Short Communication

Quantification of pelagic filamentous microorganisms in aquatic environments using the line-intercept method

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Abstract

The line-intercept method was adopted for quantification of aquatic filamentous microorganisms. The cumulative length of filaments in a sample is calculated from the number of intercepts between filaments and test bars of known length. The product of the cumulative length and of filament diameter reveals the total biovolume of filaments. The method is suitable for reliable and statistically correct quantification of long or dense filaments immeasurable individually. The combination of epifluorescent microscopy and image analysis speeds up sample processing and supports differentiation between heterotrophic and autotrophic filaments. Parallel testing of the line-intercept method in Utermöhl sedimentation chambers revealed tight correlation ($r^2 = 0.89$) of both methods for cyanobacterial filaments. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Bacterial filament; Cyanobacterial filament; Image analysis

1. Introduction

Filamentous microorganisms may comprise a significant part of the microbial biovolume in many aquatic ecosystems. An increasing number of studies have recently focused on their occurrence [1,2], ecological significance [3], morphology [4], phylogeny [5], and growth regulation [6–8]. While abundance and biovolume determination of small non-filamentous microbial cells is a routine process, reliable quantification of filaments is difficult.

To determine the biovolume of non-filamentous microbes, cells are stained with an appropriate fluorochrome (most frequently 4',6-diamidino-2-phenylindole, DAPI), concentrated on filters, enumerated with epifluorescent microscopy [9], and sized – usually with image analysis [10]. To avoid counting bias, cells (~400) have to be counted on randomly selected fields applying simple rules concerning cells crossing the borders of the count frame [11]. If sizing bias is to be avoided, the whole length of the measured cell must be visible – the longest cell processed must therefore be shorter than the margin between the count frame and the border of the microscope field, or, in the case of image analysis, between the measurement frame and the image border [12]. As margins wider than 10–15 μm are usually not practical, the approach based on counting and sizing of individual cells cannot be reliably applied to filaments exceeding this length. Besides, individual filaments may be indistinguishable if they branch, cross each other, or cross other objects. Heterotrophic filaments and filamentous cyanobacteria of similar morphology may also coexist in the same samples and in this case it may be necessary to examine the cells for chlorophyll autofluorescence using a specific filter set [13].

In the present paper we test the applicability of the line-intercept method for the determination of the cumulative length of linear structures, originally developed for root length measurement [14], as an alternative possibility of quantifying filamentous microorganisms in water samples. Instead of counting and sizing of individual filaments, the method measures density of filaments and converts it to their cumulative length in a sample.

2. Materials and methods

The cumulative length of linear structures randomly arranged over a given area can be calculated from the num-
ber of intercepts between these structures and test bars of known length according to the following equation [14]:

\[
L = \pi \times A \times N / 2T
\]

(1)

where \(L\) is the cumulative length of linear structures measured, \(A\) is the area, \(N\) is the number of intercepts, and \(T\) is the cumulative length of test bars (see Fig. 1A for an example). For practical measurement of the cumulative length of filaments in a sample, Eq. 1 can be rearranged as follows:

\[
\text{CLF} = \frac{\pi}{2} \times \frac{N}{T} \times A_F \times 10^{-6} / V_F
\]

(2)

where \(\text{CLF}\) (m ml\(^{-1}\)) is the cumulative length of filaments, \(N/T\) (\(\mu\)m\(^{-1}\)) is the ratio of the number of intercepts and the cumulative length of test bars, \(A_F\) (\(\mu\)m\(^2\)) is the effective filtration area of the filter examined, and \(V_F\) (ml) is the volume of the water sample filtered. Random orientation and distribution of the measured filaments over the filter area is considered.

Three acidified glacial lakes, Čertovo, Plešné, and Prášilské Lakes, located in the Bohemian Forest, Czech Republic (see [15] for detailed description), were sampled in 1998. Samples were preserved in 2% formaldehyde and stored at 4°C before processing (several days to months). Portions (2–10 ml) of samples were stained with DAPI (0.2% final concentration) for 4 min, filtered using mild vacuum (≤20 kPa) through black polycarbonate Poretics filters (effective filtration area 227 mm\(^2\)) of pore size 1 \(\mu\)m (for filament quantification) or 0.2 \(\mu\)m (for quantification of non-filamentous cells), and prepared for epifluorescent microscopy [9].

For image acquisition and processing we used the image analysis system Lucia G/F 4.11 (Laboratory Imaging, Czech Republic, http://www.lim.cz, 980×720 pixels, 256 gray levels, integrating a digital monochromatic LCD camera, Pulnix TM1001, mounted to an Olympus AX 70 microscope). For filament quantification, two images of each of 10–20 randomly selected microscope fields were captured at 400× magnification: the first using fluorescence settings specific for chlorophyll a autofluorescence (excitation/emission 510–550/≥590 nm, referred to as CHLA image, Fig. 1B), the other using fluorescence settings for DAPI (excitation/emission 360–370/≥420 nm, referred to as DAPI image, Fig. 1C).

Fig. 1. Estimation of the cumulative length of filaments with the line-intercept method. A: An example of the method principle: there are six intercepts on a field. B: Chlorophyll autofluorescence, ‘CHLA image’, taken with a monochromatic LCD camera. Plešné Lake, 20 May 1998. C: The same but DAPI fluorescence, ‘DAPI image’. D: Combination of CHLA and DAPI images, with superimposed test lines, scale bar, and highlighted autofluorescent filaments (black here but red on the computer screen; Lucia image analysis software). Scale bar = 5 \(\mu\)m.
We used the line-intercept method for quantification of filamentous microorganisms, arbitrarily defined as elongated microorganisms longer than 5 μm [7,8]. First, the matching of DAPI and CHLA images was corrected if necessary. The DAPI image was then digitally sharpened. The CHLA image was processed with the ‘Mexican Hat’ filter to enhance edge detection and automatically segmented. Finally, the composed image was constructed and displayed on screen (Fig. 1D). The image consisted of the gray DAPI image, superimposed with segmented CHLA image (chlorophyll-containing filaments were traced by red color) and with counting bars of the apparent length (i.e. the length in the scale of the image) of 757 μm. The operator marked the intersections between filaments and test bars on the computer screen with the mouse, the system registered and counted the marks.

We measured diameters of filaments manually using the built-in function of the Lucia system on DAPI images taken separately at 1000× magnification. We found that in the lakes studied there were roughly two kinds of heterotrophic filaments, with diameters of about 0.3 μm and 0.7 μm, referred to as fine and thick heterotrophic filaments, respectively, and autotrophic (cyanobacterial) filaments of about 1.3 μm in diameter. They were quantified separately and their biovolumes were calculated from their cumulative length and diameter.

We used the object-measurement method (based on measurement of individual cells, for details see [16]), to size the non-filamentous bacteria (<5 μm) in all samples and, for comparison with the line-intercept method, also the filaments in selected samples containing only short filaments (≤15 μm).

To test the principle and design of the line-intercept method, we used a scanner to take 19 images of pieces of string. The cumulative length of the string was 3 m and it had been randomly arranged over an area of 210×150 mm. The images were processed by image analysis with the line-intercept method using test bars of different apparent length (145–1599 mm) arranged either orderly (parallel horizontal lines, as on Fig. 1D), or randomly.

The filamentous cyanobacteria were quantified in Lugol-fixed water samples in Utermöhl’s sedimentation chambers (volume 13.5 ml) on an inverted microscope (Nikon 108) at 600× magnification with the line-intercept method. The lines of the ocular grid (20×20 lines of the apparent length of 1336 μm each) served as test bars.

Variability at levels of different filter pore size, filter duplicates, different operators, and images was studied on a difficult field sample with weakly stained filaments from Plešná Lake (20 May 1998) quantified with the line-intercept method. We filtered 2 ml of the sample through two parallel filters for each of the following pore sizes: 0.2, 1.0, and 2.0 μm, and we captured 20 images from each filter. The images were processed independently by four persons of varying research experience.

To study the effect of filtered volume, we filtered 1, 2 and 5 ml of each of the three samples differing in the character of filamentous microorganisms through a filter of 1.0 μm pore size.

3. Results and discussion

Fig. 2 summarizes the results of estimation of the length of randomly arranged string pieces. When using orderly arranged test bars, real string length was slightly overestimated (by 5.0–14.3%); this overestimation, as well as the variability of estimation (S.D., n = 19), decreased with increasing total length of test bars. When using randomly arranged test bars, no systematic deviation of the estimated string length from the real length was apparent. The slight overestimation occurring when using orderly arranged test bars could have been caused by the fact that the distribution of string pieces, arranged manually, tended to be lower at the edges which were not covered sufficiently by test bars. The same problem was encountered by [14,17]; however, this type of non-homogeneous distribution should not be a problem with filter measurements. No overestimation was apparent when using random arrangement of test bars (Fig. 2). Nevertheless, we decided to use orderly arranged test bars in routine measurements, as image processing was more convenient. In routine measurements, we used six horizontal parallel test bars with a total apparent length of 757 μm per computer screen.

For four natural samples from different depths of Prášilské Lake (14 September 1998), containing only short filaments, we used both methods (Fig. 3). The variability of estimations (S.D., n = 19) was 0.7% with the object-measurement method and 5.0% with the line-intercept method.

Fig. 2. Measurement of string pieces with the line-intercept method. Pieces of string of a known cumulative length of 3 m were randomly arranged over an area of 210×150 mm, scanned, and the resulting set of 19 images was processed with the line-intercept method using different cumulative lengths of test lines arranged either orderly or randomly. Averages ± S.D. of the string length estimations. Triangles: variation coefficient (right scale).
filaments (≤15 μm), no statistically significant difference (P > 0.05, Mann–Whitney) was found between the estimates of cumulative filament lengths with the line-intercept method and object measurement used as a reference method.

On average, the 1.0-μm filters apparently retained only 76% and the 2.0-μm filters only 51% of the total filaments estimated by their cumulative length as compared to the 0.2-μm filters. The estimates obtained by four different operators agreed within 24%, while the average differences in counts between parallel filters processed by one person (20 fields counted per filter) were 6.3%. The effect of filter pore size was statistically significant (P < 0.001; Kruskal–Wallis). The most important sources of variability were found at the level of image-to-image variability (52.4% of overall variability) and at the level of filter pore size (45.0%), while the contributions at the levels of operator (2.4%) and of filter replicates (0.2%) were much smaller (nested ANOVA).

The effect of the volume of the sample filtered was remarkable. In general, an increase in the filtered volume led to a decrease in the estimated cumulative length of filaments, but the effect and its statistical significance varied between samples and types of filaments (Table 1). A five-fold increase in filtered volume from 1 ml to 5 ml decreased the estimated filament cumulative length on average by 38%.

In contrast to the rather small effect of the subjective factor included in detection of filament and test bar interceptions, sample preparation (i.e. filter pore size and filtered volume) had a surprisingly large influence on the results. In some cases (Plešně Lake), higher filter pore size and larger filtered volumes led to a reduction of measured filament length by up to 50%. However, because a small volume of sample may not contain enough measured organisms and the large amount of detritus on a 0.2-μm filter may make staining and detection of filaments difficult, we decided on a practical compromise: we routinely filtered 2–3 ml of sample over 1-μm filters.

For good reproducibility, a total of ≥200 intercepts on five or more fields should be counted. For dense samples, where the number of intercepts per field (screen) exceeds

### Table 1

Effect of filtered volume on the estimation of cumulative filament length (m ml⁻¹; average ± S.E.M.; n = 15) with the line-intercept method in natural samples from Bohemian Forest lakes

<table>
<thead>
<tr>
<th>Sample</th>
<th>Filament type</th>
<th>Filtered volume</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 ml</td>
<td>2 ml</td>
</tr>
<tr>
<td>Čertovo Lake, 10 August 1998</td>
<td>Fine</td>
<td>3.45 ± 0.32</td>
<td>2.43 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>Thick</td>
<td>0.41 ± 0.17</td>
<td>0.35 ± 0.13</td>
</tr>
<tr>
<td>Pražilské Lake, 14 September 1998</td>
<td>Fine</td>
<td>2.31 ± 0.32</td>
<td>2.50 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>Thick</td>
<td>1.24 ± 0.21</td>
<td>0.97 ± 0.14</td>
</tr>
<tr>
<td>Plešně Lake, 11 June 1998</td>
<td>Fine</td>
<td>6.28 ± 0.68</td>
<td>4.33 ± 0.66</td>
</tr>
<tr>
<td></td>
<td>Thick</td>
<td>2.66 ± 0.54</td>
<td>1.67 ± 0.30</td>
</tr>
<tr>
<td></td>
<td>Autotrophic</td>
<td>1.66 ± 0.27</td>
<td>1.00 ± 0.18</td>
</tr>
<tr>
<td></td>
<td>Average %</td>
<td>100</td>
<td>77.3</td>
</tr>
</tbody>
</table>

Filter pore size was 1 μm. Differences between data from different filtered volumes were tested with the Kruskal-Wallis test.

Fig. 3. Example of a vertical profile of cumulative lengths (left) and of biovolumes (right) of filamentous microorganisms of different kinds: fine heterotrophic filaments, thick heterotrophic filaments, and cyanobacterial filaments, in Plešně Lake, 12 August 1998. The cyanobacterial filaments were determined with the line-intercept method both by autofluorescence and with the Utermöhl method.
50, the length (or number) of test lines should be decreased. For samples with sporadic filaments, the number of fields counted should be increased (but not the number of lines per field, otherwise reproducibility declines). As a rule of thumb, the distances between lines should not be lower than 1/10 of their lengths.

Fig. 3 shows an example of the application of the line-intercept method in routine sampling of acidified lakes in the Bohemian Forest. Pelagic food webs of these lakes are generally dominated by microorganisms [15], in particular by dense and long heterotrophic filaments (cf. Fig. 1), accounting for more than 50% the total bacterial biovolume on seasonal average (Nedoma et al., unpublished). The line-intercept method used also enabled the quantification of the biovolume of individual cyanobacterial species within cyanobacterial mats (Komářková, personal communication).

Total filament length can also be measured manually, with the lines of the ocular count grid serving as test bars. Fig. 3 shows good agreement of total lengths of filamentous cyanobacteria estimated either as autofluorescent filaments with image analysis or in Utermöhl chambers, both using the line-intercept method. We found a high linear correlation between estimates of cyanobacterial filament length by autofluorescence and by the Utermöhl method \( (r^2 = 0.89, P < 0.001, n = 10) \) in samples from two vertical profiles on Plešné Lake.

The present method offers a quick and reliable tool for the quantification of total length and biovolume of filaments, if values for individual filaments are not required. It allows a statistically correct quantification (i) of long filaments vastly exceeding the dimensions of count fields and (ii) of dense samples where individual filaments are indistinguishable, e.g. activated sludge, cyanobacterial or algal mats, etc.

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References


