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Use of image analysis in the study of competition between filamentous and non-filamentous bacteria

Edgardo M. Contreras[a], Leda Giannuzzi[a], Noemí E. Zaritzky[a,b,*]

[a] *Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA), CONICET—Facultad Ciencias Exactas, Universidad Nacional de La Plata, 47 y 116, La Plata 1900, Argentina*

[b] *Facultad de Ingeniería, Universidad Nacional de La Plata, 47 y 116, La Plata 1900, Argentina*

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Abstract

One of the most common problems in the operation of activated sludge plants is the inability to separate solids from treated effluents in the clarifiers caused by the overgrowth of filamentous micro-organisms (FM) with respect to floc-forming bacteria. In order to develop kinetic models that help to predict bulking events, growth kinetics of the FMs and non-filamentous bacteria (NFB) should be known. This paper addresses the competition of a FM and a NFB in a continuous stirred tank reactor. Experimental observations of the effect of the dilution rate on the composition of the mixed culture were compared to simulated results. Image analysis was used to measure NFB and FM fractions in order to evaluate the proposed mathematical model. Experimental results and numerical simulations showed that low D values favored the growth of FM; on the contrary, when high D values were applied a rapid overgrowth of the NFB were observed. Thus, high D values favored the growth of NFB minimizing the risk of filamentous bulking.

Keywords: Image analysis; Mathematical modeling; Filamentous bulking; *Sphaerotilus natans*; Floc-forming bacteria

1. Introduction

One of the most common problems in the operation of activated sludge plants (known as filamentous bulking) is the inability to separate solids from treated effluents in the clarifiers caused by excessive growth of filamentous micro-organisms (FMs). Pujol and Canler [1] found that difficulties with the settler due to FMs appear at least once a year in 70% of these plants. In England, filamentous bulking appears in 63% of the domestic wastewater treatment plants; however, this percentage in France is only 25% [2]. Recently, Eikelboom and Geurkink [3] analyzed 81 samples from 70 wastewater treatment plants (WWTP) from 70 companies in 11 types of industries in four countries (The Netherlands, Denmark, Germany, Italy); in 60% of the studied cases filamentous bulking was detected.

Bulking sludges are produced by an imbalance between floc-forming and filamentous bacteria, preventing the formation of well settling sludge flocs. Bulking by the FM *Sphaerotilus natans* has been attributed to several factors such as low dissolved oxygen in the aeration basin, treatment of wastes with high carbon to nitrogen (C:N) ratios and phosphorus limitation [4–6]. However, the occurrence of bulking by *S. natans* has been reported in potato processing wastewater treatment plants [7], meat and poultry wastewater [8] and fruit processing wastewater and brewery [9] in which the ratio C:N is variable.

*Corresponding author. Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA), CONICET—Facultad Ciencias Exactas, Universidad Nacional de La Plata, 47 y 116, La Plata 1900, Argentina. Tel.: +54-221-4254853; fax: +54-221-4249287.

E-mail address: zaritzky@volta.ing.unlp.edu.ar (N.E. Zaritzky).

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Nomenclature

A	total field area (pixels)
a_b	mean particle area (pixels/particle)
c	coefficient in Eq. (5) ($L\,(g\,COD)^{-1}$)
D	dilution rate (h^{-1})
f_a	image analysis-based fraction (dimensionless)
F_b	ratio between the occupied area by bacteria and the total area (dimensionless)
$f_{X1,2}$	biomass concentration-based fraction of micro-organisms 1, 2 (dimensionless)
h	slope ratio (dimensionless)
k_d	endogenous decay constant (h^{-1})
K_S	Monod saturation constant ($mg\,COD\,L^{-1}$)
n	number of particles within the examined field (particles)
Rg	reduced gyration radius (dimensionless)
Ro	roundness (dimensionless)
S	outlet substrate concentration ($mg\,COD\,L^{-1}$)
S_o	inlet substrate concentration ($mg\,COD\,L^{-1}$)
X	Biomass concentration ($mg\,COD\,L^{-1}$)
$Y^T_{X/S}$	true biomass yield ($mg\,COD\,(mg\,COD)^{-1}$)
μ_{max}	maximum specific growth rate (h^{-1})

Biological methods to control filamentous bulking comprise preventive actions to suppress FMs and selectively support the growth of floc-forming bacteria [2]. In order to avoid the occurrence of filamentous bulking a selector can be used. In the selector, high substrate concentrations and short hydraulic retention times (HRT) are achieved which favor the growth of floc-forming bacteria. The rationale behind this method is summarized in the kinetic selection theory [10]; floc-forming bacteria characterized by higher values of Monod equation constants K_S and μ_{max} than FM are favored at high substrate concentrations and low HRT. In this way, it is possible to control the overproduction of FM under these conditions.

Several mathematical models were developed in order to predict filamentous bulking; a brief literature review on this topic can be found in [11]. Cenens et al. [11] demonstrate that co-existence of filamentous and floc-forming bacteria competing for a single substrate growing in a continuous stirred tank reactor (CSTR) or in CSTR with an ideal settler and biomass recycling (e.g. an activated sludge system) is generically not possible. In the model developed by Hermanowicz [12], dissolved oxygen concentration was dynamically altered in order to prevent washout of one of the species. Kappeler and Gujer [13] developed a mathematical model that included three common FMs and one floc-forming micro-organism; this model was extended to describe facultative-aerobic floc-forming bacteria, obligate aerobic FM and nitrifying micro-organisms [14]. Recently, Cenens et al. [15] developed a mathematical model based on the kinetic selection and filamentous backbone theory [16] that predicts the coexistence of both FM and floc-forming bacteria for a wide range of dilution rates; this model considers that FMs are incorporated to the flocs decreasing its concentration. All these mathematical models include coefficients that describe the growth of microorganisms (e.g. μ_{max}, K_S, k_d, $Y^T_{X/S}$) which have to be determined using pure cultures.

In order to verify the accuracy of the models, the fraction of floc-forming bacteria and FM in mixed cultures should be measured for a wide range of operating conditions. Traditionally, biomass concentration was determined using lumped parameters (e.g. VSS, COD) that did not allow the calculation of floc-forming bacteria and FM fractions in mixed cultures. In this sense, the lack of a simple and accurate technique to determine microbial composition is one of the main problems when a mixed culture is analyzed [17]. In the last years, the image analysis (IA) has been widely used in all kind of applications due to the decreasing in the price/quality ratio of the IA systems [18]. IA was used to quantify different bacterial properties in both suspended and immobilized pure cultures [19–21]. Miyanaga et al. [22] applied IA to quantify pigments in vegetal cells. Cheng et al. [23] also used IA to enumerate marine viruses in various types of samples. Grijspeerdt and Verstraete [24] used low magnification microscopy combined with IA to estimate settling properties and biomass concentration in activated sludge samples. Other examples of IA applications in microbiological systems were reported by Jeison and Chamy [25], Cenens et al. [26] and da Motta et al. [27].

The objectives of the present work were: (a) to analyze the effect of the dilution rate in a CSTR on the population dynamics of a mixed culture of a filamentous (*S. natans*) and a non-filamentous bacteria (NFB, strain E932), typical micro-organisms of an activated sludge reactor; (b) to apply a quantitative technique based on IA to measure relative concentrations of these bacteria in mixed cultures; and (c) to propose a mathematical model that interprets the experimental results.

2. Materials and methods

2.1. Micro-organisms and culture conditions

The FM *S. natans* ATCC ♯29329 was obtained from the American Type Culture Collection. The NFB, strain E932, was isolated from a lab-scale-activated sludge treatment plant, fed with a model effluent from a potato processing industry. Strain E932 was identified as *Acinetobacter anitratus* using the biochemical test system

Sensident-E (Merck). The culture medium was developed in a previous work [28] and contains citrate (limiting substrate) and ammonia as carbon and nitrogen sources, respectively. Pure and mixed cultures of both strains were performed in a chemostat apparatus at 30°C, pH = 7.0 with dissolved oxygen concentration above 2 mgO_2 l^{-1}. Samples were drawn to measure the total chemical oxygen demand (COD_T). A portion was filtered using Millipore HA (0.45 μm) membranes and substrate (S) was determined as soluble chemical oxygen demand (COD_S). Biomass concentration (X) was calculated as the difference between COD_T and COD_S [29]. COD measurements were performed with a commercial kit (Hach Corp., Loveland, USA).

2.2. Effect of dilution rate on the composition of mixed cultures of FM and NFB

The effect of controlled step changes of the dilution rate (D) on mixed cultures of *S. natans* (FM) and strain E932 (NFB) was studied in the chemostat apparatus described in the previous section. Tested D values ranged between 0.03 and 0.39 h^{-1}. An IA procedure was applied to quantify the population changes due to modifications in the dilution rate values.

2.3. Image analysis

Samples were prepared by placing 10 μL of pure or mixed cultures on microscope slides. Slides were observed using a Leica DMLB microscope; phase contrast illumination system (1000×) was used. Images were acquired using a Leica DC100 camera via a Leica DC100 Version 2.51 frame-grabber system which allowed capture 768 × 582 pixels JEPG images coded on True Color, 300 pixels pda^{-1} resolution. Images were transformed to TIFF format (256 grey-scale levels) with Microsoft Photo Editor 3.0 and they were analyzed with the Global Lab Image 2.10 software. In order to identify bacteria (particles) from the background, grey-scale images were thresholded. Since particles were darker than the background, pixels with grey level lower than a critical threshold (t_C) were considered as particle pixels. For each digital image, t_C was calculated as the grey level value that corresponded to the maximum of the grey level histogram second derivative [26]. In order to filter coarse debris material (artifacts and other material that may interfere with the analysis), only particles larger than 10 pixels were considered [30]. Once t_C was selected, the IA software calculated the values a_b (mean particle area) and n (number of particles) within the analyzed field. When mixed cultures were analyzed, filamentous and floc-forming bacteria were manually classified and the values of a_b and n corresponding to each class were calculated. In all cases, approximately 200–1000 particles per image were detected. Three slides per sample were prepared and 3–5 images per slide were randomly acquired; results were expressed as the mean of all analyzed fields.

In order to classify particles as FM or NFB the following shape parameters were tested:

Roundness (Ro): Ro was defined as the ratio between the object area (A) to the area of a circle with a perimeter equal to the object perimeter (P). For a circle Ro = 1 and it decreases as more elongated the object is. Ro was calculated as follows:

$$\mathrm{Ro} = \frac{4\pi A}{P^2}. \tag{1}$$

Reduced gyration radius (*Rg*): Rg was defined as the ratio between the object gyration radius to the radius of a circle with area equal to the object area. For a circle Rg = 0.707 and it increases as more elongated the object is. Rg was calculated as follows:

$$\mathrm{Rg} = \frac{\sqrt{M_{x2} + M_{y2}}}{\sqrt{N/\pi}}, \tag{2}$$

where N is the object area (pixels) and M_{x2} and M_{y2} are the central second moments with respect to x-axis and y-axis of the image respectively:

$$M_{x2} = \frac{1}{N}\sum_{i=1}^{N}(x_i - M_{x1})^2 \tag{3}$$

$$M_{y2} = \frac{1}{N}\sum_{i=1}^{N}(y_i - M_{y1})^2 \tag{4}$$

with $M_{x1} = 1/N\sum_{i=1}^{N} x_i^2$, $M_{y1} = 1/N\sum_{i=1}^{N} y_i^2$, where (x_i, y_i) is the position of each pixel that belongs to the analyzed particle.

2.4. Biomass concentration measurements in pure and mixed cultures by IA

The IA method to measure biomass concentration was based on the correlation of the ratio between the occupied area by bacteria and the total examined area (F_b) with the real biomass concentration (X) [31]:

$$F_b = cX, \tag{5}$$

where the coefficient c depends on each micro-organism.

F_b values were calculated as follows:

$$F_b = \frac{na_b}{A}, \tag{6}$$

where a_b is the mean particle area (pixel/particle), n the number of particles within the field, and A the total field area.

In the particular case of a mixed culture with only two micro-organisms the following fractions were defined:

$$f_{X1} = \frac{X_1}{X_1 + X_2} = \frac{X_1}{X_T}, \tag{7}$$

$$f_{a1} = \frac{F_{b1}}{F_{b1} + F_{b2}}, \quad (8)$$

where f_{X1} is the biomass concentration-based fraction of FM, f_{a1} the IA-based fraction of FM, and X_T the total biomass concentration. Both fractions are related by the following expression:

$$f_{a1} = \frac{hf_{X1}}{f_{X1}(h-1)+1}, \quad (9)$$

where $h = c_1/c_2$; c_1 and c_2 are the coefficients of Eq. (5) corresponding to FM and NFB, respectively. Calibration curves of F_b as a function of biomass concentration (X) were made by progressive dilution of pure culture samples of *S. natans* (FM) or strain E932 (NFB) to determine the coefficients c (Eq. (5)) corresponding to each micro-organism. Mixed culture model systems with known fractions of each micro-organism (f_{X1},f_{X2}) were prepared by mixing different volumes of pure cultures of FM and NFB having known biomass concentrations. For each mixture, the corresponding f_{a1} value was measured and coefficient h (Eq. (9)) was determined by non-linear regression analysis using the software Sigma Plot 2.0.

2.5. Numerical simulation of the dilution rate effect on the concentration of FM and non-filamentous microorganisms

In order to model the experimental results, unsteady state mass balance equations corresponding to the simultaneous growth of FM and NFB competing for a single limiting substrate in a CSTR were solved. Numerical solutions of the coupled first-order differential equations were obtained using a fourth-order Runge–Kutta method (Sigma Plot 2.0). Kinetic and stoichiometric constants corresponding to both micro-organisms were determined in a previous work [32] and were used in the numerical solution.

3. Results and discussion

3.1. Calibration of the digital IA method to determine FM and NFB biomass concentrations

Fig. 1a shows a micrograph of a typical mixed culture of the FM *S. natans* and the NFB strain E932. *S. natans* was observed as relatively long filaments ($10-100 \times 1.3-1.6\,\mu m$) composed of round-ended, rod-shaped cells contained in a clear tightly fitting sheath [28]. Strain E932 (*A. anitratus*) was observed as little rods ($0.9-1.6 \times 1.5-2.5\,\mu m$) becoming shpericals in the stationary phase of growth. Juni [33] reported that *Acinetobacter* commonly occur in pairs and also in chains of variable length; however, only single cells and occasionally pairs were observed in this work.

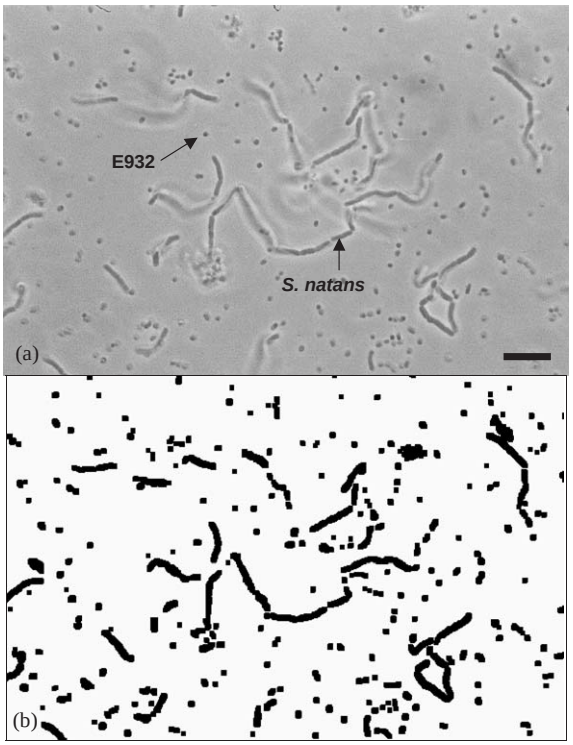

Fig. 1. (a) Typical micrograph of a mixed culture composed by the FM *S. natans* and the non-filamentous strain E932. Bar corresponds to 10 μm. (b) Binarization of Fig. 1a, threshole used = 131.

The threshold corresponding to the image in Fig. 1a was calculated as the maximum of the grey level histogram second derivative [26]; in this case a value of 131 was found. Fig. 1b shows the binary image of Fig. 1a using the obtained threshold; the binarization was performed with the Optimas 4.0 software. The observed differences in size and shape suggested that IA could be used to quantify both FM and NFB in mixed cultures.

In order to obtain statistically significant results, the minimal number of images to capture was determined following a similar procedure developed by Drouin et al. [19]. Five samples of a pure culture of *S. natans* were prepared and 10 images per sample were randomly captured; Fig. 2 shows that similar F_b values were obtained in the five analyzed samples. Fig. 3 shows that mean value and 95% confidence interval (IC 95%) reached approximately constant values when the number of analyzed images was greater than 10. Therefore, 100 sets of 10 images were randomly built from the initial series of 50 images. Fig. 4 shows that 91 sets were within the $\pm 15\%$ band around the mean value calculated on 50 images; in addition, the error was

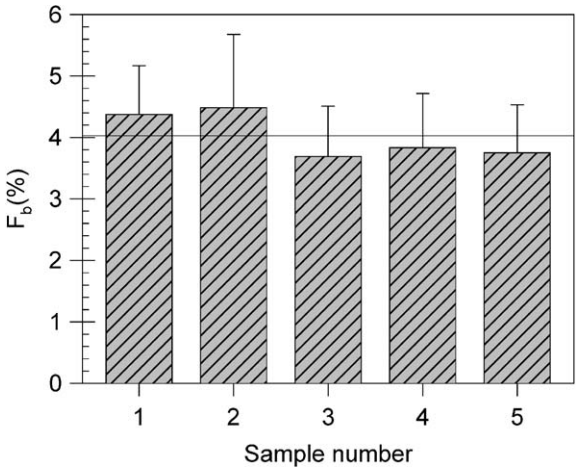

Fig. 2. Ratio between the occupied area by bacteria and the total examined area (F_b). Results correspond to five samples and 10 images per sample were acquired. Bars indicate the 95% confidence interval), (—) Overall F_b mean value.

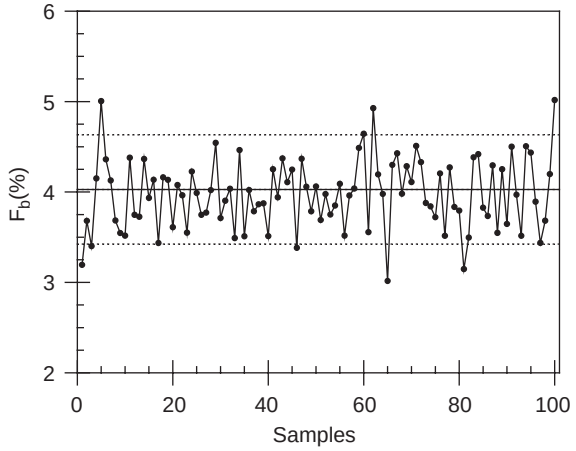

Fig. 4. Statistical validation by random selection of groups of 10 images out of 50. (—) Overall F_b mean value, (- - -) ±15% band around the mean value.

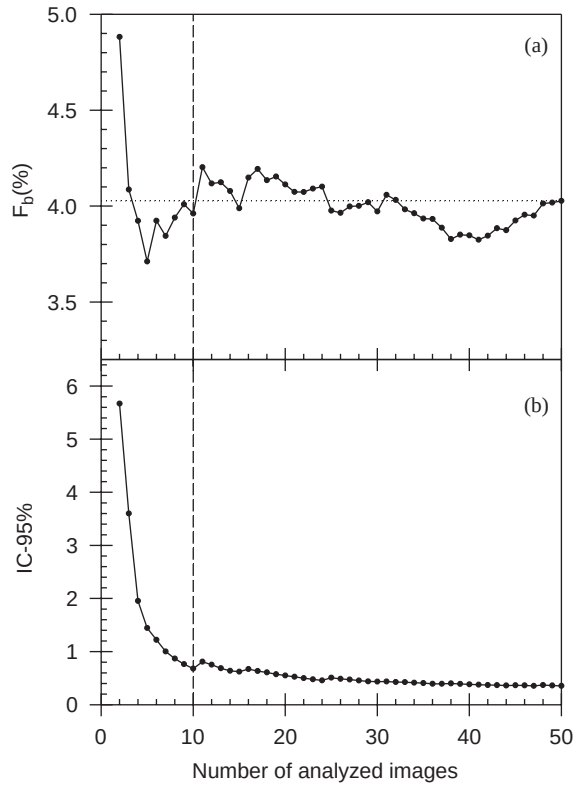

Fig. 3. (a) Ratio between the occupied area by bacteria and the total examined area (F_b) and (b) 95% confidence interval (IC95%) as a function of the number of analyzed images. (- - -) Overall F_b mean value.

always lower than 25%. Based on these results, 10 images per sample were considered enough to obtain statistically significant results. Since there were at least 200 bacteria per image, the total number of analyzed particles were more than 2000.

Drouin et al. [19] studied the differentiation of *Sreptomyces ambofaciens* using IA; these authors reported a minimum of 40 processed images (each image containing generally one filament) to obtain statistically relevant results. Grijspeerdt and Verstraete [24] analyzed the morphology of activated sludge finding that at least 150 objects need to be processed. Spicer and Pratsinis [34] studied the flocculation of polystyrene particles; these authors counted a minimum of 500 particles per sample during the determination of the particle size distribution. Jenné et al. [35] developed a fully automatic IA method for recognizing flocs and filaments in an activated sludge sample; they reported that a minimum of 50 images were sufficient for representative quantification of flocs and filaments. However, these authors did not report the mean number of particles per image; therefore, no estimation of the number of analyzed objects can be made.

3.1.1. IA method calibration with pure cultures

The calibration of the IA method to determine biomass concentration (X) was performed measuring F_b as a function of X in samples of pure cultures of *S. natans* and strain E932. In both cases, a satisfactory linear correlation between X and F_b was found (Fig. 5). From the slopes of the linear regression, coefficients c (Eq. (5)) were calculated. Obtained values were: $c_1 = 3.01(\pm 0.04)$ L(g COD)$^{-1}$ for *S. natans*, $c_2 = 3.63(\pm 0.08)$ L(g COD)$^{-1}$ for strain E932 being the slope ratio $h = c_1/c_2 = 0.83(\pm 0.02)$.

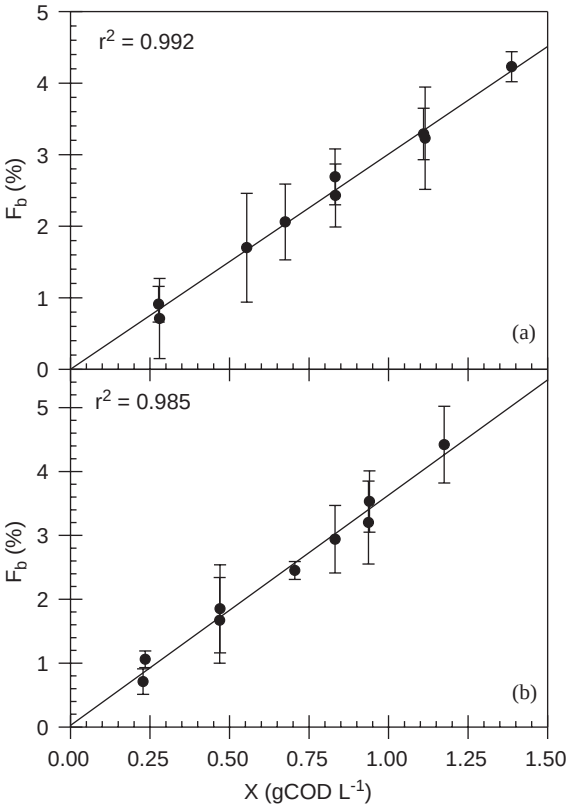

Fig. 5. Correlation between F_b (ratio of the occupied area by bacteria and the total examined area) with X (real biomass concentration) in pure cultures of (a) *S. natans*, (b) strain E932. Bars indicate the 95% confidence interval.

3.1.2. IA method validation with mixed cultures

The IA method calibrated with pure cultures of each micro-organism was validated with mixed cultures of *S. natans* and the strain E932. Mixed culture model systems with known *S. natans* and strain E932 biomass fractions (f_{X1}, f_{X2}; see Eq. (7)) were prepared and the corresponding area fraction (f_{a1}) was measured. In these experiments, manual classification (human observation) of the micro-organisms was performed. Eq. (9) was fitted to the data shown in Fig. 6 by non-linear regression analysis obtaining a slope ratio $h = 0.78(\pm 0.07)$ which was very close to the previously calculated one ($h = 0.83 \pm 0.02$) based on the analysis of pure cultures. Therefore, the proposed method of IA was used to determine biomass concentration of FM and NFB in mixed cultures.

3.1.3. Automated application of the IA method

One of the most important steps in the application of IA to analyze mixed cultures is the classification of the micro-organisms as either FM or NFB. In order to perform an automated micro-organisms classification

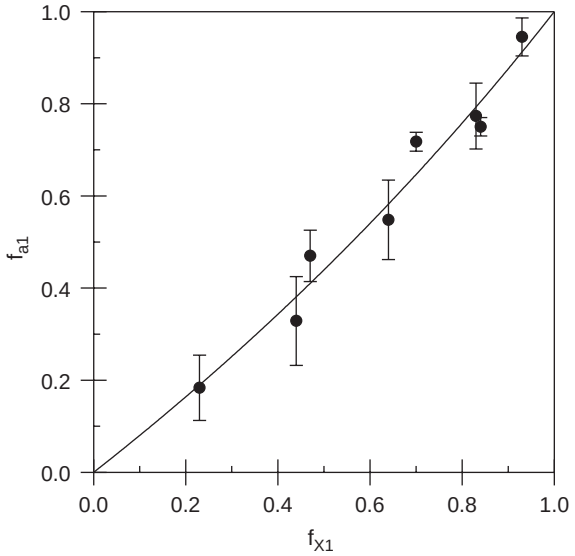

Fig. 6. *S. natans* fraction based on area measurements (f_{a1}) as a function of the fraction based on biomass concentration (f_{X1}), in mixed cultures of *S. natans* and strain E932. (•) experimental data (bars indicate the standard deviation), (—) Eq. (9).

two shape parameters were evaluated: roundness (Ro) and reduced gyration radius (Rg). Fig. 7 shows that the distribution of Ro and Rg corresponding to pure cultures of *S. natans* (FM) and strain E932 (NFB) were considerably different. Ro and Rg distributions corresponding to NFB were very sharp; they reached maximum values at Ro = 1 and Rg = 0.75, respectively, showing that NFB was almost a spherical bacteria. On the contrary, distributions of Ro and Rg for FM were wider due to the observed shape differences between both micro-organisms (Fig. 1). In this case, the position of the maximum values in the distribution corresponded to Ro = 0.16 and Rg = 1.50. Based on the obtained results, it can be stated that both shape parameters could be used to classify the micro-organisms as either FM or NFB.

The discriminating levels of Ro and Rg (Ro_C, Rg_C) were defined as the values that determine if a micro-organism is classified as FM or NFB. The best discriminating level for each shape parameter (Ro_C, Rg_C) was determined by the following procedure. For different mixed culture samples, *S. natans* area fraction using manual classification was calculated (f_{a1Man}). In addition, different Ro_C and Rg_C levels were selected and the corresponding *S. natans* area fraction was computed (f_{a1Auto}). The mean square error (MSE) corresponding to the tested discrimination levels for each parameter was calculated as $MSE = (f_{a1Man} - f_{a1Auto})^2/N$, where N was the number of analyzed images. The obtained results are shown in Fig. 8. For each shape parameter, a

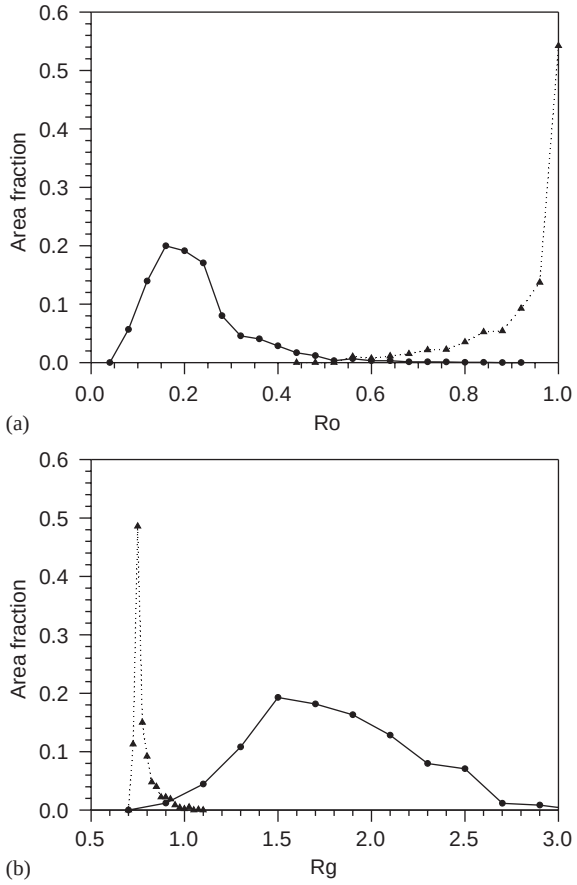

Fig. 7. Area-based histograms of (a) roundness, Ro, and (b) reduced gyration radius, Rg, corresponding to pure cultures of (—) *S. natans* and (- - -) strain E932.

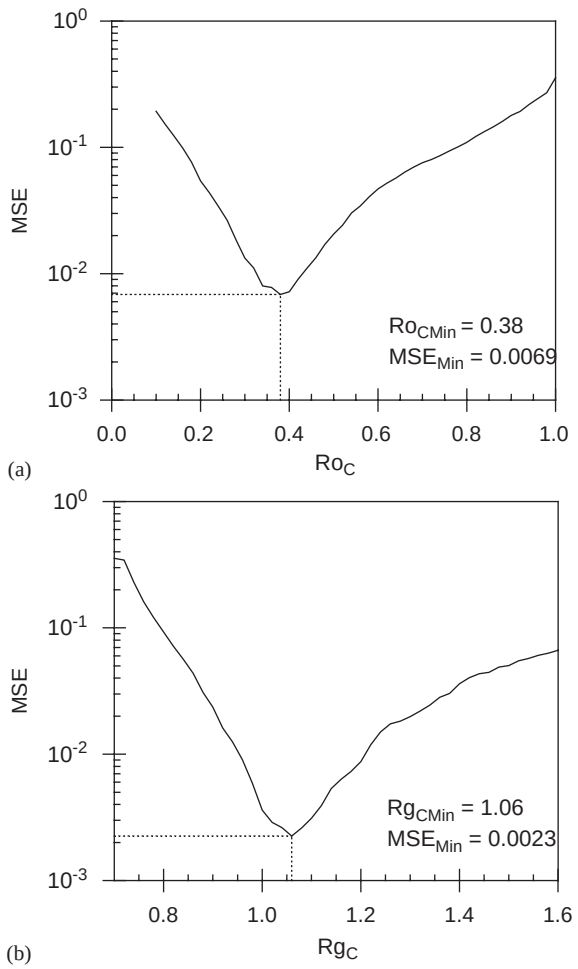

Fig. 8. Selection of the discriminating levels of (a) roundness (Ro_C) and (b) reduced gyration radius (Rg_C) by minimization of the MSE.

minimum MSE value was obtained and the corresponding values of $Ro_C = 0.38$ and $Rg_C = 1.06$ were considered as the best discriminating levels. Thus, all particles with $Ro < 0.38$ or $Rg > 1.06$ were considered as FMs.

Fig. 8 shows that the minimum value of MSE corresponding to Rg ($MSE_{Min} = 2.3 \times 10^{-3}$) was three times lower than for Ro ($MSE_{Min} = 6.9 \times 10^{-3}$). Fig. 9 shows that the correlation between f_{a1Man} and f_{a1Auto} using $Rg_C = 1.06$ ($r^2 = 0.9804$) was better than using $Ro_C = 0.38$ ($r^2 = 0.9001$). Therefore, although both shape parameters were suitable to classify particles as either FM or NFB, more accurate results were obtained using Rg than Ro. Cenens et al. [36] used computed generated objects to evaluate the robustness of five shape parameters finding that the Rg was the most suitable parameter to distinguish between flocs and filaments; in addition, these authors reported discriminating values close to those obtained in the present work ($Ro = 0.35$, $Rg = 1.10$).

The method proposed by Cenens et al. [26,36] includes several morphological operations (erosion-dilation) of the images in order to improve the identification (discrimination) of FMs from the flocs in the sludge. In our case, this operation was not necessary because a mixture of two micro-organisms that did not form flocs was analyzed. Flocs were not formed because of the high stirring velocity applied in the chemostat to achieve an adequate mixing of the fluid; besides, the reactor was operated without recycling the biomass and the cellular residence times were very low in comparison to the values in actual wastewater treatment plants. Satisfactory settling properties of activated sludge are commonly achieved above a minimum sludge age of 3 days [2]. In the present work, the minimum sludge age was 1.4 days ($D = 0.03\,h^{-1}$); however, these values permitted to accelerate the changes in the microbial flora composition.

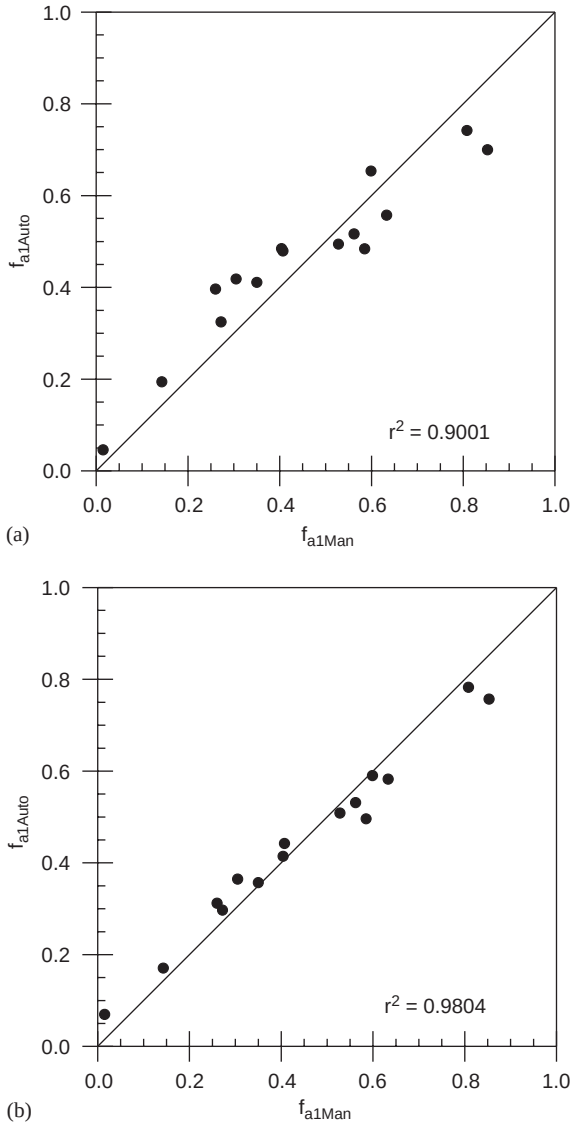

Fig. 9. Correlation between manual (f_{a1Man}) and automated (f_{a1Auto}) determination of *S. natans* fraction based on area measurements using (a) $Ro_C = 0.38$ and (b) $Rg_C = 1.06$. Straight lines indicate the perfect correlation.

3.2. Application of IA to the study of the dilution rate effect on FM and NFB mixed cultures

The IA method was applied to the study of the effect of controlled changes in D on the relative microbial composition in mixed cultures of *S. natans* (FM) and strain E932 (NFB). In these experiments, biomass concentration of each micro-organism was measured by IA combined with the total biomass concentration.

The experiment started with an FM pure culture grown in a chemostat working at $D = 0.15\,h^{-1}$. At $t = 0$ h., 500 mL was replaced by a same volume of an NFB overnight culture obtaining a mixed system with 60% (biomass-based percentage) of FM and 40% of NFB (Fig. 10). Simultaneously D was changed to $0.03\,h^{-1}$; in such condition, FM fraction increased up to 90–95% and NFB was washed out. After 135 h., D was increased to $0.39\,h^{-1}$ and a predominance of NFB (99%) was observed. At $t = 175$ h., D decreased to $0.05\,h^{-1}$; although NFB was still predominant, FM fraction increased up to 13% at $t = 260$ h. Finally, D was changed to $0.17\,h^{-1}$ at $t = 305$ h; in such condition, a predominance of NFB (90–98%) was again observed.

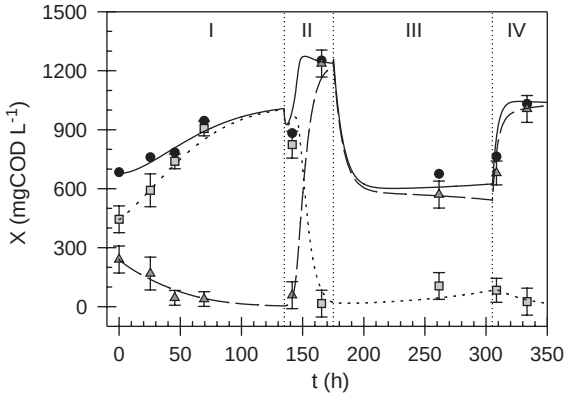

Fig. 10. Effect of dilution rate (D) on the population of filamentous and NFB in a mixed culture in a CSTR. (I) $D = 0.03\,h^{-1}$, (II) $D = 0.39\,h^{-1}$, (III) $D = 0.05\,h^{-1}$, (IV) $D = 0.17\,h^{-1}$. (●) Total biomass, (□) *S. natans*, (△) strain E932. Bars indicate the standard deviation. Lines correspond to the numerical simulation.

3.3. Numerical simulation of the effect of dilution rate on the composition of the mixed culture

In order to interpret the experimental results, the following mass balance equations describing the simultaneous growth of *S. natans* (FM) and strain E932 (NFB) growing on a common limiting substrate (citrate) in a CSTR were solved [24]:

$$\frac{dX_1}{dt} = \left(\mu_{max1}\frac{S}{K_{S1}+S} - k_{d1} - D\right)X_1, \tag{10}$$

$$\frac{dX_2}{dt} = \left(\mu_{max2}\frac{S}{K_{S2}+S} - k_{d2} - D\right)X_2, \tag{11}$$

$$\frac{dS}{dt} = D(S_0 - S) - \left(\mu_{max1}\frac{S}{K_{S1}+S}\right)\frac{X_1}{Y^T_{X/S1}} - \left(\mu_{max2}\frac{S}{K_{S2}+S}\right)\frac{X_2}{Y^T_{X/S2}}, \tag{12}$$

where subscripts 1 and 2 corresponds to *S. natans* (FM) and strain E932 (NFB), respectively.

In a previous work [32], the behavior of a mixed culture of the FM *S. natans* and the NFB strain E932 was analyzed. Based on the kinetic and stoichiometric coefficients determined in pure cultures, a critical dilution rate ($D_C = 0.185\,h^{-1}$) was found. When $D = D_c$ a co-existence of both micro-organisms under steady state conditions may be observed. However, when $D > D_c$ NFB may predominate over FM; therefore, this condition minimizes the risk of filamentous bulking. Fig. 10 shows that theoretical predictions (high values of D favored the growth of the NFB) agree with the observed qualitative behavior of the mixed culture.

The simultaneous numeical resolution of Eqs. (10)–(12) allowed to calculate the effect of D-controlled changes on FM and NFB biomass concentrations (X_1 and X_2, respectively). Kinetic and stoichiometric coefficients used in the simulation are the following [24]: $Y^T_{X/S1} = 0.575\,mg\ COD\,(mg\ COD)^{-1}$, $Y^T_{X/S2} = 0.642\,mg\ COD\,(mg\ COD)^{-1}$, $\mu_{max1} = 0.458\,h^{-1}$, $\mu_{max2} = 1.037\,h^{-1}$, $K_{S1} = 70\,mg\ COD\,L^{-1}$, $K_{S2} = 95\,mg\ COD\,L^{-1}$, $k_d = 0.01\,h^{-1}$, $k_d = 0.08\,h^{-1}$. Total biomass concentration (X_T) was calculated as $X_1 + X_2$. Feed substrate concentration was fixed at $S_0 = 2400\,mg\ COD\,L^{-1}$. The simulation starts at the time when NFB was added into the reactor ($t_0 = 165\,h.$). The values of the variables at this time (X_1, X_2 and S) were adopted as the initial simulation condition.

The numerical simulation agreed with the response of the mixed culture due to the changes of D throughout the experiment (Fig. 10). For the first 135 h. D was $0.03\,h^{-1}$ and an increase in the total biomass concentration was calculated mainly due to the predominance of the FM; the simulation showed that FM fraction at $t = 135\,h.$ was 99.6%. Between 135 and 175 h., D was shifted to $0.39\,h^{-1}$ and a rapid wash-out of the FM was predicted; the calculation showed that FM concentration dropped from $955.5\,mg\ COD\,L^{-1}$ at 141.5 h. to $87.3\,mg\ COD\,L^{-1}$ at 165.5 h. according to the experimental results. At $t = 175\,h.$, D decreased to $0.05\,h^{-1}$ and the FM concentration slowly raised from $17.3\,mg\ COD\,L^{-1}$ at 186.9 h. to $85.5\,mg\ COD\,L^{-1}$ at 307.6 h. When D was changed to $0.17\,h^{-1}$, a rapid predominance of NFB was calculated.

Both the experimental results and simulations showed that low D values (Stages I and III in Fig. 10) favored the growth of FM; on the contrary, when high D values were applied (Stages II and IV) a rapid overgrowth of the NFB were predicted. Therefore, this condition of high D values favored the growth of NFB, minimizing the risk of filamentous bulking in activated sludge facilities treating food industry wastewaters.

4. Conclusions

This paper addresses the competition of a filamentous (FM) and a non-filamentous (NFB) bacteria in a CSTR. Emphasis was placed on the comparison of simulated results with the experimental observations about the effect of the dilution rate on the composition of the mixed culture. Measurements of NFB and FM fractions by digital IA are used to evaluate the proposed mathematical model.

The IA method was calibrated using pure cultures of FM (*S. natans*) and NFB (strain E932). An automated IA procedure to classify particles as FM or NFB using two shape parameters (reduced gyration radius, Rg, and roundness, Ro) was proposed.

Experiments in a continuous stirred reactor where both micro-organisms competed for a single substrate showed that low D values favored the growth of FM; on the contrary, when high D values were applied, a rapid overgrowth of the NFB was observed.

A mathematical model, based on the solution of the coupled first-order differential equations, corresponding to mass balances for the substrate and both micro-organisms was proposed; numerical simulation agreed with the experimental results.

Further work is ongoing to apply the IA method to determine the effect of chemical agents (e.g. chlorine, ozone) on FMs in activated sludge for bulking control.

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