Dilution effects on aggregates and filaments contents in automated image analysis methodologies

O. Dias*, D. P. Mesquita*, A. L. Amaral*', E. C. Ferreira***

* IBB - Institute for Biotechnology and Bioengineering, Centre of Biological Engineering Universidade do Minho, Campus de Gualtar, 4710-057, Braga, Portugal
(E-mail: *ecferreira@deb.uminho.pt*)
** Instituto Superior de Engenharia de Coimbra, Instituto Politécnico de Coimbra, Rua Pedro Nunes, Quinta da Nora, 3030-199 Coimbra, Portugal

Abstract
Monitoring activated sludge processes by microscopic observations and image analysis is a well established technique with the utmost importance for microbial community characterization. Occasionally, there are biological systems operating with high biomass concentrations that need to be diluted, causing reproducibility problems for the sampling and image analysis methodologies. In the current work, image processing and analysis methodologies were used to determine the aggregates and filamentous bacteria contents and morphological characterization, of five dilutions from three activated sludge systems. The outcome of reducing the concentration of the samples was obtaining almost 3.5 times the pattern results for the total filamentous bacteria contents and almost the double for the total aggregates contents. Moreover, the differences were diverse for each aggregates class and, therefore, the dilution effects cannot be predicted and quantified for all of the aggregated biomass as a whole based on an overall correction factor.

Keywords
Activated sludge, image analysis, dilution, aggregates, filamentous bacteria

INTRODUCTION

Monitoring activated sludge processes by image analysis methodologies is, nowadays, a well established technique for the microbial community characterization. The balance between floc forming and filamentous bacteria is usually determined for the establishment of bulking events, which may induce biomass washout and foaming problems.
Image analysis is particularly useful in determining bulking events (Jenne *et al*., 2007), although bulking problems can be looked upon as microbiological or engineering problems (Martins *et al*., 2004).
Schuler and Jassby (2007) scrutinized the work of several authors and suggested a modified empirical model for filamentous contents and settling ability. According to Schuler and Jassby (2007) the previous belief of a threshold at approximately 150 mL/g sludge volume index (SVI) corresponding to 10000 mm/mL of filamentous bacteria is invalid and the notion of filamentous bulking above 150 mL/g of SVI is therefore, defied. However, these conclusions could be misled by incorrect evaluation of the work of the other researchers. In fact, some of the considered surveys could be conducted with diluted samples and, therefore, the end results would be inaccurate as it will be noticed further on.
Image analysis is also used to determine settling ability and concentration properties through the categorization of the aggregated flocs (Grijspeerdt and Verstraete, 1997). Occasionally, there are biological systems that operate with high biomass concentration that needs to be diluted. When the dilution is not performed, aggregates and filaments may appear linked to each other leading to misleading information (da Motta, *et al*., 2002).
Dilutions can be useful for microscopic inspection and especially for image analysis processing, leading to a more accurate evaluation of the activated sludge system. However, the dilution technique may also give raise to representative problems given the fact that the amount of screened biomass decreases.
The aim of this study was to monitor the effect on the parameters provided by image analysis of increasing dilutions: aggregates morphology, total filamentous bacteria length (TL) and total aggregates area (TA). The image processing and analysis programs were developed in the

Matlab environment and the aggregates were first classified in three categories: small (< 0.25 mm) medium (>0.025 mm and < 0.25 mm) and large (>0.25 mm).

MATERIAL AND METHODS

Experimental study

Three activated sludge samples were collected from a wastewater treatment plant at Braga, Portugal and, for each one, five dilutions were performed with distilled water. The dilutions encompass 1 fraction sludge for 1, 2, 5, 8 and 10 parts of diluted sample. Microscopic observations were undertaken, in order to estimate the contents and morphological parameters of the microbial aggregates and filamentous bacteria by image acquisition and analysis.

Furthermore, previous results acquired from two other wastewater treatment plants, with 1 fraction sludge for 10 parts of diluted sample and without dilution were also taken into account during this study.

Image Acquisition

Aggregates and filaments image acquisition. A volume of 25 µL was placed on a slide and covered with a 20x20 mm cover slip for visualization and image acquisition in bright field microscopy. Roughly 200 images were acquired per dilution sample to obtain significant data.

The bright field images were acquired using a *Leitz Laborlux S* microscope (*Leitz*, Wetzlar) with 100x magnification, coupled to a *Zeiss Axion Cam* CCD camera (*Zeiss*, Oberkochen). The images acquisition was performed in 8-bit greyscale format (1300x1030 pixels) through the commercial software *Axion Vision 3.1* (*Zeiss*, Oberkochen).

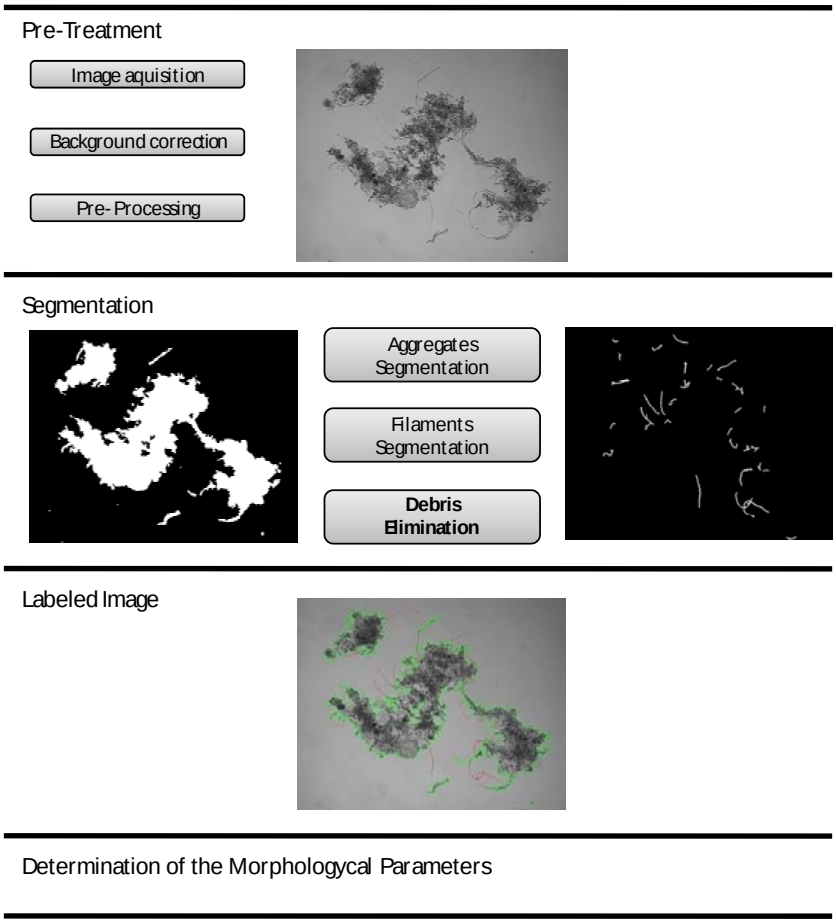

Figure 1. Main stages of the image processing program.

Image Processing and Analysis Methodology

The image processing and analysis methodology used to determine the aggregates and filaments contents and morphological descriptors was developed in *Matlab 7.3* (*The Mathworks, Inc*., Natick) language and was adapted from a previous program developed by Amaral & Ferreira (2005). Primarily, the image processing program determines and saves both the aggregated and protruding filamentous biomass binary images. The image analysis program then proceeds to determine the aggregates and filaments contents and morphological parameters. Next the raw results are multiplied by the dilution factor, as diluting a sample by two fold ought to mean that only half the original biomass will be present on the slide.

The main stages of the image processing program comprise the image pre-treatment, segmentation, and debris elimination whereas the image analysis program is oriented to the morphological parameters determination. Figure 1 represents the main stages of the image processing program.

The identified aggregates were classified into three representative classes in order to a clearer perception of the dilutions effects on the biomass estimation: small aggregates (equivalent diameter < 0.025 mm), intermediate aggregates (0.025 mm < equivalent diameter < 0.25 mm) and large aggregates (equivalent diameter > 0.25 mm).

Pre-treatment. The image pre-processing stage resides on the enhancement of the grayscale images by background removal. In this stage, the original image is first divided by a background image to minimize background light differences. The aggregates and filaments are further enhanced by using local histogram equalization in order to improve the contrast of the boundaries.

Segmentation. This stage consists primarily in the aggregates and filaments segmentation by the determination and simultaneous use of aggregates boundary and core images. After a series of morphological operations, an automatic pre-defined threshold level is used to segment the grayscale image into a binary image. Finally, a series of morphological operations and reconstructions allow for the final aggregates binary image to be determined.

With respect to the filaments segmentation, the aggregates binary image is used as a mask to eliminate the aggregates from the original grey scale image which is next segmented by a pre-defined percentile based threshold level. Furthermore, a series of erosions and dilations procedures on the aggregates binary image is then performed in order to enhance the filaments identification. Next, an automatic pre-defined threshold level allows for the determination of a filaments marker image which is later used as a mask to reconstruct the filaments more accurately. Finally, a gyration radius based procedure is implemented to discard small filamentous-like debris by the use of a 1.2 cut-off value.

Debris elimination. The elimination of residual aggregates (smaller than 3.5 µm in diameter) and debris is performed by third order erosion and reconstruction operations and all the aggregates cut off by the image boundaries are removed. Finally, the aggregated and protruding filamentous biomass binary images are saved for the morphological characterization of the activated sludge in the image analysis step.

Determination of the morphological parameters. The aggregates equivalent diameter and projected area were determined. Regarding the settling ability analysis, the total number of aggregates per volume (#/Vol), total aggregates area per volume (TA/Vol) and total protruding filaments length per volume (TL/Vol) were also determined. In Amaral (2003) can be found a detailed description of each of the above parameters.

RESULTS AND DISCUSSION

Except for the area percentages and the SVI data, all of the results presented here were obtained after compensation by multiplying with the dilution factor. The obtained results allowed establishing the trend of the total filamentous bacteria length and aggregates area with the dilutions increase. It is accepted that, when a dilution is performed, the amount of

screened biomass decreases, which may lead to an incorrect estimation of the biological system. Furthermore, the aggregates structure may deflocculate changing the aggregates size and releasing enclosed filamentous bacteria, thus, it is imperious to take some precautions to avoid wrong assessments. However, as smaller aggregates and protruding filaments may no longer be hidden by larger aggregates, as it could happen in non diluted samples, the dilutions may present an advantage in high biomass contents systems.

The total aggregates area per volume and total filamentous bacteria length per volume, for each studied sample and dilution, are depicted in Figure 2. For comparison effect, samples with no dilution were acknowledged as the pattern (100%) and all the other samples were assessed as percentages of the first.

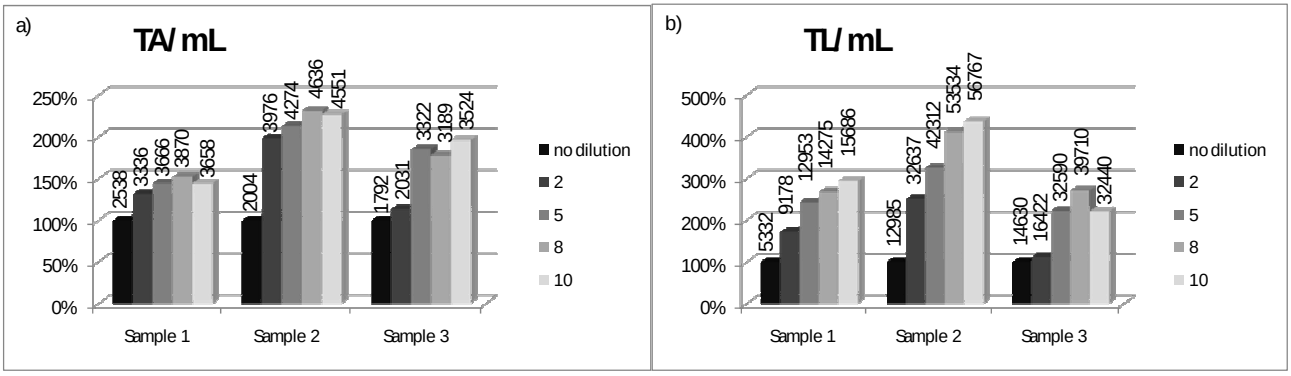

Figure 2. Results for all the 3 samples of the studied activated sludge system (values above data columns report to the measured TA/mL in mm^2/mL and TL/mL in mm/mL).

Figure 2 allowed establishing that sample dilution is likely to provide inaccurate data, foremost when assessing the total filaments length (Figure 2 b), with values up to 4.37 times greater for the tenfold dilution than the pattern. Likewise, the determination of the total area suffered from the same problem with values rising up to 2.31 times greater. Within the first sample TA values increased up to 150% and TL values reach almost 300% the pattern value for the tenfold dilution. The second sample was found to be the most susceptible to dilution, since even a twofold dilution caused the TA value to double and the TL value to raise 250%. The maximum values for this sample were slightly over 200% for TA and almost 450% for TL. Regarding the last sample, the dilutions provided values reaching up to 200% on the TA assessment and 250% for the TL estimation for the tenfold dilution. These results could be explained by the fact that reducing the concentration of the samples may expose smaller aggregates previously hidden by larger aggregates. Another possible mechanism to be considered is a deflocculating process occurring with the addition of the distilled water.

The average total aggregates area per volume and average total filamentous bacteria length per volume, for all of the studied samples and for each dilution, are depicted in Figure 3.

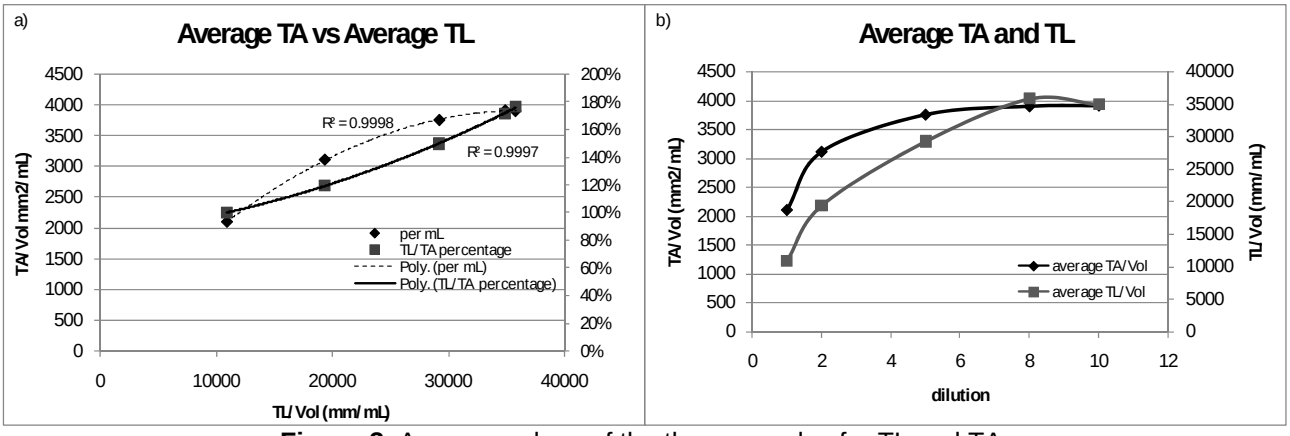

Figure 3. Average values of the three samples for TL and TA.

Concerning Figure 3, TA/Vol parameter seems to converge to a limit value after the fivefold

dilution, and TL/Vol appears to decline after the eightfold dilution. Whereas the average TA assessment was short from doubling for the tenfold dilution, TL appraisal raised to 3.26 times the non-diluted average. Hence it can be assumed that the TA measurements became constant earlier than the TL, and may have reached a threshold in the fivefold dilution, with further dilutions presenting similar values. When plotted against each other, TA and TL followed a well defined trend line (R^2 of 0.9998) modeling their inter-dependence with the dilutions. Furthermore, since the data from the fivefold and tenfold dilutions is quite similar the value of 1.80 times larger increase for the TL than for the TA seems to be the limit for above dilutions.
The aggregates number per volume, for each dilution and aggregates class and for all of the studied samples, is depicted in Figure 4.

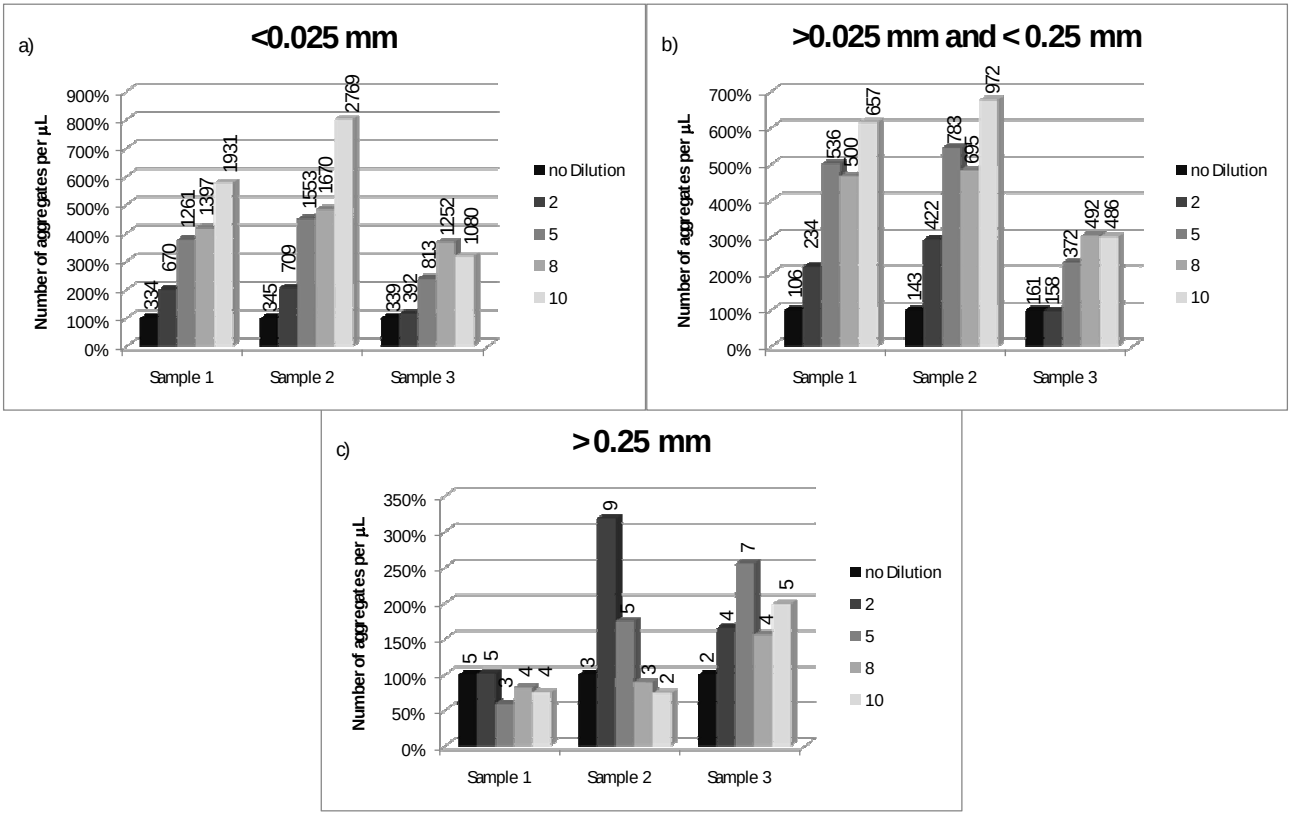

Figure 4. Number of identified aggregates per volume: small (equivalent diameter < 0.025 mm); intermediate (equivalent diameter > 0.025 mm and < 0.25 mm); large (equivalent diameter > 0.25 mm) (values above data columns report to the measured #/mL in mL^{-1}).

As expected and shown in Figure 4 a) and b), the dilution factor has a major influence in the number of identified aggregates. Figure 4 c) has to be cautiously considered as it only represents an universe of ten objects at the most, therefore it cannot be assumed that this data truly represents the expected differences between dilutions. Figure 4 a) and b) confirm that increasing dilutions led to a sharp increase on the number of recognized aggregates for both intermediate and small aggregates (from two to ten times more in the tenfold dilution). However, as it is clear on Figure 5a, such seems not to be the case for the larger aggregates with numbers stabilizing around the initial values. This fact may point towards no significant deflocculating mechanism for the larger aggregates, since their number did not decrease markedly with the dilutions. However it must be present that, as a deflocculating process occurs, the mechanical structure of the aggregate collapses, leading initially to looser larger aggregates (da Motta *et al.*, 2002). Analyzing Figure 5a, for the tenfold dilution, the aggregates number reaches a value of 8.02 times larger than the non diluted sample for the small aggregates, and 6.77 times larger for the intermediate aggregates. Furthermore, whereas for the large aggregates, their number seems to converge to a value close to the non diluted sample from the fivefold dilution onwards, for the smaller and intermediate aggregates no limiting value was found up to the tenfold dilution.

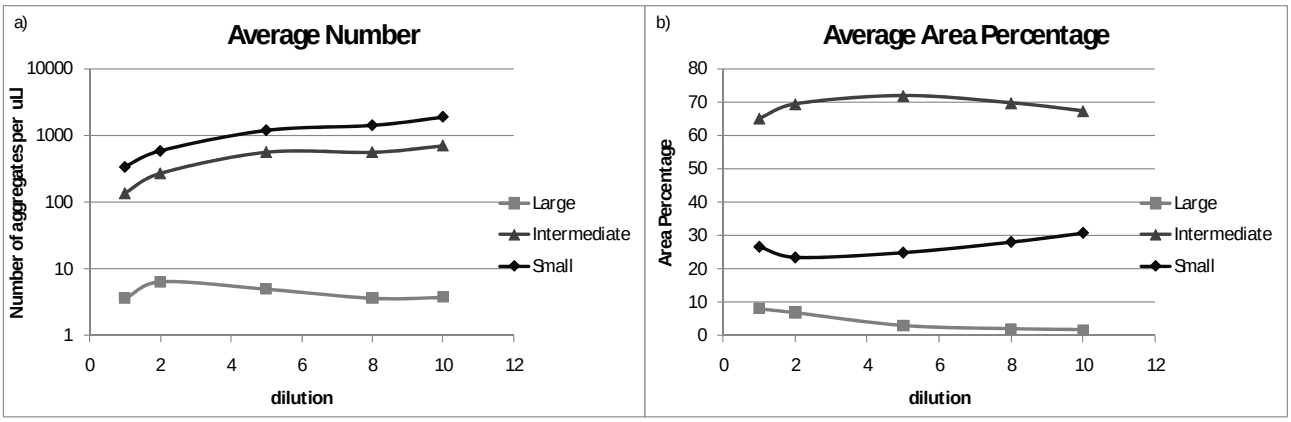

Figure 5. Average number of aggregates per volume and average area percentage for small, intermediate and large aggregates.

The area percentage, for each dilution and aggregates class and for all of the studied samples, is depicted in Figure 6.

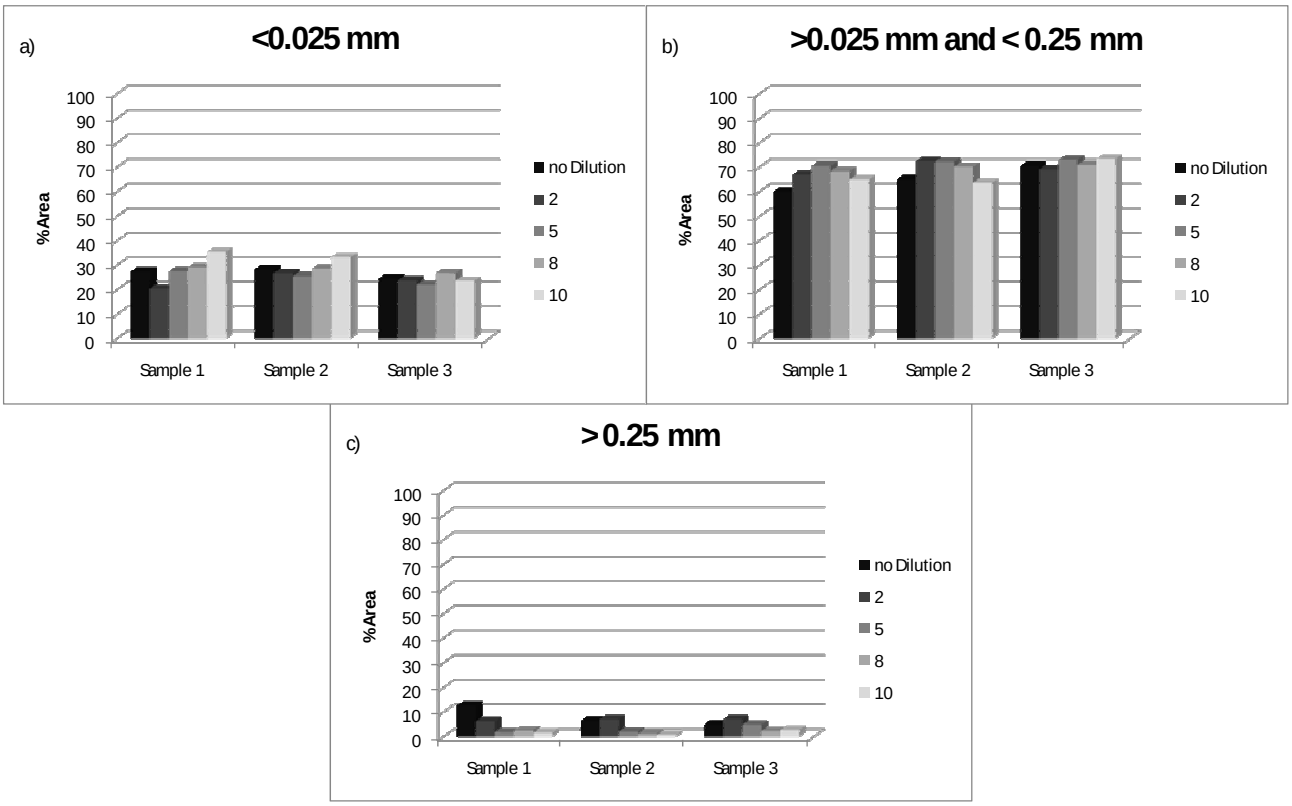

Figure 6. Area percentage: small (equivalent diameter < 0.025 mm); intermediate (equivalent diameter > 0.025 mm and < 0.25 mm) and large aggregates (equivalent diameter > 0.25 mm).

Despite of the dilution effect on the number of recognized aggregates, Figure 5b), 6a) and 6b) demonstrate that the relative area percentages of the small and intermediate aggregates remain somewhat stable.

However, an in-depth analysis suggests that the first two dilutions (two and fivefold) either disclose the concealed intermediate aggregates or deflocculates the larger aggregates, in contrast to the eight and tenfold dilutions that seem to further reveal the smaller aggregates or deflocculate the intermediate aggregates. However, the variation is too small to make a positive stand and, more data would be needed to fully confirm this trend. Figure 6 c) on the other hand points towards a progressive loss of significance of the larger aggregates, as the

dilution increases, on cause of the steady raise in the number of the other aggregates (Figure 5).
As the overall values of the area percentage of the large aggregates is quite small in the studied samples, the relative differences for these aggregates are higher than for the intermediate and small aggregates. Once again, care should be taken in the analysis of these aggregates results due to the restricted universe, in this study, of the larger aggregates.
The SVI dependence on both TL/Vol and TA/Vol for two different activated sludge systems and for non diluted and tenfold dilution samples, is depicted in Figure 7.

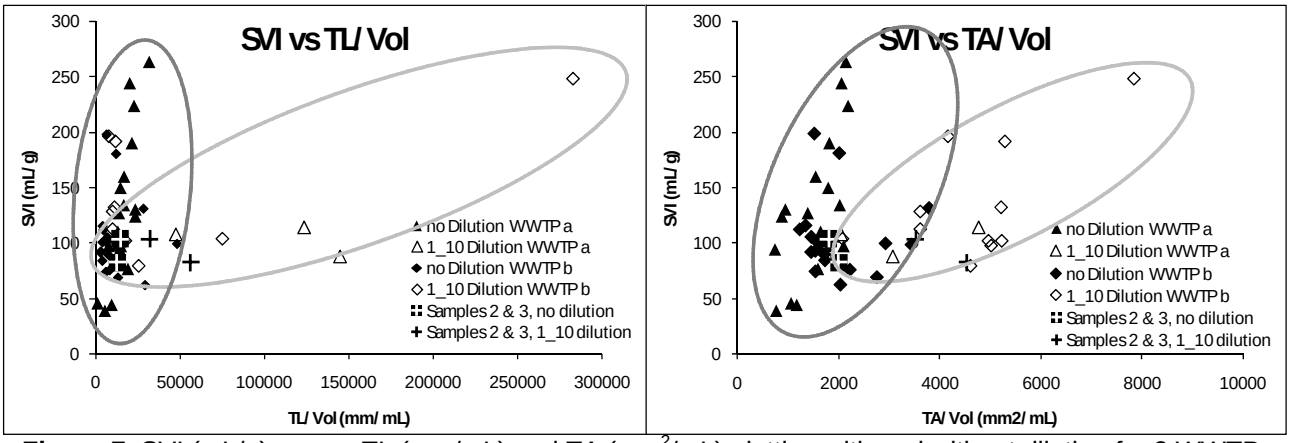

Figure 7. SVI (mL/g) versus TL (mm/mL) and TA (mm^2/mL) plotting with and without dilution for 2 WWTP. The black and the white crosses indicate the location where samples two and three of this study would be placed.

The analysis of the SVI dependence on the TA and TL, for samples with and without dilution (Figure 7), indicates that diluted samples do not pursue the trend line established by non-diluted samples. The activated sludge system *a* operates under low biomass loads (average TSS of 2533 mg/L) and normal filamentous bacteria contents. Regarding the tenfold dilution experiments, system *a* attains, for an SVI around 100 mL/g, TL values up to ten times larger than the non-diluted, and TA more than doubles the non-diluted values, within the same SVI range.
Considering the wastewater treatment plant *b*, it can be assessed that the dilution effect is more significant on the TA appraisal. This system operates under medium biomass concentrations (4802 mg/L) and normal filamentous bacteria contents. Again, for an SVI value around 100 mL/g the diluted samples presented a difference of 3.5 times larger for TA and TL. Furthermore, as the SVI contents were higher, the differences between the diluted and non-diluted TA and TL kept rising.
Two of the tenfold dilution of the three previously analyzed samples, belonging to WWTP *b*, are also presented in Figure 7 (samples 2 and 3, since sample 1 SVI was not available). The white and the black crosses represent the non-diluted and diluted (tenfold dilution) samples, respectively, confirming the trend of diluted samples to overestimate both TA and TL parameters.

CONCLUSIONS

The obtained results reveal the vulnerability of image analysis to the dilution effects. When a dilution is performed, representative problems may arise given the fact that the amount of screened biomass decreases and deflocculating phenomena may occur, however on the opposite sense, small aggregates and protruding filaments may no longer be hidden by larger aggregates as it could happen in non diluted samples. Furthermore, the aggregates structure may deflocculate changing the aggregates size and releasing enclosed filamentous bacteria. This study confirmed the importance of the questions that are raised, when a dilution is performed, other than the dilution factor.
Total filamentous bacteria length (TL) and total aggregates (TA) were shown to be severely influenced by dilution. The outcome of reducing the concentration of the samples was

obtaining almost 3.5 times the pattern results for the TL and almost the double for the TA. When plotted against each other, TA and TL seemed to follow a well defined trend line modeling their inter-dependence with the dilutions with a limit value of 1.75 times larger increase for the TL than for the TA, from the fivefold dilution onwards.
The number of identified aggregates increased significantly with dilution for both small and intermediate aggregates, contrary to the larger aggregates that remained somewhat stable. These results seems to point to either the disclosure of concealed intermediate aggregates or deflocculating of larger aggregates on the first two dilutions (two and fivefold), in contrast to the eight and tenfold dilutions that seem to further reveal the smaller aggregates or deflocculate the intermediate aggregates.
The study also seems to point that the lower the biomass concentration is, in a given activated sludge system, the more susceptible the TL assessment is to the dilution effects, which is not a pressing problem since lower biomass concentrations are less likely to be diluted. In the case being, the lower biomass system gave rise to larger discrepancies when appraising TL than when appraising TA, between the diluted and non-diluted samples.
However, the obtained results also appear to indicate that the dilution of samples with high biomass concentrations is more likely to affect the TA assessment. This can pose a problem since these samples are more likely to be diluted and as seen in this study the difference may more than double the non diluted sample.
Furthermore, the effect of assessing a diluted sample by image analysis cannot be predicted or quantified for all of the aggregated biomass as a whole, needing specific correction factors for each aggregates class, as shown in this study. Moreover, the correction factors were shown to depend on the dilution factor, and on the smaller aggregates and filamentous bacteria contents exposed in each dilution.

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REFERENCES

Amaral A.L. (2003). Image Analysis in Biotechnological Processes: Applications to Wastewater Treatment. PhD. Thesis, University of Minho - (http://hdl.handle.net/1822/4506).
Amaral A.L. and Ferreira E.C. (2005). Activated sludge monitoring of a wastewater treatment plant using image analysis and partial least squares regression. Anal. Chim. Acta, **544**, 246–253.
da Motta M., Amaral A.L., Neves L., Araya-Koff P., Ferreira E.C., Alves M.M., Mota M., Roche N., Vivier H. and Pons M.N. (2002). Efeito da Diluição na Caracterização da Biomassa de Sistemas de Tratamentos de Efluentes por Análise de Imagem. Proceedings of the 14th Brazilian Congress on Chemical Engineering, CD-ROM (p. 9), Natal (Brazil).
Grijspeerdt K. and Verstraete W. (1997). Image analysis to estimate the settleability and concentration of activated sludge. *Water Res.*, **31**, 1126–1134.
Jenné R., Banadda E.N., Smets I.Y., Deurinck J. and Van Impe J.F. (2007). Detection of Filamentous Bulking Problems: Developing an Image Analysis System for Sludge Composition Monitoring. Micros. Microanal., **13**, 36-41.
Martins A.M.P., Pagilla K.R., Heijnena J.J., and Loosdrecht M.C.M. (2004). Filamentous bulking sludge— a critical review. Water Res., **38**(4) 793-817.
Schuler A.J. and Jassby D. (2007). Filament content threshold for activated sludge bulking: Artifact or reality? Water Res., **41**, 4349-4356.