In situ microscopy as a tool for the monitoring of filamentous bacteria: a case study in an industrial activated sludge system dominated by *M. parvicella*

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**ABSTRACT**

The present study demonstrates the application of in situ microscopy for monitoring the growth of filamentous bacteria which can induce disturbances in an industrial activated sludge process. An in situ microscope (ISM) is immersed directly into samples of activated sludge with *Microthrix parvicella* as dominating species. Without needing further preparatory steps, the automatic evaluation of the ISM-images generates two signals: the number of individual filaments per image (ISM–filament counting) and the total extended filament length (TEFL) per image (ISM–online TEFL). In this first version of the image-processing algorithm, closely spaced crossing filament-segments or filaments within bulk material are not detected. The signals show highly linear correlation both with the standard filament index and the TEFL. Correlations were further substantiated by comparison with real-time polymerase chain reaction (real-time PCR) measurements of *M. parvicella* and of the diluted sludge volume index. In this case study, in situ microscopy proved to be a suitable tool for straightforward online-monitoring of filamentous bacteria in activated sludge systems. With future adaptation of the system to different filament morphologies, including cross-linking filaments, bundles, and attached growth, the system will be applicable to other wastewater treatment plants.

**Key words** | filament index, image analysis, in situ microscope, light microscopy, total extended filament length

**INTRODUCTION**

Filamentous bacteria are considered to be the backbone for floc formation and serve as a structure to other floc-forming bacteria (Seviour & Nielsen 2010). Under specific conditions, these bacteria can outcompete and overgrow others, leading to poor sludge settling ability and poor compaction properties (Mesquita et al. 2011). Filamentous bulking and foaming are the most studied problems regarding this phenomenon (Jenné et al. 2001, 2007; Seviour & Nielsen 2010; Mesquita et al. 2011). Filamentous bulking occurs when a large number of extended filamentous bacteria, e.g. *Sphaerotilus natans*, ‘Candidatus Microthrix parvicella’ (*M. parvicella*) (Seviour & Nielsen 2010), generates inter-floc bridging and diffuse sludge flocs with poor settling characteristics. This situation is characterized by a high sludge volume index (SVI) > 150 ml g⁻¹ (Jenkins et al. 2004). Filamentous foaming is based on a flotation process induced by hydrophobic bacteria such as *Gordonia* spp., *M. parvicella* or Type 1863 (Jenkins et al. 2004, Paris 2004). Gas bubbles attach to hydrophobic surfaces and thus increase the buoyancy of sludge flocs. Surfactants or bio-surfactants can increase the hydrophobicity of sludge flocs and stabilize the foam layer due to micelle formation.

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Filamentous bulking and foaming always have negative economic and environmental consequences, hence the importance of better monitoring the filamentous bacteria.

Conventional optical microscopy has become a standard technique for the characterization of filamentous bacteria and microbial aggregates (Tchobanoglous et al. 2004). However, this technique is time-consuming and suffers from subjective interpretation (Belini et al. 2013). Over the past few years, the practical power of microscopic visualization has been much enhanced by combining it with image analysis (Seviour & Nielsen 2010; Mesquita et al. 2011) allowing a better and more reproducible evaluation of the process (Tchobanoglous et al. 2004). Previous studies have shown that image analysis is a feasible method for the identification of disturbances, e.g. bulking events (Jenné et al. 2001; Banadda et al. 2005; Mesquita et al. 2011). The development of online quantitative image analysis systems could dramatically change the usefulness of real-time process monitoring and controlling in wastewater treatment processes (Costa et al. 2013).

In this context, Koivuranta et al. (2013, 2014) demonstrated an imaging system for analysis of sludge flocs and filaments to evaluate their influence on sludge settling properties. This system uses dilution of the activated sludge sample with deionized water, thereby changing the matrix of the samples. Due to limited optical resolution of the Koivuranta system, bacterial filaments with diameters in the order of µm were not visualized. Jenné et al. (2001, 2004, 2007) applied a discontinuous microscopy setup to activated sludge samples which were diluted with filtered supernatants in order to obtain a constant mixed liquor suspended solids (MLSS) concentration of 1 g L⁻¹.

In this study, we aim to avoid dilution steps and to get sufficient resolution to visualize individual filamentous bacteria. Therefore, we apply a high resolution in situ microscope (ISM) combined with a suitable image analysis. The ISM is inserted into the activated sludge without any pre-treatment. Due to its high frequency of uncorrelated images, the ISM produces a large amount of image data per time (Wiedemann et al. 2011), leading to a small margin of statistical errors. Thus, a high sensitivity for detecting small changes in the amount of filamentous bacteria is to be expected. For reference, the total extended filament length (TEFL) by Sezgin et al. (1978) and the filament index (FI) by Kunst et al. (2000) is measured. Real-time polymerase chain reaction (real-time PCR) for the quantification of M. parvicella is used as additional reference. Activated sludge samples from an industrial wastewater treatment plant (WWTP) in Leverkusen (Germany) are used. In these samples, M. parvicella was identified as the dominant filamentous bacterium (see Appendix A, available with the online version of this paper) using fluorescence in situ hybridization (Dunkel et al. 2015).

### MATERIAL AND METHODS

#### Samples

Activated sludge samples were collected weekly at an industrial WWTP in Leverkusen, Germany (Currenta GmbH & Co. OHG) from the second biological treatment step (cascade biology). For real-time PCR analysis, samples were kept at −20 °C immediately after sampling.

#### Standard process monitoring parameters

The organic dry matter (oDM) was measured according to DIN EN 12879. Sludge settleability was evaluated using the diluted sludge volume index (DSVI; Jenkins et al. 2004).

#### Conventional microscopic techniques for filamentous counting

The FI by Kunst et al. (2000) was used to determine filamentous bacteria growing outside the sludge flocs: after crystal violet staining (carbol-gentian violet solution CN00.1, Carl Roth GmbH + Co. KG, Germany), images were obtained using dark field microscopy (10-fold magnification; Axiostar plus; Carl Zeiss Microscopy GmbH, Germany). The analysis of the samples was conducted in triplicates and 10 images were taken each time. An average value per sample was calculated and used for comparisons.

Standard total extended filaments length (TEFL) values were calculated according to Sezgin et al. (1978). A 30 mL sample was diluted in 1 L distilled water and stirred for 1 minute at 95 rpm. 20 µL of sample was transferred and analyzed in a Thoma chamber (Superior Marienfeld GmbH, Germany) at 100-fold magnification (Axiostar plus; Carl Zeiss Microscopy GmbH, Germany). The TEFL was calculated using Equation (1) and the measurements were performed in quadruplicates.

\[
\text{TEFL} \left[ \mu m \right] = \frac{\text{total extended filament length } \left[ \mu m \right]}{\text{sample volume } \left[ mL \right]} \quad (1)
\]
Experimental setup for *in situ* microscopy

The *in situ* microscopic monitoring of the samples was carried out using the ISM (Figure 1) previously described by Belini *et al.* (2015) and Wiedemann *et al.* (2011). In order to verify our findings, the ISM was used off-line. The front end of the ISM was submerged into the activated sludge sample (150 mL, without any pre-treatment) which was agitated by a magnetic stirrer at 500 rpm in a 200 mL beaker (see Appendices B and C, available with the online version of this paper).

**ISM and image acquisition**

The ISM is a transmitted light microscope. A pulse generates short (~3 μs) LED-pulses (wavelength = 660 nm) which are transmitted by an optical fiber. Short pulses are necessary for still imaging of moving objects (0.1 to 1 m/s) in bioreactors. The fiber ending is positioned approximately 0.3 mm above a quartz glass window separating suspension from objective. Particles in suspension flow through this gap, provided that their size does not exceed the gap’s width. An objective (40-fold magnification, numerical aperture 0.75) is attached to the inner tube, which is optically coupled to the quartz-window by water immersion.

The object field (0.16 × 0.22 mm²) is imaged onto a CCD-sensor (Basler A102f). Up to 10 monochromatic images per second are acquired (1,392 × 1,040 pixels resolution; pixel size 6.45 × 6.45 μm²).

**Image processing**

For this study, an algorithm was developed using the MATLAB image evaluation toolbox (Version 8.4, The MathWorks Inc., Natick, MA, USA, 2014). The algorithm generates two signals which monitor the number and the total length of filamentous bacteria. 500 ISM-images are analyzed within 7 minutes. This large amount of image data in a short period of time enables online-monitoring with small statistic uncertainty. Four groups of operations are applied to each image, as follows.

**Enhancement and binarization**

The original image is smoothened by 2D Gaussian filter. Subsequent contrast enhancement is achieved by a combination of top-hat and bottom-hat operations. The resulting image is shown in Figure 2(a). This image is binarized (Figure 2(b)) through variance thresholding, so that all remaining objects appear as regions of connected white pixels on a black background.

**Removal of bulky structures**

A value is assigned to each white pixel, corresponding to the distance between that pixel and the nearest background pixel of the image. As a result, bulky kernels present higher values (Figure 2(c), in red) than thinner objects. Thresholding generates binarized images containing only
bulky objects, somewhat eroded. The original floc-shape is approximately recovered by morphological dilation and then subtracted from the previously binarized image obtained in step 1. The result is a binarized image containing only filaments and some noise components (Figure 2(d)).

**Skeletonization and reduced radius of gyration filtering**

Skeletonization by morphological operations results in suitable objects for length measurements. Skeleton branches are eliminated using a pruning technique. Amongst the remaining lines of pixels (Figure 2(e)) there are some which originate not from filaments but from sharp borders of bulky objects. They tend to change direction more often than the skeletons of true filamentous species. To eliminate such objects, their reduced radius of gyration (RRG; Jenné et al. 2001) is computed. After thresholding the RRG, the remaining skeletonized objects are likely to represent filaments (see Figure 2(f)). The RRG criterion is disadvantageous insofar as it tends to eliminate crossing filament-images because they represent different directions and therefore generate smaller RRG values (see Appendices D and E, available with the online version of this paper). A potential alternative is to implement a neural network trained for the detection of such image-objects. However, we found the RRG to be a sufficient criterion for this first study (see also below). Lastly, the length of the detected filament-segments is computed by adding the Euclidean distances between pixel centers.

The final image of detected filaments (Figure 2(e)) shows that the algorithm identifies segments of long curved individual filaments which randomly drift through the sharply imaged object field of the ISM. Representing long and curved filaments by one or more detected partial segments instead of depicting them as a whole is an intrinsic feature of contact-free in situ microscopy. Another feature of the present stage algorithm is the above-mentioned exclusion of closely spaced cross-linking filaments. However, this principle of measurement is based on the fact that the statistical distribution of detected segments is caused by the true filament distribution. Therefore, the measured histogram of filament-lengths monitors the true/real amount of individual filamentous bacteria.

**Quantification of detected filament-segments**

Each ISM-image generates a number of filament-segments and their corresponding length-values. Two ISM-signals are computed:

(a) ISM-filament counting (ISM-FC): the mean of the number of filaments per ISM-image;
(b) ISM-online TEFL (ISM-oTEFL): the mean of the total extended length of filament segments per ISM-image.

Quantification of *M. parvicella* using real-time PCR

DNA extraction

Genomic DNA was isolated from activated sludge samples using the FastDNA SPIN Kit for Soil (MP Biomedicals, Eschwege, Germany) following the manufacturer's instructions.

Real-time PCR

Quantification of *M. parvicella* was performed with the 16S rRNA primer set S-S-M.par-0828-S-21 (5'GGTGTTGGGA-GAACTCAACTC-3') and S-S-M.par-1018-A-17 (5'-GACCCGAAGGACACCG-3') (Kaetzke et al. 2005) using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad; Munich, Germany). Method evaluation was based on the MIQE-guideline (Bustin et al. 2009). Details: initial denaturation (95°C, 5 min); 40 cycles of denaturation (95°C, 0.5 min), annealing (54.5°C, 1.25 min), amplification (72°C, 1 min); and final amplification (72°C, 10 min). Reactions (in triplicate): 25 µL (5 µL template, 12.5 µL MESA GREEN qPCR Master Mix Plus (Eurogentec, Belgium), 1 µL of each primer (100 mM; 4 mM MgCl₂), 5.5 µL nuclease-free water). The calibration curve for the quantification of *M. parvicella* showed an efficiency of 106.6% and a linearity of $R^2 = 0.995$ (see Appendix F, available with the online version of this paper). Level of detection and level of quantification were 20 GU µL⁻¹ and 200 GU µL⁻¹, respectively.

For *M. parvicella* quantification, a customized Micro-thrix-DNA-standard (Life Technologies; Darmstadt, Germany) containing $3.4 \times 10^{10}$ GU µL⁻¹ was used. Genomic units (GUs) were calculated as described by Vanysacker et al. (2014) considering a plasmid length of 2.674 bp and the DNA concentration of 0.1 µg µL⁻¹ for the standard.

Statistical analysis

To evaluate the significance level of correlations of ISM-signals and reference data, a single factor analysis of variance is applied using the statistical software tool R (version 3.1.0). The Granger causality test, performed in R (version 3.1.0) using the package lmtest (Zeleis & Hothorn 2002), is carried out to evaluate the similarity of ISM signals to sludge settleability related parameters.

RESULTS AND DISCUSSION

Filament counting and length-measurement

The main subject of this study was to validate the ISM-signals (ISM-FC and ISM-oTEFL) in comparison to the standard microscopic measurements FI and TEFL.

The first ISM-signal represents the average number of filament-segments per image (ISM-FC). Figure 5 (left) shows ISM-FC and FI values from February 2014 to March 2015. A linear relationship between the two data sets is indicated by a Pearson correlation coefficient of $r = 0.74$. Both signals FI and ISM-FC exhibit a similar trend. High abundance of filamentous bacteria in February is followed by a decrease until the beginning of April. Afterwards, two local maxima were observed in June and October. A dramatic proliferation of filamentous bacteria began at the end of November 2014. Within three months, it caused foam to rise from 0 to 0.88 m, resulting in an overflow from the cascade biology basin to the surrounding area. The Granger causality test further substantiated the relationship of ISM-FC and the height of the foam layer with a $P$-value of 0.023, suggesting that the ISM-FC can be used for monitoring the foam formation.

The second ISM-signal represents a measurement of the average TEFL per image (ISM-oTEFL). It is displayed in Figure 4 (left) in comparison with the conventional TEFL. Both signals display a similar trend: During the first month, the values are decreasing. Two maxima appear in September and in October. Similar to the ISM-FC signal, also the ISM-oTEFL signal monitors a dramatic increase of filamentous bacteria in the last three months of the observation period, which agrees with the conventional TEFL measurements. The linear relationship between both signals is confirmed by a Pearson correlation coefficient of $r = 0.87$.

As additional reference method for the assessment of in situ microscopy, a real-time PCR SYBR green assay for determining *M. parvicella* was carried out. In Figure 4 (right) the ISM-oTEFL, normalized with oDM is compared to the *M. parvicella* concentration per total DNA. With the exception of one data point in December, both signals show similar trends including the strong increase in *M. parvicella* from January 2015 to March 2015 in agreement with the foaming event. The Pearson correlation coefficient of these two signals is $r = 0.55$. 
The relationship of the TEFL with the SVI was first investigated by Sezgin et al. (1978), Palm et al. (1980), and Lee et al. (1982). These studies underline the influence exerted by filamentous bacteria on the sludge-settling behavior. Finstein & Heukelekian (1967) pointed out that the quotient of TEFL value per sludge floc should be used for correlation with the SVI. Banadda et al. (2005) showed the positive correlation of SVI and TEFL for the first time, using conventional microscopy combined with image analysis. The DSVI, a modification of the conventional SVI, was proposed by Stöbbe (1964).

Figure 5 shows a comparison of the ISM-oTEFL-signal (after normalization with the oDM) with the measured DSVI. Similar trends in both signals were obtained, resulting in a Pearson correlation coefficient of $r = 0.7$. In particular, the increase of filamentous bacteria at the end of the measurement period is monitored by both signals. By applying the Granger causality test it was found that the normalized ISM-oTEFL is Granger causing the DSVI ($P = 0.0026$), which indicates that \textit{in situ} microscopy can be used to monitor the sludge settling properties.
Seven independent biological samples \( (n = 7) \) were used in order to determine ISM-signals and reference signals (FI and TEFL) simultaneously, with confidence intervals (CIs) of 95%. The relative uncertainty of each signal was computed by dividing the half width of the CI by the mean value of the seven sampled values. In doing so, we obtain 4.4% relative uncertainty for the ISM-FC, 2.2% for the FI, 4.3% for the ISM-oTEFL, and 12.4% for the TEFL. The low relative uncertainty of the FI was surprising, given the subjective scale used for this measurement. Potential sources of deviations in all signals are sampling, preparation and manual measurement.

Future online application of an ISM will make sampling obsolete and should reduce the uncertainty to deviations between image-samples. Due to the large amount of image data, e.g. 500 images per data point, the ISM-CIs are expected to be markedly smaller than the CIs presented here.

**CONCLUSIONS**

In this study, the application of an ISM combined with image analysis for specific detection of filamentous bacteria (mainly *M. parvicella*) is shown. Two signals were extracted from the ISM-images: the filament count per image (ISM-FC) and the TEFL per image (ISM-oTEFL). They showed positive correlation when compared to the conventional filament counting parameters TEFL and FI. Moreover, significant correlations were obtained with the DSVI and the amount of *M. parvicella* assessed by real-time PCR. These results support the suitability of ISM data for monitoring disturbances in the activated sludge process. This performance is also expected in other WWTPs with different dominant filamentous species which generate a certain amount of individual filaments outside of bulk material. These filaments can be detected by a filament-specific algorithm, optimized with respect to specific morphologies and indices of refraction.

In order to enhance the filament-caused ISM-signal, we aim at developing the algorithm so that it also detects cross-linking filaments, filament bundles and filaments with attached growth. Apart from filamentous bacteria, the floc size, floc shape and stability of the sludge flocs influence sludge settleability. Further ISM studies will include these parameters in the image evaluation. Measures against bulking and foaming of activated sludge in WWTPs might thus be supported by an online monitoring system based on *in situ* microscopy.
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