Improvement of the intersection method for the quantification of filamentous organisms: basis and practice for bulking and foaming bioindication purposes

Humbert Salvadó

ABSTRACT

Bulking and foaming phenomena in activated sludge wastewater treatment plants are in most cases related to the abundance of filamentous microorganisms. Quantifying these microorganisms should be a preliminary stage in their control. In this paper, the simplicity of quantifying them based on the intersection method is demonstrated, by redescribing the theory and applying a new improved protocol; new data of interest are also provided. The improved method allows us to use it with stained smears, including epifluorescence techniques. The error that could be made, when considering the distribution of filamentous bacteria in fresh microscope preparations in two dimensions rather than three is negligible. The effect of the different types of filamentous microorganisms on the settleability was also studied. The effect of the total extended filament length on the sludge settleability was shown to depend on the type of filamentous organism and how it aggregates. When these groups of filamentous organisms are found in small aggregations and there is an increase in the number of filamentous organisms, the sludge volume index (SVI) increases proportionally to the filament length. However, when aggregation increases, the impact on the SVI is significantly lower.

Key words | activated sludge, sludge volume index, total extended filament length

INTRODUCTION

Massive increases in filamentous organisms lead to serious problems in activated sludge wastewater treatment plants (WWTPs). One of the main problems is due to the bulking phenomenon, which has been defined as a massive increase in particular species of filamentous microorganisms that form three-dimensional networks, which obstruct the sedimentation process and lead to high values of the sludge volume index (SVI) (Sezgin et al. 1978; Palm et al. 1980; Eikelboom 2000). Another problem is the formation of foam caused by an increase of certain filamentous microorganisms. Different types of filamentous organisms have different effects on sludge settleability and may or may not be related to foam formation (Wanner 1994; Jenkins et al. 2003). Quantification of the filamentous organisms which cause these problems is vital for proper management in WWTPs (Wanner & Grau 1989; De Los Reyes et al. 1997).

Different techniques have been developed for measuring or estimating the abundance of filamentous organisms. Pipes (1979) determined the number of filamentous organisms using a Neubauer chamber, by establishing the relationship between the number of filaments found and the SVI observed. Meanwhile, Sezgin et al. (1978) proposed a method which involves size classification to measure the length of each individual filament. This latter method is accurate and enables the filamentous organisms to be expressed as a length/volume, but it is extremely laborious. Jenkins et al. (1986) described a technique which provides relative results expressed as intersections per unit volume, based on counting the filament intersections found along a line drawn on the eyepiece. This technique can be quite accurate; however, the results are not expressed in units of length. Using this technique as a basis, Pitt & Jenkins (1990) developed a technique for counting Gordonia amarae (formerly Nocardia amarae), which consisted of counting the number of branched Gram-positive filaments that were more than 1 μm wide in a diluted Gram-stained
sample of mixed liquor. The result can also be expressed as intersections per unit volume. The identification and in situ detection of filamentous bacteria in activated sludge using fluorescent labeled oligonucleotide probes have been reported for some filamentous bacteria: Gram-negative bacteria (Wagner et al. 1994), and Nocardiaceae and Mycobacteriaceae actinomycetes (De Los Reyes et al. 1997). The last technique cited involves a laborious procedure and large-scale equipment; although quantification is usually good, with accurate identification of the filamentous species following an image analysis procedure (Liao et al. 2004). Image analysis, with or without epifluorescence methods, is a promising technique for quantifying filamentous bacteria (Mesquita et al. 2013; Liwarska-Bizukojc et al. 2014), although specific software is needed attached to camera, to count the samples. Finally, only a few morphotypes can be distinguished in a single slide.

A previous study by Salvadó (1990) shows that the abundance of the different filaments expressed in lengths could be obtained from the intersections between the filaments. The basic equipment required is a compound microscope without any special accessories, such as a camera or computer required for other methods. The length can be converted into total biomass and then compared with results from other techniques. In contrast with epifluorescence techniques, this technique allows morphological patterns to be differentiated in living samples. However, the aggregated spread of filaments could make the counting difficult. Aggregation could also cause variations in the relationships of filaments of sludge settleability. The combination of in situ hybridization with fluorescent-labeled rRNA probes and filament intersection counting, as explained here, will improve the currently available tools for analyzing filamentous organisms in wastewater treatment processes.

Thus, the aims of the present study are to improve the technique for quantifying the total extended filament length, to determine the relationship between different filamentous organisms, aggregation and sludge settleability, and to provide a straightforward and comprehensive method for research and WWTP control.

MATERIAL AND METHODS

Sampling

A total of 290 samples were collected over a 4 year period from five conventional urban WWTPs in Catalonia, NE Spain: four from the metropolitan area of Barcelona and the fifth from the city of Reus. The WWTPs had mean cellular retention times of 2.5 to 15 days, between 0.80 and 3.50 g MLVSS l⁻¹ and 1.5–3 mg DO l⁻¹ (MLVSS: mixed liquor volatile suspended solids; DO: dissolved oxygen). Periods when chlorine or ferric chloride were added for bulking control were taken into account. To analyze the sludge settleability, the parameters Settling Volume 30 (SV30) and SVI were used. Filamentous organisms were identified according to Eikelboom (2000), Jenkins et al. (2007) and Seviour & Nielsen (2010).

Basis of the technique

The technique used, the basis of which is described by Salvadó (1990), is based on the relationship between the average number of intersections between filamentous organisms and the length of the segment along which they are counted. Some improvements have been made; here a more accurate method of application is described and some problems that may arise are commented on.

The theoretical basis of the method is summarized in the fact that the average number of intersections of a series of parallel lines with a perpendicular rectilinear segment (S) is directly proportional to the number of lines or the length...
of S. So more intersections are observed as the length of the segment or the number of lines is increased (Figure 1(a) and 1(b)). The density of the lines is determined by moving S, of known length, at random, but always perpendicularly, as the quotient: average number of intersections/segment length.

If S and the parallel lines are not perpendicular, the number of intersections decreases (Figure 1(c)). In other words, the angle (α) between the parallel lines and S must be taken into account; in each case, the effective length of S will be H, which is obtained by multiplying the length of the segment by the sine of the angle (α). However, if we consider that the distribution of lines and segments is random, the mean value of sin α between 0 and π/2 rad (90°) or π rad (180°) is calculated by means of the integration between 0 and π/2 rad, and is: 2/π = 0.63662.

\[ \int_{0}^{\frac{\pi}{2}} \sin \alpha \, d\alpha = \frac{2}{\pi} \]

A straight line of known length could be used as S; however, if the filaments are parallel, semi parallel or not distributed at random with respect to the angle of S, the measurement would be incorrect (Figure 2(a)). To solve this problem, a circumference can be used as S, the segment of known length, since it possesses all the angles with equal probability and can be used to measure lines or filaments distributed in any arrangement (Figure 2(b)). So for the same density, lines that have the same number of intersections will be the same length, independently of whether the lines are parallel, distributed at different angles, straight or curved. The circumference of the field of vision of the microscope can be used as the segment of known length (Figure 2(a) and 2(b)).

The specific area under study must always be defined when the length of the filaments is calculated. This area (A), in the case of microscopic analysis of water samples, may be, for example, the size of the coverslip (Figure 2(c)). The total length of filaments can be calculated using Equation (1):

\[ \text{Total length of filaments} = \frac{N_i \cdot A}{H} \]  

(1)

\[ N_i = \text{average number of intersections} = \frac{\text{number of intersections}}{\text{number of observed fields}} \]

\[ A = \text{area} \]

\[ H = (\text{length of S}) \times (\text{average sine } \alpha) = (L) \cdot \frac{2}{\pi}. \]

Checking the basis of the theory

I now report a practical method for simply checking the theory.

1. A sheet of lined paper (Figure 3) was selected, in this case with 5 mm between the lines (the usual spacing of lined paper).
2. A = the area of this sheet of paper; in this case, for DinA4, A = (210 mm × 297 mm).
3. TL = the total length of all the lines on the sheet; 
   \[ TL = (210 \text{ mm}/5 \text{ mm}) \cdot 297 \text{ mm} = 12,474 \text{ mm} . \]

4. Then circles of known diameter (in this case 50 mm) were drawn on the paper, positioned at random on the sheet: 
   \[ \Omega = 50 \text{ mm} \] (Figure 3), where 
   \[ H = (2\pi) \cdot (2/\pi) = 2\Omega . \]

5. Equation (1) was applied to find \( N_i \); remembering that 
   \[ N_i = \text{average number of intersections that can be found} \]
   between each circle and the lines.

\[ \text{Total length of lines} = \frac{N_i \cdot A}{H} = \frac{N_i \cdot A}{2\Omega} = \frac{N_i \cdot 62,370 \text{ mm}^2}{100 \text{ mm}} = 12,474 \text{ mm} \]

6. As observed, \( N_i = 20 \) intersections on average. We can also calculate the average number of intersections in the drawing; in this case what we observe is that a circle crosses each line twice.

Using the microscope, an alternative way to check the basis of this method would be to measure the length of the grids of a Neubauer chamber or other similar device.

**Area and volume**

The results are given in units of length. The next step is to consider the number of filaments in a given area, or volume, giving a final result in Length/Area or Length/Volume. In the case of water samples, it can be referred to a specific volume (\( V_m \)). The total length of filaments/Volume can be calculated using Equation (2):

\[ \text{Total length of filaments/Volume} = \frac{N_i \cdot A}{H \cdot V_m} \]

in which:

- \( N_i \) = average number of intersections = number of intersections/number of observed fields
- \( A \) = area occupied by the ‘drop’ sample (the coverslip)
- \( H \) = (length of segment drawn on the microscope eyepiece) \times (average \( \sin \alpha \)) = (\( L \)) \cdot (2/\pi)
- \( V_m \) = volume of the drop of sample.

**Depth**

When the sample is placed between the slide and the coverslip, a new angle appears, expressed as the depth of field, so 
\[ H = (L) \cdot (\text{average } \sin \alpha)^2 . \]

The space between the two glass slides in a fresh sample may be some tens of micrometres. In this space, the filaments may be arranged in three dimensions; however, we have observed that filaments tend to look flat, either because of their weight or because some stick to the surface of the coverslip as in the case of nocardiform actinomycetes or Type 1863. Figure 4 shows the possible effect of the angle on the underestimation of length.

However, to determine the angle of the filaments with respect to the axis of the depth, several repetitions were performed. Twelve series of photographs were taken in different fresh sludges. In each series, 70 consecutive photographs were taken along the depth axis. To prevent desiccation, the samples were sealed with acrylic nail polish. Between each consecutive photograph, one micron was moved in the depth field by an automated Z-axis step. Then, all the filaments in the photographs (as a two-dimensional projection on the plane X Y-axis) were manually measured. Short filament portions, from 5 to 30 \( \mu \)m, of each filament were measured taking depth variation between the ends as a variable. Using classic trigonometry, the angle of each short portion of the filament was calculated. This measurement is repeated until the whole length of each filament is measured.

Four types of filamentous bacteria were analyzed. The results show that the average filament angle ranges from 6 to 16° from the horizontal plane of the slide. According to the
type of filament, the distribution of each portion of each type of filament is summarized in Table 1 and Figure 5.

The variation in the angle of the Z-axis represents an underestimation of just 2% to 9%. This angle (on average) increases slightly with the presence of dense bundles with coiled filaments to $10^\circ$, as in Type 021N. Moreover, to prevent bundle density, it is advisable to dilute the samples when the number of filaments in the sample is high. In any case, in the counting protocol, the samples are not necessarily sealed, and the water quickly evaporated, about 50% of the water evaporated in 20 minutes under conditions of 20°C and 25%–45% moisture, reducing the thickness of the water sheet and enhancing the underestimation error greatly. For these reasons, Equation (2) was used. In addition, if we count in Gram- or Neisser-stained smears, the angle is much smaller than its counterpart when using fresh samples, and the underestimation is negligible, even in Gordonia amarae-like organisms (GALO) <1%.

**Application of the method for measuring filamentous organ abundance**

1. Twenty microlitres of a well-mixed liquor sample was transferred to a slide.
2. The slide was completely covered with a $20 \times 20$ mm coverslip, depending on the volume used. In order to spread the microorganisms in the sample homogeneously, the slide was moved in circles just before the coverslip was placed over it. Any badly spread samples, in which sludge is seen to have accumulated in a particular area, were discarded. Any liquid not covered by the coverslip is a potential source of error. To remove it, thin coverslips, <0.16 mm thick, were used and the coverslip was placed carefully. (Leftover liquid was quantified, and never exceeded 0.5% of the total volume.)
3. Using non-immersion lenses and a phase contrast microscope, in this case with ×400 magnification, several fields

![Figure 5](https://iwaponline.com/wst/article-pdf/74/6/1274/458641/wst074061274.pdf)

**Table 1 | Average angle between filaments and slide, according to the type of filamentous bacteria**

<table>
<thead>
<tr>
<th>Type</th>
<th>Average angle (min and max)</th>
<th>Underestimation of length in %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microthrix parvicella</td>
<td>7.7 (0–42')</td>
<td>2.04</td>
</tr>
<tr>
<td>Type 021N</td>
<td>7.1 (0–55')</td>
<td>1.62</td>
</tr>
<tr>
<td>Type 1851</td>
<td>6.7 (0–50')</td>
<td>1.3</td>
</tr>
<tr>
<td>GALO</td>
<td>16 (0–72')</td>
<td>9.07</td>
</tr>
</tbody>
</table>

(some 15–30) were observed. The fields were well distributed randomly across the area of the entire coverslip. The number of intersections with the line of the circle was counted for each filament type.

4. The average number of intersections was calculated for each filament type and Equation (2) was applied. The results were expressed in metres of total extended filament length per millilitre.

5. For greater accuracy, this operation was repeated several times. In routine WWTP checks, two sub-samples are enough; but for this study, at least four samples, with a total of 60 fields, were taken. The samples were diluted when they were particularly dense forming co-axial bundles or more than 300 mm ml$^{-1}$. In this case it was enough to dilute the sample 1:2 or 1:4. If the count did not improve (which never happened in this study) we would then have a minimum value and so the result could be expressed as: ‘over x.’

During the count, when distinguishing types of filamentous organisms was difficult, the overall number was calculated at ×400, and later the percentage of each type was counted at a higher magnification; usually ×1,000, either using fresh samples or fixed Gram or Neisser staining. The samples were stained in some cases in order to count more easily; in which case, the sample was spread homogeneously over a known area on a slide, as suggested by Pitt & Jenkins (1990). The count was then performed using immersion lenses, following the steps described above. Since the aim of this method is to quantify the filamentous forms, when some filamentous bacteria fragment into individual cells, as can occur in the actinobacterial Mycolata GALO, for example, (Seviour & Nielsen 2010), the individual cells obviously are not included as filaments because they no longer are.
Application for stained smears or FISH to quantify filamentous bacteria

Two alternatives for stained smears or the fluorescence in situ hybridization (FISH) application are known; however, here, they were not necessary. Intersections can be counted on the stained smears, for example, Gram and Neisser, positive or negative. In this case, however, the entire area occupied by the extent of the drop of sample has to be considered. Then a drop of distilled water is added to the slide and covered with a large, 24×60 mm, coverslip. Clearly, the entire sample placed on slides must remain within the area the coverslip covers. Then, we should move across the area of the coverslip randomly by applying the same steps described above. In this case, we must include the volume of the sample used for the smear (for example 0.02 or 0.04 ml) and the surface of the coverslip. The second application is when using FISH, taking into account the volume of and the area occupied by the sample; so we can obtain the intersections inside or outside the flocs. Then we can distinguish between the total extended filament length and the total filament length, as proposed by Liao et al. (2004). As a complementary technique, if we use alternatively the phase contrast and epifluorescence microscopy, we can quantify the proportion of the filaments that emit fluorescence.

Statistical data analysis

Aggregation

As a measurement of accuracy in the study of aggregation, the coefficient of variation was calculated on the basis of the results for each microscope field. The average number of intersections in each field and the standard deviation of the 60 fields counted for each sample were used. The coefficient of variation was expressed as a percentage thus: (100-standard deviation)/(average).

Correlations

Data were analyzed statistically using the Statgraphics Centurion XVI software package. Normality of the variables was determined using the Shapiro Wilks test. The Spearman correlation coefficient was used to test the statistical relationship between the total extended length of filamentous microorganisms and settling parameters (SVI, SV30). Statistical significance was considered at \( p \leq 0.01 \).

RESULTS AND DISCUSSION

In the samples analyzed, the most frequent filamentous bacteria observed were Type 021N, Haliscomenobacter hydrossis, Type 1701, Microthrix parvicella, Type 1851, Type 1863 and GALO. Other types of filamentous organisms appeared sporadically.

As Sezgin et al. (1978), Palm et al. (1980) and Lee et al. (1982) attempted on previous occasions, this study aims to relate the total extended length of filamentous organisms to the SVI. However, the analysis of all 290 samples did not give a high correlation coefficient (Table 2). This problem was noted by Salvadó (1990), Liao et al. (2004) and Schüler & Jassby (2007). Not all species of filamentous bacteria affect the SVI in the same way; so their morphology could be the cause of the low correlation. In order to observe the specific effect of each type of filamentous organism on the SVI, sludge samples with only one species should be analyzed. However, it is difficult to obtain samples with only one type of organism from full-scale WWTPs. So,

**Table 2** | Results of the Spearman correlations between total extended filament length (F) and SVI or SV30.

<table>
<thead>
<tr>
<th>Type of samples</th>
<th>All samples</th>
<th>Less than 5%</th>
<th>Less than 5%</th>
<th>Less than 5%</th>
<th>Less than 5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>GALO or Type 1863 abundance in %</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Additional chemicals</td>
<td>with</td>
<td>with</td>
<td>without</td>
<td>without</td>
<td>without</td>
</tr>
<tr>
<td>Coefficient of Spearman correlation</td>
<td>r</td>
<td>r</td>
<td>r</td>
<td>r</td>
<td>r</td>
</tr>
<tr>
<td>SVI and F</td>
<td>F in m ml(^{-1})</td>
<td>0.23</td>
<td>0.64</td>
<td>0.66</td>
<td>0.77</td>
</tr>
<tr>
<td></td>
<td>F in m mg(^{-1})</td>
<td>0.55</td>
<td>0.72</td>
<td>0.72</td>
<td>0.88</td>
</tr>
<tr>
<td>SV30 and F</td>
<td>F in m ml(^{-1})</td>
<td>0.49</td>
<td>0.67</td>
<td>0.7</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>F in m mg(^{-1})</td>
<td>0.51</td>
<td>0.59</td>
<td>0.69</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Values of SVI in ml g\(^{-1}\), SV30 in ml l\(^{-1}\), and filamentous microorganisms in m ml\(^{-1}\) or in m mg\(^{-1}\). CV – Coefficient of variation as a percentage. \( p < 0.01 \).
samples in which one type of filamentous bacteria predominated, with a relative abundance of over 90%, were selected and related to the SVI (Figure 6). It can be observed that the increase of filamentous bacteria implies a gradual rise in the SVI up to 200 m g$^{-1}$. From 200–400 m g$^{-1}$, there was a clear separation into two groups. Organisms which cause bulking, such as Type 021N, Haliscomenobacter hydrossis or Microthrix parvicella, continued to make the SVI rise while their total length increased. However, with the nocar-dioform organisms GALO, only slight increases occurred compared to the former three types. Type 1863 should be included in this second group, as the SVI remained below 270 ml g$^{-1}$ when more than 90% of the filaments belonged to Type 1863. This SVI value matched the maximum abundance of this filament at 400 m g$^{-1}$. However, abundance of Type 1863 of over 300 m ml$^{-1}$ resulted in very poor quality effluent, due to the large number of free and suspended filaments.

Secondly, the relationship between the total extended filament length and the SVI was studied, regardless of the species found in the sample. Taking into consideration the above results, to analyze the interrelationship between these parameters better, samples in which the presence of GALO or Type 1863 was under 5% of the total were considered (Table 2). Samples with abundant Zoogloea were discarded, as just a few of these bacteria cause the SVI to rise rapidly. Thus, it could be observed that the SVI rose when there was an increase in the abundance of filamentous microorganisms (Figure 7). This increase had already been observed by Sezgin et al. (1978), Palm et al. (1980), Lee et al. (1982), Barber & Veenstra (1986) and Liao et al. (2004). Unlike those studies, in which a threshold value at which the SVI suddenly increased was proposed, the increase (Figure 7) was continuous, as also found by Schüler & Jassby (2007). In contrast, the addition of reagents such as chlorine (3–12 mg Cl$_2$ g$^{-1}$ MLVSS day) or coagulants such
as ferric chloride (100–200 mg Cl₂Fe g⁻¹ MLVSS day) to the sludge, decreases the coefficient of correlation between the SVI and filamentous organisms, and may lower the SVI for the same abundance of filamentous bacteria. Samples taken during periods when bulking was treated with chemical reagents (such as chlorine) were also discarded (Table 2), correlations between coefficients suffered a small increase.

Previous findings indicate that the shape and distribution of filamentous bacteria or the condition of the sludge may cause irregularities in the relationship between their length/volume ratio and the SVI. Some filamentous organisms such as Microthrix parvicella or Type 021N may group together forming bundles; while some may be distributed more uniformly. As shown in Figures 6 and 7, the highest values of filamentous organisms occur in samples with abundant Microthrix and Type 021N grouped mainly in bundles. The variation coefficient between the standard deviations of the intersections found in each field and their mean gives an idea of how much they vary. Variation coefficients of over 150% indicate that aggregation is high. Over 300% means that most of the filamentous microorganisms have formed thick bundles, which make counting difficult as they increase the margin of error. Most variation coefficient values over 300% were found to the right of the correlation straight line between the two parameters. This clearly demonstrates that filamentous bacteria grouped together in bundles affect the SVI less than uniformly spread bacteria do. In addition, closer correlation between the SVI and the total length of extended filamentous organisms was found in samples with lower variation coefficients (Table 2). In order to improve the correlation between filamentous organisms and the SVI, samples from periods when chemical treatment was employed for bulking control and samples with variation coefficients of over 80% were disregarded; the correlation coefficient increased significantly (r = 0.96). In whichever case, the accuracy of the results depends on the number of samples analyzed and the variation coefficient found. In this way, the variation coefficient can be used as a way of distinguishing data and as a parameter to describe and indicate when filamentous microorganisms are aggregated in bundles.

### FINAL REMARKS

Therefore, using only techniques like the SVI to control the increase or decrease of filamentous organisms does not always lead to good results, since the relationship between filamentous bacteria and the SVI is variable. The SVI depends on various parameters such as aggregation, type of filament, or the addition of chemical substances such as chlorine or coagulants. If the outcome of a treatment of filamentous bacteria, whether in a real process or in the laboratory, is to be completely clear, then the abundance of filamentous organisms must be found.

The results obtained through applying this method are precise, and never less significant than the results obtained with other techniques such as those of Sezgin et al. (1978), Palm et al. (1980), De Los Reyes et al. (1997) and Mesquita et al. (2013). In addition, this technique is quick and the samples are easy to count using it, as it does not require any additional material apart from a simple microscope. As we know, not all microorganisms are filamentous, and the total count of flocculants or dispersed bacteria using microscopy depends on techniques that are more laborious; based, for example, on epifluorescence or molecular methods that are not easy to apply in a laboratory of a WWTP.

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