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# ACTIVATED SLUDGE MORPHOLOGY CHARACTERIZATION THROUGH AN IMAGE ANALYSIS PROCEDURE

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**Abstract -** This work deals with the development of a digital image analysis procedure to characterize microbial flocs obtained in three different WWTP: a bench-scale Sequencing Batch Reactor (SBR) dealing with phenol and nitrogen biological removal, a municipal treatment unit (Ilha do Governador, Rio de Janeiro, Brazil) and an industrial wastewater treatment plant (Ciba – Estrada do Colégio, Rio de Janeiro, Brazil). The developed procedure permits to obtain its morphological parameters like equivalent diameter, compactness, roundness and porosity properties as well as the fractal dimension. This procedure was validated and lead to identify the major relationships between the analysed morphological parameters. A minimum of 300 flocs should be included in the image analysis and a significant influence of the sample dilution step on the mean size of the flocs was verified. The porosity parameter positively correlated with the fractal dimension of microbial aggregates indicating the that highly porous flocs are very irregular.
*Keywords*: Activated Sludge; Image Analysis; Monitoring; Characterization.

## INTRODUCTION

Nowadays, the aerobic activated sludge systems constitute one of the most used technologies for the secondary treatment of wastewaters. In general, these systems consist in a two stage wastewater processing: (1) the biotransformation and (2) the biomass separation. Great attention has been given to the biotransformation stage and the results of different studies have been contributed to its optimization and control. However, the biomass separation from the effluent purified by gravity settling frequently becomes the critical stage of the process, due to the different problems that severely affect the settling and compaction of sludge (Eriksson *et al*., 1992). Consequently, considerable alterations in the quality of the final effluent and in the economy of the process are obtained, impairing the overall performance and efficiency of the treatment process (Wilén *et al*., 2000).

In activated sludge system the bacteria responsible for the removal of organic matter are aggregated, through a process known as flocculation, forming microbial flocs. This active process takes place as a result of the network formation of extracellular polymeric substances (EPS) excreted by floc-forming bacteria in which the bacteria and other organic and inorganic particles are embedded, and of the filaments from filamentous bacteria that serve as a "backbone" of the microbial floc. (Jenkins *et al*.,1993)

Several studies have shown that the settling and the compaction properties of the activated sludge are directly related to the flocs structure, which depends on a group of chemical, physical and biological factors that significantly influence the balance between filamentous and floc-forming bacteria (Pujols

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and Canler, 1992), leading to changes in the structure and, thus, in the morphological properties of microbial aggregates. In this way, it is possible to establish relationships between sludge settling indexes and several parameters that characterize the morphology of microbial flocs (Námer and Ganczarczyk, 1993; Li and Ganczarczyk, 1987, 1988, 1990, 1992), being these relationships useful for monitoring the settling stage in activated sludge systems.

A good balance between filamentous and floc-forming bacteria favors the formation of large, dense and strong flocs desirable for adequate settling and compaction of the activated sludge. Misbalance could induce filamentous bulking caused by an overgrowth of filamentous bacteria or disperse growth (pin point floc) provoked by a scarce growth of floc-forming bacteria. The filamentous bulking promotes the formation of highly irregular flocs causing a decrease of settling speed as well as low sludge compaction, while the disperse growth leads to the formation of small and lights flocs that not settle, resulting in a very turbid effluent with high concentration of suspended matter. (Jenkins *et al*., 1993)

Several techniques have been proposed in literature in order to describe the complex structure of the flocs in terms of the material organization within the aggregates. These techniques have allowed to known the physical aspect of the floc (filament size and fractal dimension), the granulometric distribution of the floc sizes (measured by photographic technique in free settling, Coulter Counter, laser diffraction and Malvern counter, etc) and the consequences of bio-flocculation on flow properties (rheological measurements and settling rates).

The recent development of image analysis technique has enabled a more complete understanding of the aggregates physical structure and morphology. Image analysis has become a fundamental tool with great applications within the Environmental Science. In aerobic activated sludge systems, it has been applied for morphological characterization of microbial flocs, allowing the estimation of different parameters of the Euclidian geometry (Grisjpeerdt and Verstraete, 1996, 1997, Jin *et al*, 2003; Amaral 2003), the fractal analysis of contour of these aggregates and other aspects such as detection and counting of filaments (Li and Gaczarczyk, 1989, da Motta *et al*., 2001). These morphological parameters have been correlated with settling properties of activated sludge, estimated as Sludge Volume Index (SVI) (Grijspeerdt and Verstratete, 1997; da Motta *et al*., 2001; Amaral 2003), in order to monitor filamentous bulking in wastewater treatment plants.

The aim of the present work was the development of a digital image analysis procedure to characterize microbial flocs obtained from a Sequencing Batch Reactor (SBR) set-up, permiting its morphological parameters estimation like the porosity property. Additionally, a validation of the image analysis procedure developed was carried out with activated sludges from a municipal and industrial wastewater treatment plants, leading to identify the major relationships between the analysed morphological parameters.

## MATERIAL AND METHODS

### Microorganisms and Culture Conditions

The biomass used along the experimental work was courteously provided by the wastewater treatment plant of REPAR refinery (PETROBRAS S.A., Brazil).

The culture was maintained in a well-mixed Sequencing Batch Reactor with a working volume of 15 L containing an inoculum of approximately 5 L. The reactor was operated at 25 °C in fill-and-reaction periods according to the operational strategy described by Souza (2000). The overall cycle consisted of 360 minutes being 260 minutes in aerobic conditions and the last 100 minutes in anoxic one. The reactor was fed with a synthetic wastewater presenting the following composition: $NH_4Cl$ – 76.1 $mg.L^{-1}$; $Na_2HPO_4.12H_2O$ – 46.2 $mg.L^{-1}$; NaCl – 10.1 $mg.L^{-1}$; KCl – 4.7 $mg.L^{-1}$; $CaCl_2.H_2O$ – 4.7 $mg.L^{-1}$; $MgSO_4.7H_2O$ – 16.7 $mg.L^{-1}$, $NaHCO_3$ – 243.3 $mg.L^{-1}$; $Na_2CO_3$ – 162.2 $mg.L^{-1}$; glicose – 300 $mg.L^{-1}$. Trace elements ($Fe_2Cl_3.6H_2O$, $ZnSO_4$; $MnSO_4.H_2O$, $CuSO_4$) for biomass maintenance were added in lower concentrations (less than 0.2 $mg.L^{-1}$). At the end of the treatment the purified effluent was separated from sludge by gravity settling.

Samples were taken during the treatment process to realize microscopic observations in order to estimate the morphological parameters of these microbial aggregates by using image digital analysis.

Activated sludge samples were also collected from the aeration basins of a municipal watewater treatment plant at Ilha do Governador (Rio de Janeiro, Brazil) and of an industrial one at CIBA - Estrada do Colégio (Rio de janeiro, Brazil) in order to validate the procedure developed.

### Physico-Chemical Analyses

The Total Suspended Solids (TSS) and Volatile Suspended Solids (VSS) of the samples were determined according to the Standard Methods (APHA, 1992) and are presented in Table 1.

**Table 1: Average characteristics of activated sludge samples from the WWTPs.**

| WWTP | TSS (mg/L) | VSS (mg/L) |
|---|---|---|
| REPAR | $3.6 \times 10^3$ | $3.0 \times 10^3$ |
| Ilha do Governador | $6.2 \times 10^3$ | $4.7 \times 10^3$ |
| CIBA | $2.8 \times 10^3$ | $2.3 \times 10^3$ |

## Microbial Flocs Images Acquisition

For the aggregates images acquisition a drop of sludge was deposited on a slide and carefully covered with a cover slip. Image acquisition of the flocs on the slide was carried by the bright field microscopic technique (magnification 120x) using a photonic microscope (Nikon Eclipse 200) and keeping the illumination constant for all samples. The microscope was coupled to a Nikon CCD (Coolpix 990) camera, being the images grabbing performed in 24 bit (16 million of colors approximately), 2048x1536 pixels matrix. Around 50 digital images were acquired for each sample and stored in JPEG format. The calibration of the metric unit dimension was made by means of a micrometer slide.

## Image Analysis

The digital image processing was carried out by using the commercial software *Image-Pro Plus*® (version 4.5) (*Media Cybernetic Inc.*). The analysis of the processed images was carried by quantification of several Euclidian geometry parameters and contour fractal dimension of the microbial flocs. The schematic representation of image processing and analysis procedure is illustrated in Figure 1.

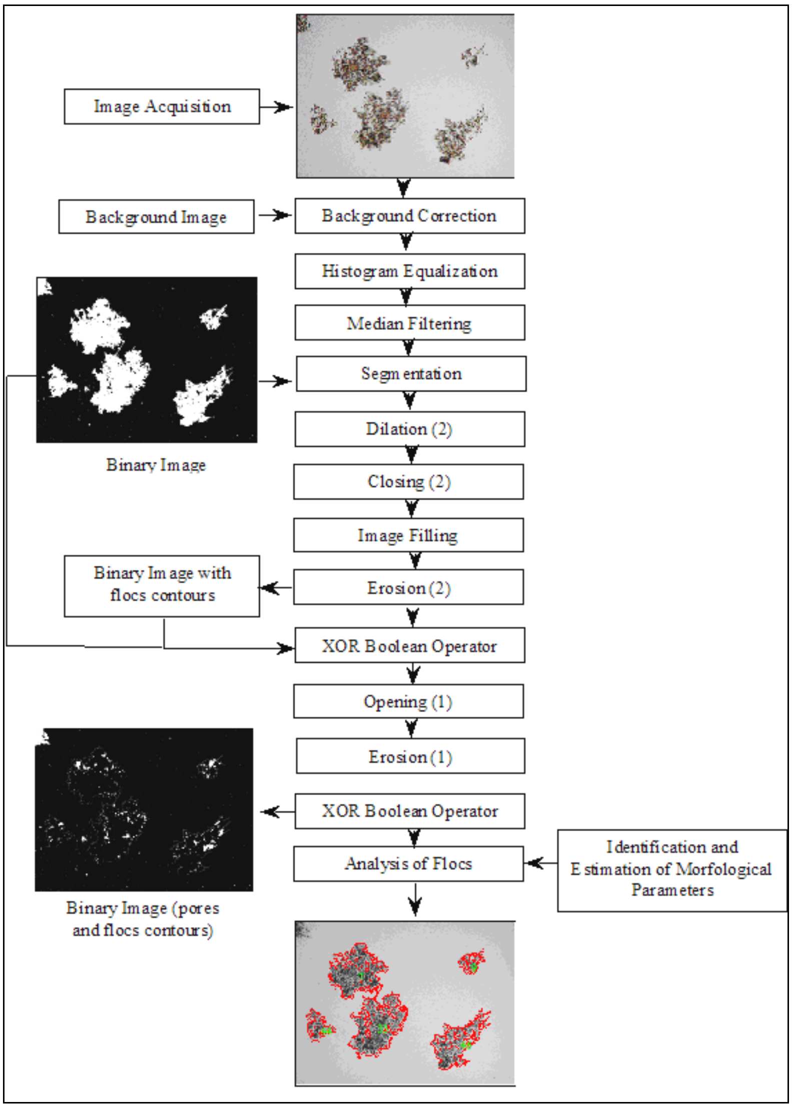



**Figure 1:** Schematic representation of the image analysis procedure

The first step of the procedure consisted on the conversion of RGB images into grey-level images and the subsequent background correction to reduce the uneven background intensities caused by uneven lighting from image acquisition system. The grey-level images were then enhanced by means of the image processing tools (histogram equalization and median filtering). Once the digital images were improved, the next step consisted on the application of a segmentation step based in Zack algorithm (Rossin, 2001) in order to select the microbial flocs, resulting in binary images. After segmentation the binary images were additionally enhanced by means of morphological operations (dilation, closing, filling) to facilitate the images analysis. In this step the porous were separated from flocs, using a Boolean operator, improved with the help of morphological filters (erosion and opening) and joined again to the flocs obtaining images with enhanced flocs and porous.

## Morphological Parameters

Once the images were treated by the procedure described above, several morphological parameters of both Euclidian (Convexity, Compactness, Roundness, Number of porous, 2D-Porosity, Equivalent Diameter) and Fractal geometry (Fractal Dimension of the Contour) were estimated by the own software.

### 1. Euclidian Geometry

Size - The size distribution of the microbial flocs was determined as the equivalent circle diameter *Deq*, calculated from the projected area as:

$$\mathrm{Deq} = 2.\sqrt{\mathrm{Area}/\pi}$$

Shape quantification - The Convexity, Compactness and Roundness were implemented to quantify and describe the shape of microbial flocs. The advantage in using these parameters is the fact they are not related with aspects such as orientation, size and location of the objects which allows comparing flocs that differ in these aspects but have the same shape.

Compactness is defined as the ratio between the area of the objects and the area of a circle with the same perimeter. This parameter takes the value 1 for circular objects and values lower than 1 for non-circular ones.

Roundness is estimated by the software through the following expression:

$$\mathrm{Round} = \frac{\mathrm{Perimeter}^2}{4.\pi.\mathrm{Area}}$$

According to this parameter a circle has a Roundness of 1 and higher values for objects with shape different of a circle.

Convexity was also quantified in the present study and is defined as the ratio between the convex perimeter of an object and its perimeter, both geometric parameters measured by the *Image Pro* software. The convex perimeter is defined as the perimeter of the convex outline of the object. The Convexity varies between 0 for irregular object and 1 for convex objects.

The 2D porosity parameter was also included in this study. It was estimated from the Hole ratio parameter which is described as the ratio of the object area excluding holes to the total area of the object. Then the 2D-Porosity can be defined as:

$$\mathrm{2D-Porosity} = 100 \times (1 - \mathrm{HoleRatio})$$

### 2. Fractal Geometry

Fractal dimension of the flocs contour was also employed to characterize the bacterial aggregates. This parameter has been used in some studies to characterize the contour irregularity of the microbial aggregates generated in water and wastewater treatment processes. In the present study the image analysis software estimated directly the fractal dimension of flocs applying the box counting method (Liebovitch and Toth, 1989) extensively used in literature to estimate the aggregates fractal dimension. The values for this parameter are between 1 and 2, being smaller values for objects with irregular contour and higher values for highly irregular objects (Obert *et al*, 1990).

## Statistical Processing of Results

The estimated morphological parameters were analyzed applying the commercial software Statistica (*StatSoft Inc., 2000*) to determine the size distribution function of the microbial aggregates as well as for verification of sample dilution effects on flocs size through the one-way ANOVA statistical technique. To identify the major cause and effect relationships between the morphological parameters analyzed in this study, univariate linear correlation were used. Correlations were considered statistically significant at 95 % confidence level ($p < 0.05$).

## RESULTS AND DISCUSSION

A visual microscopic analysis of the aggregates from the three wastewater treatment plants evaluated in the present study was firstly carried in order to verify their morphological state, being a crucial step to prepare a sufficiently robust procedure able to process digital images of microbial flocs independently of the physical aspect.

Figure 2 illustrates the digital images of microbial flocs belonging to activated sludge from each wastewater treatment plant evaluated.

The images in Figure 2 reveal that activated sludge flocs under study exhibited marked physical differences which might be due to the different composition of the effluent usually treated in each one of these wastewater treatment plants. It can be observed that the sludge flocs from Ilha do Governador municipal plant seem to be more resistant and consistent than the other aggregates. It could be explained by the fact of effluents in domestic wastewater treatment plants usually present high organic loads what, according to Barbusisnki and Koscielniak (1995), favors the increasing of the floc size which is attributed to the higher production of microbial exopolysaccharides under such conditions. Jin *et al.* (2003) during a settleability comparison study of activated sludges from 7 different wastewater treatment plants also observed that the sludge flocs from domestic wastewater treatment systems presented average sizes higher than those ones belonging to industrial systems.

On the other hand, it is also verified that the microbial aggregates from REPAR and CIBA are less consistent than those ones from Ilha do Governador WWTP. This difference is mainly due to their wastewater composition including a broad variety of chemicals originated from industrial process which have been pointed out as one of the main factors affecting not only the microbial activity but also the bio-flocculation phenomenon. These characteristics result in the breakage of large flocs and the formation of small and structurally weak flocs that will barely settle in the clarifier (or no at all) leading to a final effluent with high turbity and organic matter contents.

(a) (b) (c)

**Figure 2:** Microscopic images in bright field (total magnification 120x) of microbial flocs from wastewater treatment plants, (a) flocs from REPAR sludge, (b) flocs from Ilha do Governador sludge, (c) flocs from CIBA sludge

### Image Analysis Procedure Validation

The Image Analysis (IA) procedure (Figure 1) consisting by different image processing tools was validated according with its effectiveness to identify microbial flocs and pores/floc against a manual identification. This test was performed for digital images of activated sludge flocs from the three treatment plants under study.

Table 2 shows the total number of flocs considered in the validation test and the number of flocs correctly identified by the developed procedure after processing each group of activated sludge images assessed. The results revealed that the IA procedure exhibited the highest identification error (4.92%) when applied to images from REPAR activated sludge. The error decreased to 2% when images from Ilha do Governador flocs were processed. The lowest value of this parameter (1%) was achieved after application of the IA procedure to the flocs images belonging to CIBA WWTP which means that the proposed procedure allowed the adequately identification of 95% of the flocs included in the validation test.

The IA procedure suitability to identify the relation pores/floc can be verified in Figure 3 which illustrates the correlation between manual and automatic pores/floc identification. It was verified that for digital images from REPAR and Ilha do Governador samples, the slope values of linear correlation were very close to 1 (0.98 and 0.95, respectively) indicating the high effectiveness of the procedure in identifying the pores/floc parameter. A decrease in the slope (0.87) of the linear correlation was obtained after processing the images from CIBA samples and thus in the effectiveness of pores/floc identification

**Table 2: Effectiveness of the IA procedure to identify the microbial flocs.**

| Wastewater Treatment Plant | Total number of flocs | Number of flocs correctly identified | Identification error (%) | Effectiveness of identification (%) |
|---|---|---|---|---|
| REPAR | 305 | 290 | 4.92 | 95.08 |
| Ilha Governador | 300 | 294 | 2.00 | 98.00 |
| CIBA | 315 | 312 | 1.00 | 99.00 |

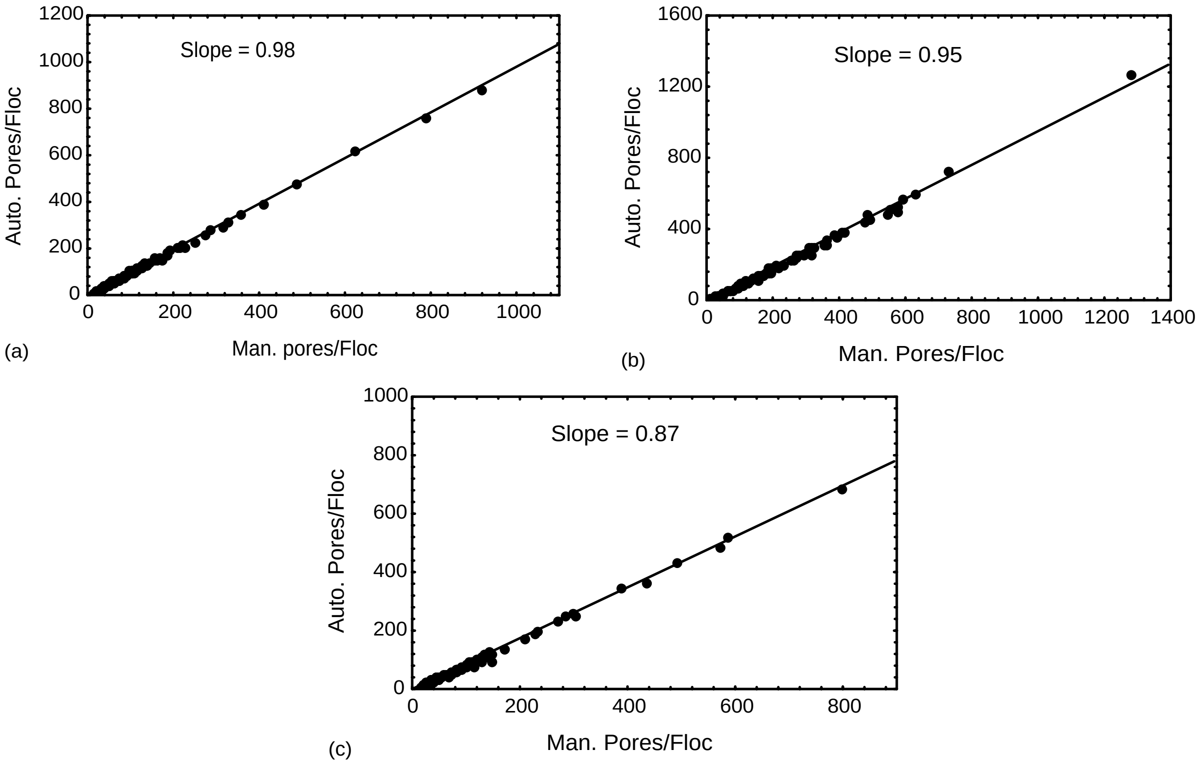



**Figure 3:** Number of pores/floc in function of Deq for the three sludge samples evaluated: (a) REPAR samples, (b) Ilha do Governador samples, (c) CIBA samples.

The differences in the identification of flocs and pores/flocs obtained might be explained if it is taken into account that these aggregates are not only different in their morphology but also in their color which could interfere in the segmentation step and thus in the software effectiveness for extracting the objects of interest. Nevertheless 87% of identification effectiveness of pores/floc can be considered adequate due to the complex nature of microbial aggregates presented in wastewater treatment processes.

Other aspect herein evaluated was the minimal number of flocs that should be included in the image analysis to obtain statistically significant results. For this study a number of 70 images of sludge flocs from REPAR WWTP was acquired and randomly processed until the total number of the flocs reached approximately 600. In this analysis were considered the measurements corresponding to Convexity, Compactness and Roundness since such morphological parameters are independent of the orientation, size and location of the objects which allows the results extrapolation obtained for REPAR sludge flocs to other WWTPs.

Figure 4 shows that the standard error on the measurements achieved a constant value when the number of flocs included in the analysis was approximately 300 which means that this is the minimum number of objects necessary to attain significant results. To obtain this number is necessary to process a mean number of 25 images of sludge flocs from REPAR containing about 10 to 20 objects each one. A similar number of images should be processed for CIBA sludge. However a number of images considerably higher (100 images) is required to include 300 objects in the processing of images from domestic wastewater treatment plant since each image contains approximately 2 to 3 flocs due to its larger flocs. These results proved that the size of the activated sludge flocs determines the number of images to be processed to attain representative morphological quantification of these microbial aggregates.

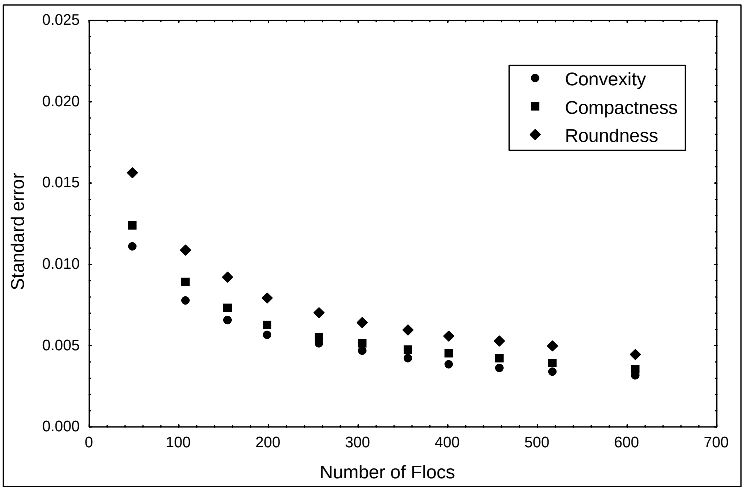



**Figure 4:** Standard error of the Convexity, Compactness and Roundness as a function of the number of flocs.

Grijspeerdt and Verstraete (1997), studying the activated sludge morphology, found that at least 150 flocs were required. Jenné *et al*. (2002) developed an automatic IA method to recognize flocs and filaments in an activated sludge sample and reported that a minimum of 50 images (corresponding to around 100 objects) were sufficient to quantify both flocs and filaments.

The differences in the results could be explained on the basis of the conditions used for images acquisition such as the microscopic technique and the magnification which could influence the standard error of the measured morphological parameters. These aspects were investigated by Carrete (1995) who verified a significant influence of the magnification on various morphological parameters measurements and noted the necessity of considering the same magnification in the measurement procedure. In this study the sludge flocs images were acquired using the bright field microscopic technique with a total magnification of 120X while, in the studies conducted by Grijspeerdt and Verstraete (1997), the dark field technique and a magnification of 40X were used. Jenné *et al.* (2002) also used different acquisition conditions: phase contrast technique and a magnification of 100X. It is interesting to remark that the acquisition conditions also determine the IA procedure to be applied.

## Floc Size Distribution

Since smaller objects produce higher measurements errors, it is necessary to establish a lower limit in the IA procedure in order to exclude such objects from the analysis. Russ (1995) observed that objects with a number of pixels smaller than 0.1% of the total number of pixels of an image should not be taken into account. In accordance with this evidence in the present study a floc size range between 65 and 2000 µm was considered for the morphological analysis. For each sample three replicates were performed and in all cases the size distribution histogram reflected a log-normal distribution described by

$$f(x) = \frac{1}{\sigma_{\ln x}\sqrt{2\pi}} \exp\left[-\frac{\left(\ln x - \mu_{\ln x}\right)^2}{2\sigma_{\ln x}{}^2}\right]$$

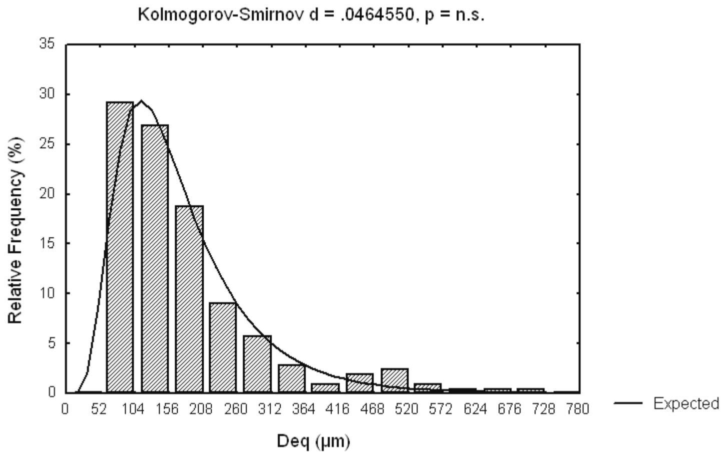
where $x$ is the floc size, $\mu_{lnx}$ and $\sigma_{lnx}$ are respectively the logarithmic average and the logarithmic standard deviation of a floc size.

The Kolmogorov-Smirnov test for distributional adequacy was not statistically significant at 95% confidence level indicating an adequate fitting of experimental data to a log-normal distribution. In previous investigations, sluge floc size distribution has also been described with the log-normal expression (Grijspeerdt and Verstraete, 1997; Barbusinki and Koscielniak, 1995; Li and Ganczarczyk, 1991; Námer and Ganczarczyk, 1993). According to Li and Ganczarczyk (1991), this kind of distribution may be expected for data sets in which part of the values (in this case smaller than 65 µm) has been arbitrarily removed.

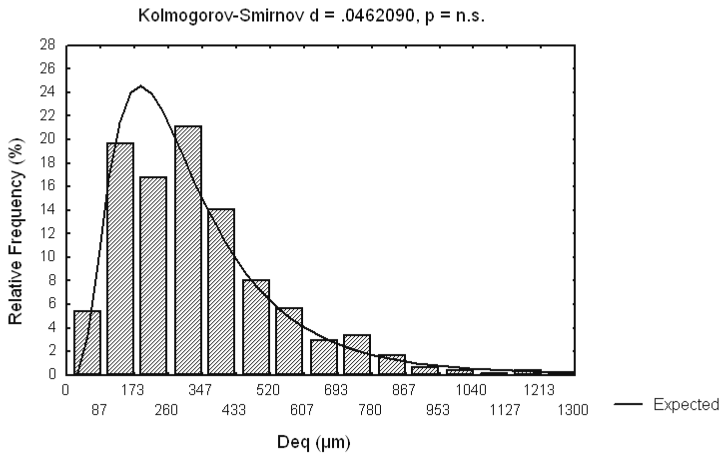
Figure 5 shows the floc size distributions expressed as the frequency of occurrence achieved for the three activated sludges under evaluation. The measured aggregate sizes of REPAR samples ranged from 68 to 712 µm. For CIBA and Ilha do Governador samples the upper limit of the floc size range gradually shifted to 920 and 1162 µm, respectively. It is also verified from Figure 5 that for REPAR samples the highest frequency corresponded to the size range 52-104 µm (29%) while for CIBA and ILHA do Governador samples, the highest frequency peak (36 and 21 %, respectively) shifted gradually towards larger sizes: up to 147-220 µm for

Ciba and 260-346 μm for Ilha do Governador samples. In general, the herein obtained range for the flocs size correspond to microbial flocs usually obtained in wastewater treatment plants, as quantified by image analysis technique and/or other methods reported in literature.

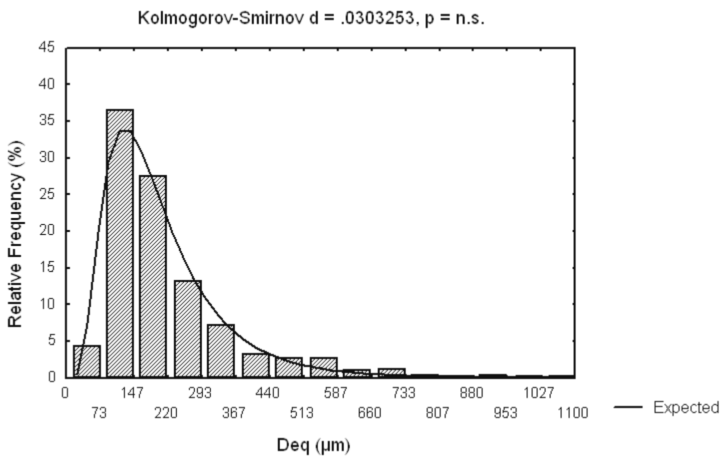
Table 3 presents the mean size values assessed for the microbial flocs, as well as the standard deviation of the measurements effectuated. The results proved that the activated sludge from Ilha do Governador WWTP are the largest flocs among the three samples studied, followed by the ones from CIBA and REPAR as observed in previous microscopic observation.




(a)




(b)




(c)

**Figure 5:** Frequency distribution histogram for Equivalent Diameter (*Deq*) of (a) REPAR samples, (b) Ilha do Governador samples, (c) CIBA samples.

**Table 3: Mean size values and respective standard deviation for the WWTP studied.**

| WWTP | Number of flocs | Mean (µm) | Std. Dev. (µm) |
|---|---|---|---|
| **REPAR** | 309 | 177.4 | 116.2 |
| **Ilha do Governador** | 300 | 328.6 | 194.8 |
| **CIBA** | 304 | 195.4 | 125.4 |

## Floc Morphology

The morphological parameters estimated from different activated sludge samples after image processing were statistically compared in order to identify the major relationships between them. The Pearson's product momentum correlation coefficient ($r_p$) was used to estimate linear correlations. The values of Pearson's coefficient range between -1 and +1, where -1 corresponds to a perfect negative correlation and +1 corresponds to perfect positive correlation. The value 0 means in this case absence of relationship. The results of the correlation analysis are summarized in Table 4.

**Table 4: The correlation matrix for settling and morphological parameters measured after applying the IA procedure**

| | Deq | 2-Dε | Convex. | Comp. | Round. | FD |
|---|---|---|---|---|---|---|
| Deq | 1 | -0.0045* | -0.1139 | -0.0758 | 0.0741 | 0.0827 |
| 2-Dε | -0.0045* | 1 | -0.3255 | -0.019* | 0.2058 | 0.4135 |
| Convex. | -0.1139 | -0.3255 | 1 | 0.2248 | -0.7421 | -0.7987 |
| Comp. | -0.0758 | -0.019* | 0.2248 | 1 | -0.6678 | -0.2132 |
| Round. | 0.0741 | 0.2058 | -0.7421 | -0.6678 | 1 | 0.6129 |
| FD | 0.0827 | 0.4135 | -0.7987 | -0.2132 | 0.6129 | 1 |

*no significant correlation at $p < 0.05$

It can be seen that the morphological parameter Roundness correlated well with the Convexity, Compactness and, in a lesser degree, with the Fractal Dimension of the microbial flocs with Pearson's correlations of -0.7421 -0.6678 and 0.6129, respectively. These values indicate, as expected, that more compact and regular flocs trend to exhibit a more spherical shape. On the other hand, a highly irregular structure may represent a lesser spherical shape of the bioaggregates. It should be remarked here that a good correlation between such parameters is desirable since they can be used to infer the shape of microbial aggregates and predict the settleability of activated sludge which is very influenced by the aggregates shape (Grijspeerdt and Verstraete, 1997, Jin *et al*., 2003; Námer and Ganczarczyk, 1993, Amaral *et al*., 2003).

A good correlation was founded between Fractal Dimension and Convexity which means the regularity of flocs boundaries increase as their Convexity approaches 1 (one), corresponding to a circle. It is well known that the highly inhomogeneous structure of the bioaggregates is difficult to be described using Euclidian geometry which is mainly due to their fractal nature. So the fractal dimension, emerged from the fractal theory, has been used to overcome these difficulties being possible to explain some unexpected phenomena. Logan and Kilps (1995) have proved that fractal dimension reflects the hydrodynamic environment in which the aggregates are formed being possible to use this parameter to study the aggregation process.

A lesser but significant correlation ($r_p$ = 0.4135) between Porosity (*2-Dε)* and the Fractal Dimension of the aggregates was also observed as shown in Table 4 suggesting that more irregular flocs present a porous structure.

Although the pores/floc increased with the increase of the flocs size (data not presented), no statistically significant correlation was found between the *Deq* and the *2-D* porosity. In spite of early studies on the porosity of sludge aggregates, using free settling test and expressions derived from Stokes' law, have indicated the existence of a relationship between the flocs size and porosity, recent investigations have proved that the estimation of this parameter presents a certain difficulty degree which is mainly due to the highly complex structure of microbial aggregates (Chu and Lee, in press). Jonhson *et al.* (1996) verified that aggregate porosity is overestimated from settling velocity data and from Stokes'law. Chung and Lee (2003) also estimated the activated sludge flocs porosity based on free settling test, buoyant weight measurements and confocal laser scanning microscope tests and proved the existence of discrepancy between the porosity values estimated by such methods.

On the other hand, in the analysis herein described the internal pores of the flocs were not considered including only the pores externally visible in the corresponding digital images. Moreover, the projected area was assumed as the total area of the flocs. Certainly these restrictions could have considerably affected the estimation of this parameter.

With respect to *Deq*, in spite of the statistical significance at 95% of confidence level, except for the 2-D porosity, the linear correlations obtained between this parameter and the other ones studied, were not strong as evidenced from the $r_p$ coefficient values. It must be kept in mind that this analysis was carried on sludges from different sources which could have introduced a high level of noise in the measurements. An individual analysis of each sludge sample should be performed in order to reduce the noise level of the measurements.

## Effect of Sample Dilution

In order to know the influence of the sample previous dilution step used for digital images acquisition on the microbial flocs morphology, a study of this effect on size distribution was realized. For this, a dilution series of the REPAR and Ilha do Governador samples were prepared and two replicates were done per dilution.

As shown in Table 5 the dilution of both samples caused a decrease in the flocs mean size. This effect was most marked when the original samples were submitted to a dilution of 1:1 (equal parts of sample:water) which resulted in a decrease of 24% and 30% in the floc size for REPAR and Ilha do Governador samples, respectively.

The experimental data were statistically processed by applying the one-way analysis of variance (anova-analysis) in order to confirm the significant influence of sample dilution on the floc size. The results of this analysis for both sludge samples are presented in Tables 6 and 7.

The results of ANOVA analysis show the existence of significant differences between the dilutions series evaluated at a 95% confidence level as indicated by the p-value $< 0.05$. These results are not in agreement with that obtained by Carrete (1995) who observed that the measurements of morphological parameters including the floc size were independent of the dilution of the sludge sample in a concentration range between 0.5 and 4.0 g/L. Although the concentrations of REPAR and Ilha do Governador sludge samples were in the range evaluated by these authors (3 and 4.7 g/L, respectively), the considerable effect of dilution was verified as soon as the original sludge samples were submitted to the first dilution (1:1). This result allowed to conclude that the sludge sample for digital images acquisition should be prepared without previous dilution in order to obtain accurate measurements of the microbial flocs size.

**Table 5: Summary of the mean values of flocs *Deq* resulting from dilutions for both activated sludges under study.**

| Dilution | REPAR | Ilha do Governador |
|---|---|---|
| not diluted | 181.4 ± 117.1 | 328.6 ± 194.8 |
| 1:1 | 138.1 ± 58.1 | 229.5 ±137.7 |
| 1:2 | 137.7 ± 59.7 | 213.0 ±142.9 |
| 1:3 | 144.2 ± 66.8 | 221.8 ±104.9 |
| 1:4 | 139.3 ± 77.6 | 204.5 ±140.4 |

**Table 6: Dilution step influence on the flocs size for REPAR activated sludge**

| Source | Sum of Squares | Degree of freedom | Weighted Sum of squares | F | p-value |
|---|---|---|---|---|---|
| Effect | 431926 | 4 | 107982 | 18.78 | 0.00 |
| Error | 8804486 | 1531 | 5751 | - | - |
| Total | 9236412 | 1535 | - | - | - |

**Table 7: Dilution step influence on the flocs size for Ilha do Governador activated sludge**

| Source | Sum of Squares | Degree of freedom | Weighted Sum of squares | F | p-value |
|---|---|---|---|---|---|
| Effect | 3027880 | 4 | 756970 | 33.11 | 0.00 |
| Error | 30704448 | 1343 | 22863 | - | - |
| Total | 33732328 | 1347 | - | - | - |

## CONCLUSIONS

The results obtained in this work can be summarized as follow:

1. The image analysis procedure obtained for processing digital images of sludge flocs is reproducible and adequate to characterize the morphology of different sludge types as demonstrated by the validation tests.
2. As minimum a total of 300 flocs should be included in the image analysis in order to obtain statistically significant results in the morphological quantifications.
3. The size distribution histogram reflected a log-normal distribution for all sludge samples tested.
4. Morphological quantification of the bioaggregates after processing digital images demonstrated that the sludge flocs from a municipal WWTP (Ilha do Governador) presented the highest mean size.
5. The Roundness parameter correlated well with the Convexity, Compactness and, in a lesser degree, with the Fractal Dimension parameters, being these correlations very important to describe the microbial aggregates shape and predict the activated sludge settleability.
6. Porosity parameter positively correlated in a lesser extension with the Fractal Dimension of the microbial aggregates indicating the highly porous flocs are very irregular.
7. It was verified a significant influence of the sample dilution step on the mean size of the flocs for the sludge samples analysed obtaining a decrease in the floc size with the increase of the sample dilution.

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