both important descriptors of the granule morphology. In the first stages of granulation an aggregate grows on the matrix of filamentous nuclei, showing small projected areas and leading to big values of L_fA (Figure 5a). A mature aggregate shows a defined surface with more or less short filaments, leading to small L_fA values (Figure 5b).

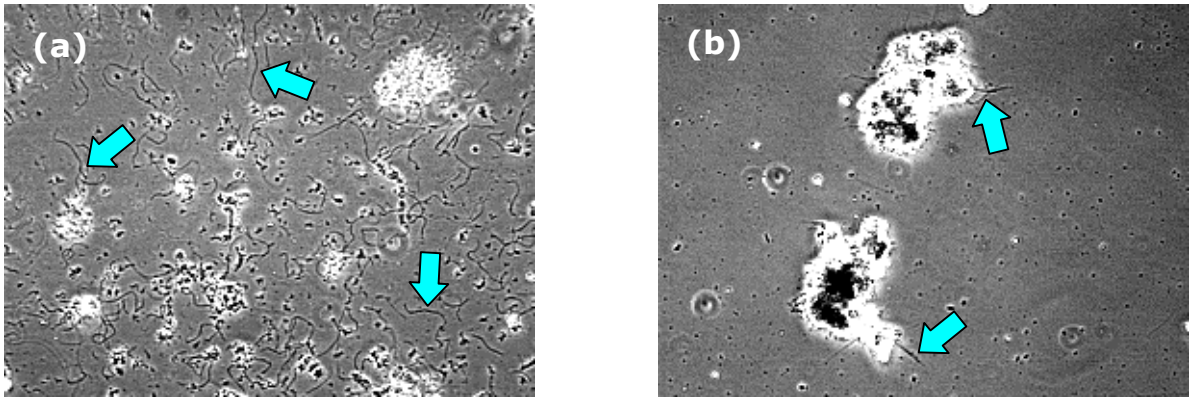

Figure 5 – Image of samples from day 70 (a) and from day 200 (b). Dilution: 20x, phase contrast and 100x magnification. Arrows indicate free filaments like the ones used for the calculation of L_fA.

In this way, and regardless the intrinsic evolution of the both parameters involved in the definition of L_fA, there are two moments that have to be taken into account: the time where the L_fA values begin to decrease, indicating a change in the filaments growth possibly due to washout and/or growth in diameter and consistency of the flocs. The second moment is where the L_fA stops decreasing and become stable, revealing that the growing granule has achieved a state where the above mentioned growth dynamic is established. This last instance could be pointed out as θ_g (120 days). The dynamics of aggregation and granules maturation in the reactor may also be described by the percentage of total area of each size range (Figure 4b). Although it was observed that the highest fraction in number concerned the smaller size range (more than 80% - data not shown), Figure 4b shows that after θ_g, the aggregates in the range size [112.8-356.8 μm] kept growing, as granules (indicated by the increase of its percentage in total area). The changes in the values of the other size ranges suggest that the medium aggregates are growing into big ones, and that the small aggregates are growing into medium ones. Near day 200 the percentage in total area in the range size of [112.8-356.8] decreased considerably together with an increase in the % of total area of the aggregates larger than 356.8 mm. This suggests that aggregates in this range are growing to the big ones, but this growth was not compensated by the increase in size of the smaller size ranges. From θ_g, on the aggregates larger than 112.8 mm became the dominant fraction of the total projected area. Considering that this area is

directly related to the biomass quantity, this clearly evidences that after θ_g, most of the sludge became structured into large aggregates.

The rise in granules size was also accompanied by a change in their shape. The P_2A factor showed a noticeable evolution, denoting the changes suffered by the shape of the granules. From the factor definition (Eq. 1) it can be seen that the more the roundness of the projected area of a particle, the nearer its P_2A value to the unit. Figure 4c shows that the shape of the projected area of the granules in all the particle size ranges tended to a more round form along the granulation process. Before θ_g the shape of the projected area of the aggregates corresponding to the two bigger equivalent diameter sizes ([112.8-356.8] and [>356.8]) was far from circular, probably due to the irregular shape of the early filamentous web structures. After θ_g the value of P_2A decreased markedly, suggesting that the growth around the formed structures is affected also by an abrasive and/or erosive effect due to the velocity of the upflow liquid, the collisions with other forming granules and the evolution of the gas loading rate.

The size distribution within the size range of the largest aggregates [>356.8 μm] can be seen in Figures 6a and 6b for days 70 and 200, respectively. There is a noticeable increase of the average equivalent diameter from samples taken in the early stage (Figure 6a) and the ones taken later on (Figure 6b). About 5% of the granules had equivalent diameters around 2500 μm.

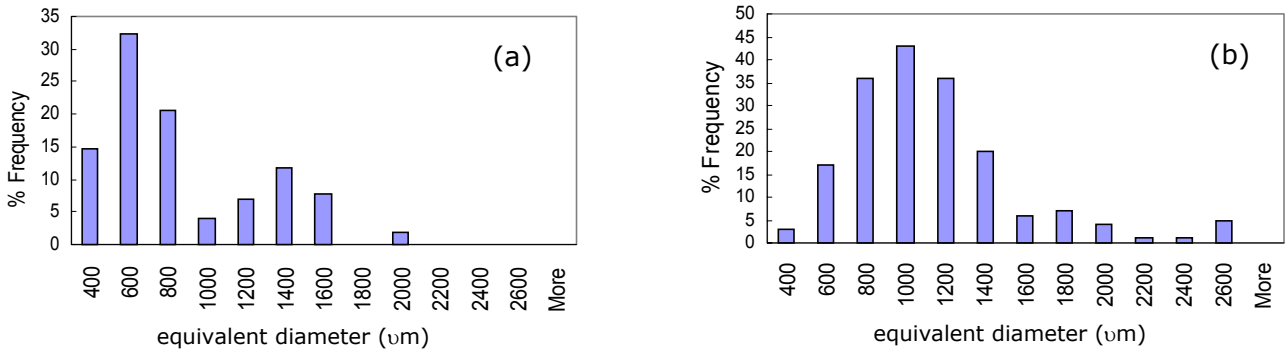

Figure 6 – Histograms show the corresponding size distribution for particles with equivalent diameter higher than 356.8 μm. Samples were taken on day 250 (a) and 400 (b).

The data showed in Figure 6a and 6b is, notwithstanding its accuracy in the defined ranges, limited by the hardware used during the image acquisition. Particles with one of its dimensions larger than the field of view of the microscope were not considered by the program, neither in the count of total particles, nor in parameters like the equivalent diameter or the P_2A factor. As a matter of fact there was a fraction of granules with diameters higher than 3 mm, but it only represented a small fraction of the total biomass. These large particles were only considered in the L_fA and in the total floc area calculations.

SEM

The EGSB inoculum was completely dispersed biomass mixed with other fine solids present in the sludge. SEM observations clearly showed the development of aggregates to a granular form (Figures 7a to 7f). They have growth not only to simple aggregates, but to homogeneous and complex structures. Figure 7a shows the heterogeneity of the inoculum, consisting mainly of residual mineral fragments, and a diversity of recognizable shaped microorganisms. Figure 7b shows a remarkable change in biomass composition. It can be clearly distinguished the filamentous nature of the bacteria and the intricate matrix that is being formed to became later on the base of the granules structure. The compaction of this structure can be seen in Figure 7c were the previously formed matrix shows less dead spaces and also the incorporation of calcium and calcium bicarbonate crystals. The energy dispersion spectrography analysis (data not shown) showed that along the operation, the Calcium content of the growing aggregates increased, in both crystalline and carbonate forms. This mineralization phenomenon could have contributed to the settling characteristics of the granules which in the final period reached settling velocities over 100 m/h (data not shown), and to the development of a pH protected microenvironment. High OLR values and the rise in the VFA profile could have promoted acidification, but the pH and the COD removal efficiencies remained stable (Figure 3a). Figure 7d shows one of the granules in their initial stages of aggregation. Figure 7e shows a granule in a later state of aggregation. As can be seen, the roughness of the surface has clearly decreased and the shape of the granules

is changing to become round-shaped. In this stage, the incorporation of calcium and calcium carbonate was intense. Mature granules from the latest period of operation of the reactor showed a dense and homogeneous structure (Figure 7f).

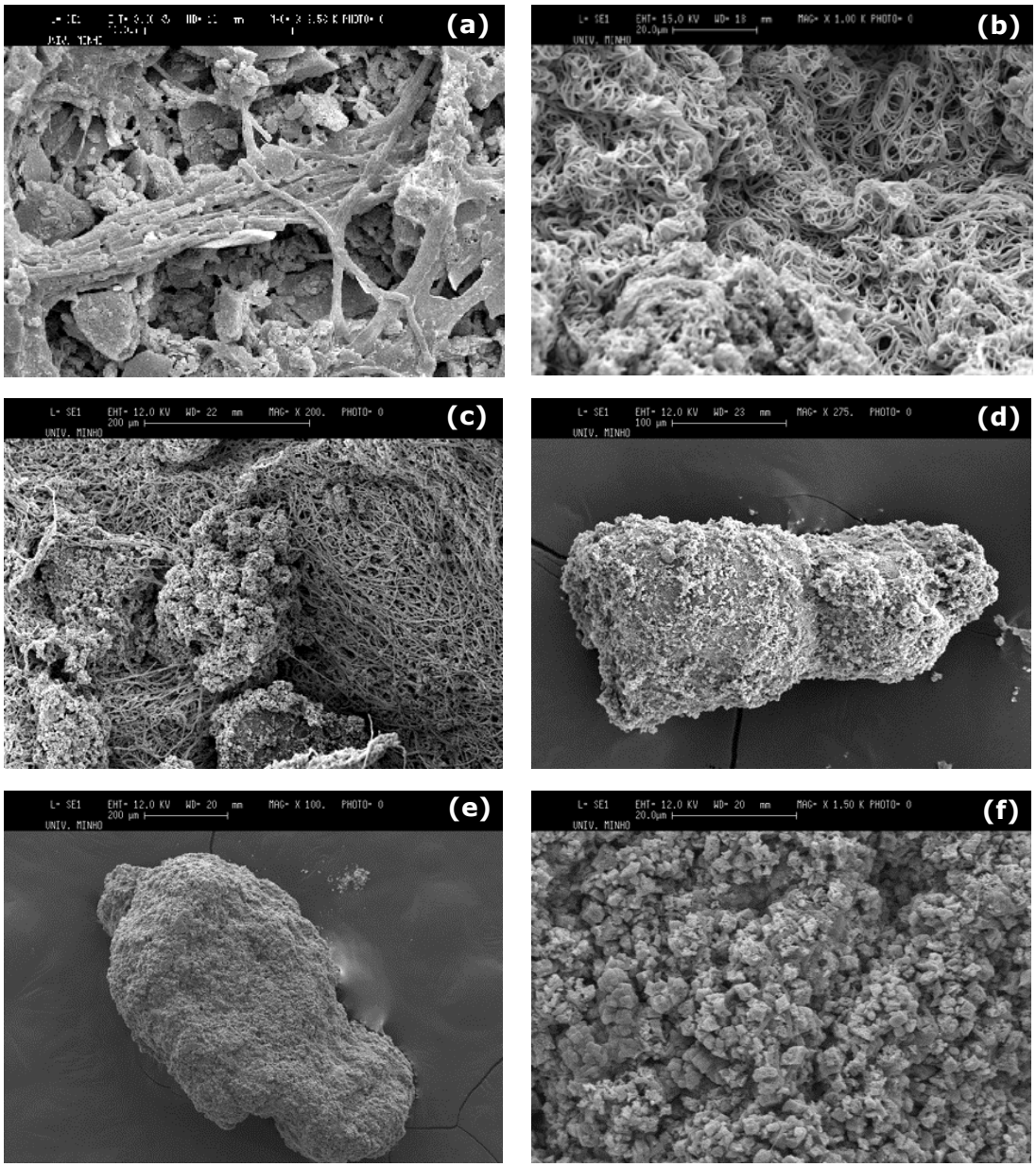

Figure 7: SEM photographs from different stages of the operation period: inoculum (a), early formed web structure (b), middle-aged granule showing dense matrix (c), first granules formed (d), later granules showing smoothed surface (e) and complex and homogeneous mineralized granule structure (f)

CONCLUSIONS

1. A highly active granular sludge with good settling characteristics was developed under the hydraulic and organic conditions maintained during the operation.
2. All along the operation of the EGSB reactor, the specific methanogenic activity (SMA) of three different trophic groups (syntrophic, acetoclastic and hydrogenotrophic bacteria) has raised over their initial values. The changes in the SMA for each group were different, indicating that each group reacts in a different manner to the applied environmental conditions.
3. Both the hydraulic loading rate and the composition of the feed on their own were less significant to the increase in the SMA of the sludge than the increase in the organic loading rate.
4. A new image analysis parameter defined as the ratio filament length/total floc (L_fA) area has proven to be sensitive to changes in the aggregation status of the anaerobic sludge.

5. L_fA allowed the recognition of the granulation time, based upon a given granule definition. P_2A was useful parameter accompanying L_fA monitoring the granulation process, and supporting the selection of L_fA as a granulation time monitoring parameter.
6. The SEM observations demonstrated that the formed structures present enough homogeneity, consistency and complexity to be described as granules.
7. Image analysis proved to be a suitable technique that allows the monitorization of the dynamics of changes of the granules sizes and a better understanding of the process dynamics.

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