Validation of a new image analysis procedure for quantifying filamentous bacteria in activated sludge

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ABSTRACT

Quantification of filamentous bacteria in activated sludge systems can be made by manual counting under a microscope or by the application of various automated image analysis procedures. The latter has been significantly developed in the last two decades. In this work a new method based upon automated image analysis techniques was elaborated and presented. It consisted of three stages: (a) Neisser staining, (b) grabbing of microscopic images, and (c) digital image processing and analysis. This automated image analysis procedure possessed the features of novelty. It simultaneously delivered data about aggregates and filaments in an individual calculation routine, which is seldom met in the procedures described in the literature so far. What is more important, the macroprogram performing image processing and calculation of morphological parameters was written in the same software which was used for grabbing of images. Previously published procedures required using two different types of software, one for image grabbing and another one for image processing and analysis. Application of this new procedure for the quantification of filamentous bacteria in the full-scale as well as laboratory activated sludge systems proved that it was simple, fast and delivered reliable results.

Key words | activated sludge, filamentous bacteria, image analysis techniques, morphology, quantification

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INTRODUCTION

Filamentous bacteria make up backbones to which zoogleal (floc-forming) bacteria attach to form flocs (Bitton 2010). However, over-growth of filamentous bacteria contributes to the decrease of settling ability of sludge and, as a result, the aggravation of effluent quality (Bitton 2010; Jones & Schuler 2010; Guo *et al*. 2012; Mielczarek *et al*. 2012; Yang *et al*. 2013). The quantity of filamentous bacteria can be estimated by manual counting under a microscope or by the application of the automated image analysis procedures.

One of the first image analysis procedures was elaborated by da Motta *et al*. (2001a, b). It consisted of the following stages: (a) preparation of vital slides, (b) grabbing of images, (c) transformation of red–green–blue (RGB) images to the greyscale, (d) image pre-treatment, (e) segmentation, (f) discrimination between aggregates and filaments by the use of morphological filters, (g) discrimination between filamentous bacteria and debris based upon the size and reduced gyration radius, and (h) measurement of the total filament length per image, the number of filaments per image and the surface ratio filaments/flocs. The images were collected by a Meteor grabbing board, while the program for image processing and analysis was developed within the framework of Visilog 5 and called FlocMorph. The procedure was used for the monitoring of activated sludge in full-scale wastewater treatment plants (WWTPs) in Nancy–Maxéville in France (da Motta *et al*. 2001b) and in pilot-plant reactors (da Motta *et al*. 2001b, 2003). No global relation between sludge volume index (SVI) and the total filament length per image was established. Nevertheless, the increase of SVI was preceded by the increase of the total filament length per image.

Another image analysis procedure was elaborated by Amaral & Ferreira (2005). Initially, vital slides were made and images were grabbed with the help of a phase contrast microscope to obtain images of filaments. At the same time a stereomicroscope was used for imaging of aggregates. Image Pro Plus software was employed for grabbing of images. Then, two programs written in Matlab 6.5 were

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applied in order to calculate the descriptors of aggregates and filamentous bacteria. The main steps of the first program was the image pre-treatment, image segmentation and debris elimination, while the second program was mainly used for the determination of morphological parameters (Amaral & Ferreira 2005). This procedure was further modified and applied in many works presenting the results from laboratory as well as full-scale wastewater treatment plants (Mesquita *et al*. 2009a, b, 2010, 2011a, b). The modification dealt with the application of an individual bright-field image to enumerate both aggregates and filaments by means of two calculation routines programmed in Matlab (Mesquita *et al*. 2010).

In both procedures described above different software was used for grabbing of images and for image analysis. What is more, the images were snapped using various imaging techniques (phase contrast and/or bright field) and magnifications.

In this work a new procedure for quantifying filamentous bacteria and basic morphological parameters of aggregates in activated sludge is proposed. All steps of this procedure from grabbing of images to the calculation of the morphological parameters were performed with the use of the individual software. So the aim of this work was to present the proposed procedure and check its applicability for laboratory- and full-scale activated sludge systems.

METHODS

Activated sludge from the full-scale WWTPs

Activated sludge was taken from two full-scale municipal WWTPs, that is, the wastewater treatment plant in Zgierz and the combined wastewater treatment plant in Lodz, in which the average pollutant load to the plant corresponded to approximately 94,000 and 1×10^6 population equivalent, respectively. In the WWTP Zgierz the biological step consists of one five-zone bioreactor and a secondary clarifier running in the Phoredox process configuration, whereas in the combined wastewater treatment plant it consists of seven lines of three-zone bioreactors and a secondary clarifier running in the UCT (University of Cape Town) process configuration. Activated sludge was sampled once per month from the anaerobic and aerobic zone of the activated sludge chamber. The characteristics of activated sludge from both WWTPs studied is presented in Table 1.

Activated sludge from the laboratory system

The laboratory activated sludge system consisted of an aeration chamber coupled with a clarifier of total working volume equal to 7.8 l. The influent was synthetic wastewater prepared as described by Liwarska-Bizukojc & Bizukojc (2006). It was delivered continuously to the aeration chambers at different volumetric flow rates from 0.133 to 0.416 l h^{-1} and the effluent was also continuously removed. More details about the laboratory setup used here were presented elsewhere (Liwarska-Bizukojc & Bizukojc 2006). Four different hydraulic retention times (HRTs) were tested: 16, 21, 48 and 55 h. Activated sludge was taken from the aeration chamber when at least four HRTs had passed. The experiments were conducted at ambient temperature ($22 \pm 2\ ^\circ C$) and at constant aeration flow rate in each experimental run (0.321 vvm). Table 1 shows the properties of activated sludge from the laboratory system.

Image analysis procedure to measure filaments and aggregates descriptors

The procedure consisted of three stages: (a) Neisser staining, (b) grabbing of microscope images, and (c) digital image processing and analysis. First of all, a known amount (about 0.05 ml) of fresh activated sludge was put on the slide and smeared. Three independent fixed smears were prepared from each activated sludge sample. Then, they were stained

Table 1 | Characteristics of activated sludge systems

Characteristics	WWTP Zgierz	Combined WWTP Lodz	Laboratory activated sludge system
Total suspended solids (TSS) (g kg^{-1})	4.7–6.2	2.8–4.1	3.5–4.6
Volatile suspended solids (g kg^{-1})	2.5–4.6	1.8–2.9	1.9–3.2
SVI (ml g TSS^{-1})	44–101	90–246	80–96
Sludge retention time (d)	9–13.5	7.3–12.4	25–27
Mean projected area of aggregates (μm^2)	5100–11,340	6750–19,460	17,080–20,780
Mean diameter of aggregates (μm)	63–98	76–118	124–138

in accordance with Neisser method described by Eikelboom & van Buijsen (1992). As a result, Neisser-positive bacteria were blue, while the colour of Neisser-negative varied from yellow to brown. Staining protocols are recommended and widely used for the characterisation of filamentous bacteria (Eikelboom & van Buijsen 1992, Jenkins *et al*. 2003, Bitton 2010). They improve the visualisation of the objects of interest, that is, filamentous bacteria, and their quantification by image analysis techniques (Pandolfi & Pons 2004).

The dried slides were observed in bright field using a 10× objective (Nikon Eclipse N*i* microscope). Due to the application of Neisser staining and selection of the proper conditions of microscopic observation (magnification, enlightment) both aggregates and filaments were well visible (Figure 1). The RGB images were grabbed with the help of NIS Elements AR software (Nikon, Japan) and saved in *tif* format (1280 × 960).

It was checked how many images should be collected for the analysis in order to receive statistically reliable data. Thus, the changes of the mean value of TL/image and confidence bands with an increasing number of images taken for the analysis were studied (Figure 2(a)). It occurred that the collection of 45 images allowed us to receive stable values of the mean total filamentous length per image (TL/image) and ensured the reliability of the image analysis. Therefore, at least 45 images were snapped and saved from each activated sludge sample. Then, all of them were processed and analysed with the use of the macroprogram written in the internal macro-language of NIS Elements AR software and operating in the environment of this software. The procedure comprised the following stages: (a) segmentation of the blue plane of the image and selection of all objects, (b) removal of the objects that are out of interest (debris), (c) transformation of the greyscale image into the binary image and calculation of the area of aggregates and filaments, (d) application of morphological filters to obtain the binary image of aggregates (without filaments) and calculation of the area of aggregates, (e) obtaining of the binary image of filaments by the subtraction of the binary image of aggregates from the binary image containing both aggregates and filaments, and (f) skeletonisation of the binary image of filaments and calculation of the length of filamentous bacteria (Figure 1).

Segmentation was made in blue plane and as a result the greyscale image was obtained. A very important issue of the segmentation was to establish such threshold level so as to obtain the proper discrimination of the objects being analysed from the background. Due to differences in the enlightment of the observed field this threshold level was set individually but automatically for each image. The objects, which were neither flocs nor filaments, were removed automatically using the size and shape filters. It concerned the objects whose projected area was below 20 μm^2 or circularity was higher than 0.85. Additionally, the operator could remove an object manually, if required.

The 'purified' greyscale image was then transformed into the binary image. The value 0 (white) was ascribed to pixels belonging to the objects, while the value 1 (black) was ascribed to the background. The aim of further processing was to discriminate filaments from the aggregates. For this purpose the morphological filters were used. This was done in two steps. First, all filaments were removed from the binary image by means of the sequence of closing and opening (two passes). Both filters had a circular 12-pixel kernel. Next, the resulting binary image of aggregates was subtracted (logical XOR function, i.e. exclusive disjunction) from the image containing both aggregates and filaments. The result of this logical operation was a binary image of filaments. Any objects smaller than 5 μm^2 or of circularity higher than 0.65 were treated as debris and removed from it. Then, from the binary images with aggregates and filaments the mean projected areas of aggregates and filaments were respectively calculated. The binary image of filaments was further skeletonised and pruned to obtain one-pixel-wide filaments in the image. From this image the length of filaments was calculated.

Physicochemical analyses

Total suspended solids (TSS), volatile suspended solids (VSS), SVI and chemical oxygen demand were determined in agreement with standard procedures (APHA 2012).

Statistical measures

The basic statistical analysis comprising the calculation of mean values, standard deviations (σ) and confidence intervals of the measured morphological parameters was made with the use of MS Excel.

RESULTS AND DISCUSSION

In Table 2 the procedure elaborated in this study is compared to the two other procedures widely used for the quantification of filamentous bacteria in activated sludge

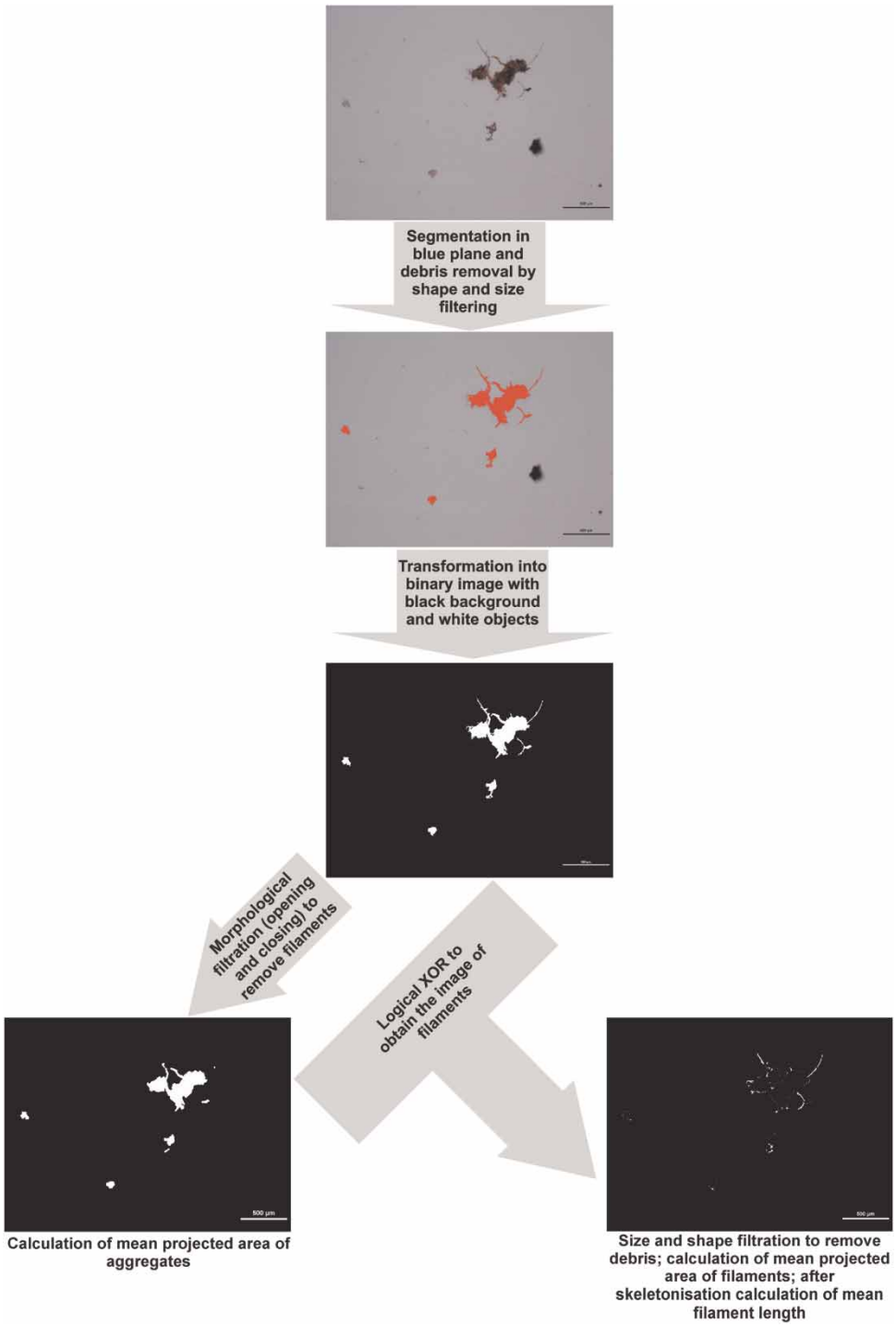

Figure 1 | Stages of the newly elaborated image processing and analysis procedure for filamentous bacteria quantification.

(da Motta *et al*. 2001b; Amaral & Ferreira 2005). A disadvantage of the procedure demonstrated here was the necessity of staining. However, the process of staining made the filamentous bacteria more visible and improved their detection by automated image analysis. It is beneficial for the quality of measurements. In this work the inexpensive and fast Neisser-staining was selected, nevertheless any other staining protocol that facilitates the contrast between all filamentous bacteria and background (e.g. Gram staining) can be helpful (Pandolfi & Pons 2004).

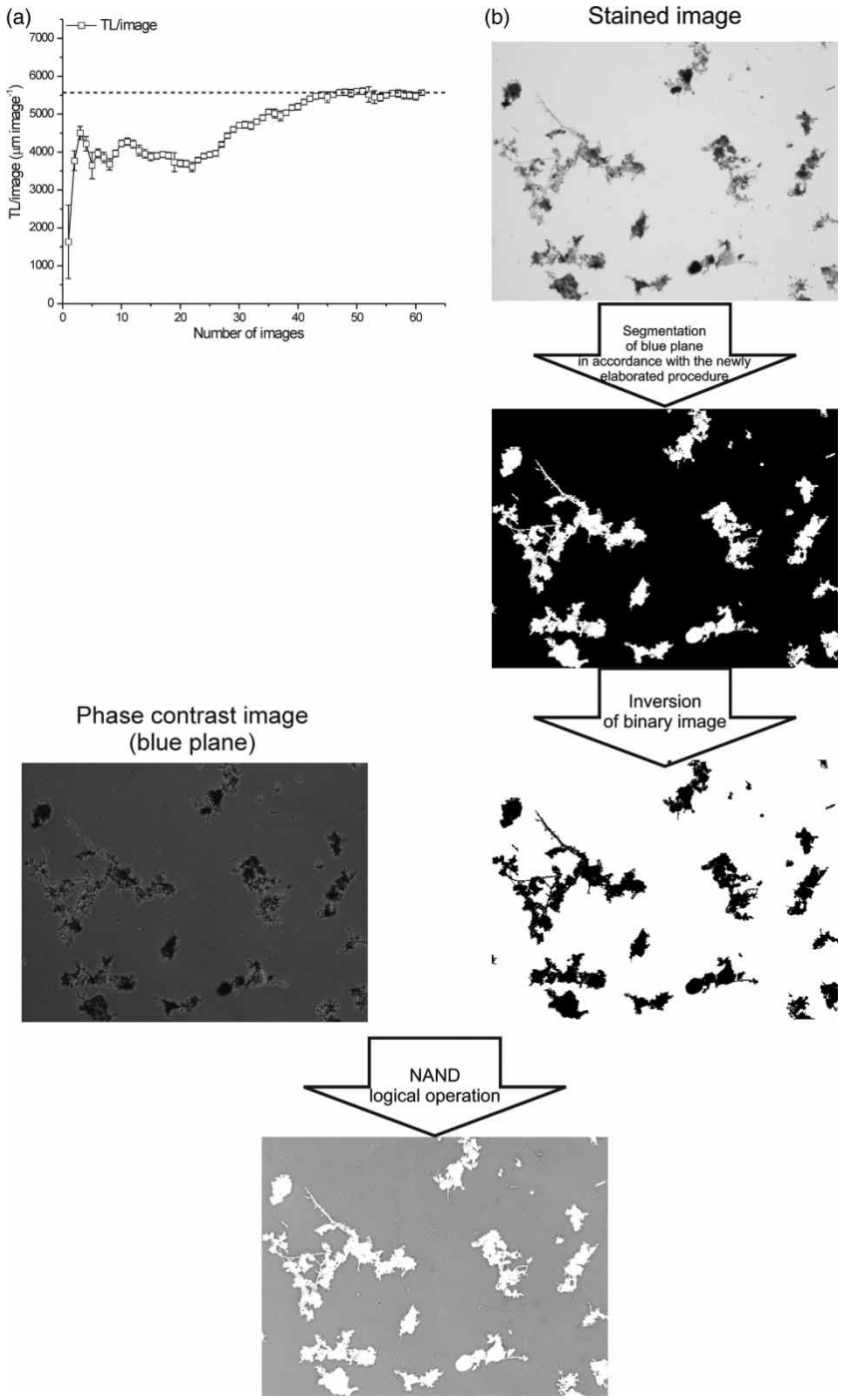

Figure 2 | Testing the reliability of the newly elaborated image processing and analysis procedure: (a) estimation of the sufficient number of images to be processed and analysed, error bars reflect the confidence bands calculated with Student *t*-test at 95% probability; (b) image analysis procedure to check the visibility of filamentous bacteria in the stained images.

The strength of the proposed procedure is the fact that it was designed to grab, process and analyse the images with the use of the individual software, that is, NIS Elements AR. As a result, it is simpler, cheaper and faster than the previous procedures.

Before the application of the proposed procedure to quantify filaments in different activated sludge systems, two issues were thoroughly studied.

Firstly, the number of images required to obtain the statistically relevant results was estimated (refer to Figure 2(a) and

Table 2 | Comparison of the automated image analysis procedures aiming at the quantification of filamentous bacteria in activated sludge systems

Feature	da Motta *et al.* (2001b)	Amaral & Ferreira (2005)	This work
Type of slide	vital	vital	stained
Conditions of microscopic observation (objective)	×10, bright field	×10, phase contrast	×10, bright field
Software use for image grabbing	Meteor Matrox	Image Pro Plus	NIS Elements AR
Stages of image processing	(1) image pre-treatment, (2) segmentation, (3) discrimination between aggregates and filaments using morphological filters, (4) discrimination between filamentous bacteria and debris using morphological filters, (5) measurement of morphological parameters	(1) image pre-treatment, (2) aggregate segmentation, (3) filament segmentation, (4) debris elimination, (5) measurement of morphological parameters	(1) segmentation, (2) removal of debris, (3) transformation of the greyscale image into a binary image, (4) application of morphological filters to obtain the binary image of aggregates, (5) subtraction to obtain the binary image of filaments, (6) skeletonisation of the binary image of filaments
Measured descriptors regarding filamentous bacteria	Total filament length per image; number of filaments per image; surface ratio of filaments/flocs	Total filamentous bacteria number (TL Nb); filamentous bacteria mean length (L); total filamentous bacteria length (TL); the filaments vs. aggregates content (TL/TA); TL vs. solids content (TL/TSS)	Total filamentous bacteria length per image (TL/image); total filamentous bacteria length per volume (TL/vol); the ratio of the area of filaments to the area of aggregates (Fil/Agg)
Software used for image processing and analysis	VisilogTM 5	Matlab 6.5 (two programs)	NIS Elements AR

'Materials and methods' section) by the adoption of the previously used methodology (Liwarska-Bizukojc & Bizukojc 2006). It occurred that the collection of 45 images ensured the reliability of the elaborated image analysis procedure. In the works of other authors the number of snapped and analysed images varied from 30 to even 200. For example, Amaral & Ferreira (2005) recommended the collection of 30 images, while Mesquita *et al.* (2011a, b) suggested grabbing of 150 images. Although the number of images in the aforementioned papers was often higher in comparison to this work, it was not explicitly shown that the used number of images was required for obtaining statistically relevant results.

Secondly, it was tested whether all filamentous bacteria, Neisser positive as well as Neisser negative, were detected in the course of the automated image processing and analysis. For this purpose, apart from the stained image made in bright field, the additional image of the same area of interest was made in phase contrast. In phase contrast images all filamentous bacteria are visible irrespective of whether they are stained or not. This imaging technique assures this. The above-mentioned stained image was subjected to the newly elaborated procedure; that is, a binary image with aggregates and filaments was obtained (Figure 2(b)). Next, this binary image was subjected to further operations. It was first inverted. As a result, the objects of interest turned black and the background turned white. The phase contrast image was neither processed by any filters nor segmented to avoid any changes in it. Only the blue plane was extracted from it. It should be mentioned that any plane of this RGB image could have been used because all elements were equally well visible in them. The aforementioned binary image and phase contrast image were finally subjected to NAND (not and) logical function. It caused that all black pixels from the binary image became white in the resulting image; namely all segmented objects from the stained image were cut from the phase contrast image. The background and any elements not detected by the segmentation remained in the resulting image. Looking carefully at Figure 2(b), it can be seen that the proposed staining and image processing procedure, including its segmentation, took into account practically all the required objects, that is, aggregates and, above all, filaments.

Different measures have been used for the quantification of filaments in the activated sludge so far. They

Table 3 | Measures of filamentous bacteria quantity. Literature data vs. this work

Filaments descriptor	da Motta *et al.* (2001b)	da Motta *et al.* (2003)	Amaral & Ferreira (2005)	Mesquita *et al.* (2011a, b)	This work
TL/image (μm image^{-1})	228–3734	100–8000	–	–	100–7500
TL/vol (mm μl^{-1})	–	–	10–120	1–100	1–17
Fil/Agg (%)	0–18	–	–	–	0–14

can be divided into two groups: direct and indirect. The first group includes, for example, total length of filaments per image, average length of filaments on the image, number of filaments per image, while the second one comprises total length of filaments per volume, total length of filaments to TSS, total filaments length per total area of aggregates. All of them were regarded as reliable and adequate to express the amount of filamentous bacteria in activated sludge (da Motta *et al.* 2001a, b, 2003; Mesquita *et al.* 2009a, b, 2011a, b). However, some of them were used more often than the other ones and, which is also very important, their values were presented explicitly in the published papers listed above. Taking this into account, three indicators for the quantification of filamentous bacteria were selected and calculated. These were the total filamentous length per image (TL/image), the total filamentous bacteria length per volume (TL/vol) and the ratio of filaments to aggregate area (Fil/Agg). The calculated values of these indicators of filaments in the activated sludge systems were compared with literature data (Table 3). It occurred that the values obtained with the help of the newly elaborated procedure were of the same order of magnitude as the values calculated due to application of the procedures published earlier (da Motta *et al.* 2001b, 2003; Amaral & Ferreira 2005). A more detailed comparison of the literature data with the ones obtained here is

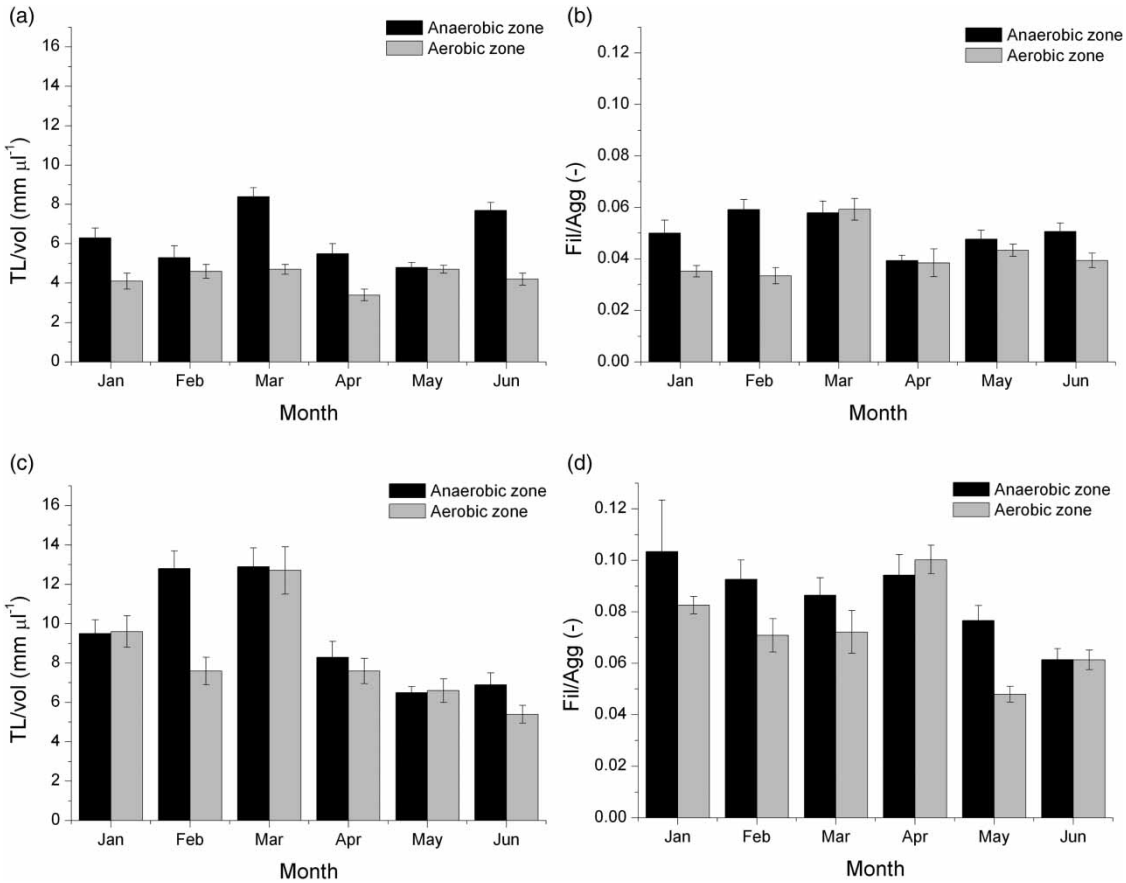

Figure 3 | Variations of TL/vol (± σ/2) and Fil/Agg (± σ/2) ratio in the WWTP Zgierz (a) and (b), and in the combined WWTP Lodz (c) and (d).

difficult due to the fact that different descriptors for the quantification of filaments in activated sludge were used by different authors.

The newly elaborated image analysis procedure was applied to estimate the quantity of filaments in two full-scale WWTPs (Figure 3). All selected descriptors of filaments, that is, TL/vol, Fil/Agg and TL/image (the latter not shown), indicated that more filamentous bacteria were present in the combined WWTP Lodz than in the WWTP Zgierz. It was in agreement with microscopic observations made by the operators and macroscopic parameters, for example, SVI (Table 1). The values of SVI were usually higher in the combined WWTP Lodz in comparison to the WWTP Zgierz and they often surpassed the limit of 150 ml g TSS^{-1} (Table 1). In both WWTPs tested, the quantity of filaments in the anaerobic zone exceeded usually their amount in the aerobic zone. This is connected with the fact that filamentous bacteria are characterised by lower values of oxygen saturation constants (about 0.01 mg O_2 l^{-1}) than the floc-forming bacteria (Bitton 2010).

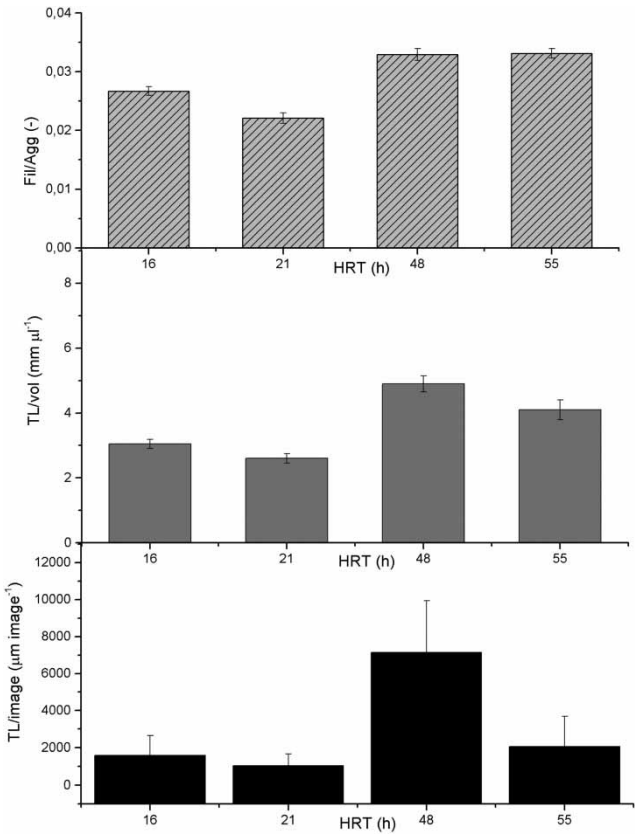

Figure 4 | Quantification of filamentous bacteria in the laboratory activated sludge system.

The elaborated procedure was also applied for the quantification of filamentous bacteria in the laboratory activated sludge system. It occurred that the character of changes of each of three descriptors, that is, TL/image, TL/vol and Fil/Agg, is similar, too (Figure 4). More filaments were found at longer HRTs. It was best reflected by the parameter TL/vol. Mesquita *et al.* (2011a, b) regarded this parameter (TL/vol) as the most adequate and it was best correlated with SVI out of several morphological descriptors used for the quantification of filamentous bacteria in activated sludge. More filamentous bacteria found at longer HRT were associated with the higher affinity for nutrients of several filamentous bacteria, that is, *Sphaerotilus natans*, *Thiothrix* sp. and Type 021N.1

CONCLUSIONS

Summing up, the elaborated procedure enables fast and reliable estimation of the quantity of filamentous bacteria in activated sludge. Its advantage, in comparison to the previously elaborated procedures, is that both aggregates and filaments are easily visible in the individual image. Owing to this, processing and analysis are performed simultaneously and operators may gain knowledge about the size of aggregates and quantity of filamentous bacteria. Moreover, in this procedure grabbing of images and image processing and analysis are performed using the same software. It makes the proposed procedure simpler, cheaper and easier for users compared to other available procedures.

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