First publ. in: Harmful algae 5 (2006), 3, pp. 281-289

Determination of the filamentous cyanobacteria *Planktothrix rubescens* in environmental water samples using an image processing system

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Abstract

Cyanobacteria occur in surface waters worldwide. Many of these produce peptides and/or alkaloids, which can present a risk for animal and human health. Effective risk assessment and management requires continuous and precise observation and quantification of cyanobacterial cell densities. In this respect, quantification of filamentous *Planktothrix* species is problematic. The aim of this study was to develop an automated system to count filamentous *Planktothrix rubescens* using image processing. Furthermore, this study aimed to assess optimum sample volumes and filament density for measurement precision and to validate image processing measurement of *P. rubescens* for an effective risk assessment.

Three environmental samples and one cultured sample of *P. rubescens* were collected by filtration onto nitrocellulose filters. Filament lengths were determined using fluorescence microscopy combined with an image processor. Cell density could be calculated from the resulting images. Cyanobacteria could easily be discriminated from algae via their fluorescence properties. The results were found to be independent of the mode of image acquisition. The precision of total filament length determination was dependent on the total filament length on the filter, i.e. analyses of highest precision could be expected for filters containing 2000–20,000 μm filaments per mm^2. When using suitable filtration volumes, the detection limits of the described method are sufficient for an effective risk assessment. To summarise, this procedure is a fast, easy and accurate method to determine cell densities of filamentous *P. rubescens* in water samples without costly and tedious manual handling.

Keywords: Cyanobacteria; Filament; Image processing; *Planktothrix*; Cell quantification; Risk assessment

1. Introduction

Cyanobacteria occur worldwide in coastal and surface waters. Surveys in various countries have demonstrated that about 75% of samples containing cyanobacteria are toxic. Due to nutritional enrichment (eutrophication), occurrences of toxic cyanobacterial blooms in surface waters, e.g. species of the genera *Microcystis*, *Anabaena*, *Planktothrix* and *Aphanizomenon* are becoming a growing problem (Bartram et al., 1999). In addition, albeit in contrast to this situation, the intentional nutritional re-depletion of eutrophic surface waters (re-oligotrophication) resulted in regular blooms

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of *Planktothrix rubescens* in several European prealpine lakes (Mez, 1998; Ernst et al., 2001; Morabito et al., 2002; Jacquet et al., 2005).

P. rubescens is a low light adapted, filamentous cyanobacterium, made up of cells, which contain gas vesicles enabling the filaments to adjust their buoyancy in the water column in order to achieve optimal use of the ambient environment (Walsby et al., 1998). Consequently, *P. rubescens* builds blooms distributed over the whole vertical water column during winter circulation and in metalimnic layers during summer stratification. Furthermore, buoyancy disturbances can result in *P. rubescens* blooms at the lake surface (Ernst et al., 2001; Jacquet et al., 2005). *P. rubescens* blooms and layers can attain densities of up to 150,000 cells/ml (Hoeger et al., 2005).

At least 46 cyanobacterial species are able to produce neurotoxins, e.g. anatoxin-a, anatoxin-a(s) and saxitoxin, a range of dermatoxins and/or predominantly potent protein phosphatase inhibitors, such as microcystins and nodularins (Sivonen and Jones, 1999; Chorus et al., 2000). In addition to producing a range of other metabolites with unknown toxicological potential, including anabaenopeptins, microviridins and cyanopeptolins (Blom et al., 2003), species of the genera *Planktothrix* have been shown to contain the highest amounts of microcystin (<5.6 mg/g dry weight) (Fastner et al., 1999). The release of these cyanobacterial toxins may present a serious risk for wild and domestic animals as well as for human health, as recently reviewed by Dietrich and Hoeger (2005). As a result of incidents attributed to toxic cyanobacteria the World Health Organisation (WHO) and several national authorities worldwide have recommended risk assessment plans and safety levels to include cyanobacteria as a parameter, which must be monitored for water quality control (Chorus et al., 2000; Azevedo, 2001; Falconer, 2001; Codd et al., 2005).

Effective risk assessment and management requires continuous and precise observations of cyanobacterial biomass and/or cell densities (Chorus and Bartram, 1999). Quantification of *Planktothrix* species is difficult as the individual cells arranged to form a filament are hardly distinguishable. Furthermore, filament counts cannot be automatically correlated to biomass or cell densities because *Planktothrix* species exhibit large variations in filament length and filaments overlay one another and are often curved in a given sample when observed on a slide or filter, making measurement of filament length difficult and inaccurate. Quantification of cell volumes is difficult because centrifugation is laborious due to the gas vesicles incorporated in *Planktothrix* cells for buoyancy. Furthermore, quantification via determination of photopigments, e.g. chlorophyll and/or phycobilliproteins is not reliable due to regulation of pigments with various growth conditions (Feuillade, 1994) and false positive results due to pigments of eukaryotic algae and zooplankton coexisting within the same environment. Gjolme et al. (2004) demonstrated protein concentrations to best reflect cyanobacterial biomass. However, as pigment and biomass parameters, protein measurement may easily be overestimated in environmental seston samples due to the coexistence of eukaryotic algae and zooplankton within the same environment.

As many of the lakes containing *Planktothrix* species are used for recreational purposes and several even as drinking water reservoirs (Hitzfeld et al., 2000; Hoeger et al., 2005), a rapid and precise procedure for quantification of *Planktothrix* species is essential.

For cell quantification of filamentous cyanobacteria most methods of choice are based on microscopic identification and counting (Olson, 1950; Bailey-Watts and Kirka, 1981; Hoogveld and Moed, 1993). This approach has the caveat of increased demand on both manpower and skill of the personnel as well as limitations in the speed with which filament densities can be determined.

Cyanobacterial species use the biliproteins phycocyanin and allophycocyanin to harvest light for photosynthesis. Some species, including *Planktothrix* species, additionally contain the biliprotein phycoerythrin (Glazer, 1985; Anagnostidis and Komárek, 1988). When examined under blue light excitation, phycoerythrin and phycocyanin fluoresce orange and red, respectively. Therefore, cyanobacteria can be enumerated by visualising the autofluorescence of phycoerythrin and/or phycocyanin using epifluorescence microscopy (Walsby and Avery, 1996; Sieracki and Wah Wong, 1999).

Walsby and Avery (1996) designed and described a semi-automated procedure to count *Planktothrix* cell densities. This method involves the transfer of epifluorescent microscope images of filter to a computer, followed by determination of filament length via computer image analysis. This is a fast and accurate method, which measures the length of several filaments simultaneously.

The aim of our study was to develop automation in counting filamentous *P. rubescens* using arrays of filament images, i.e. to improve and expand on the method of Walsby and Avery, reducing the manual interactions required for measurement and thus reducing overall time per sample. Furthermore, this study

aimed to asses optimum sample volumes and filament density for measurement precision and to validate image processing measurement of *P. rubescens* for an effective risk assessment.

2. Materials and methods

2.1. Samples

P. rubescens samples 1–3 were environmental seston samples of various *P. rubescens* densities collected from Lake Ammersee, Germany in July 2001. They were taken from the metalimnion (10–12 m depth) using a Ruttner flask sampler. The taxonomy of each sample was determined via light microscopy and classification was according to Anagnostidis and Komárek (1988). Samples were fixed with Lugol's solution (iodine–potassium iodide solution) and stored in darkness at room temperature for 24 h until filtration. Samples 1–3 were used for method validation. Sample 4 was a culture sample of *P. rubescens*, isolated from a Lake Ammersee seston sample in autumn 2002 and cultivated in BG11 medium according to Rippka et al. (1979). Sample 4 was used to investigate the roboustness of the method and to compare cell counts and chlorophyll a measurements.

2.2. Filtration, epifluorescence microscopy, video transfer and analysis mode

Defined volumes of the samples were filtered onto nitrocellulose filters (pore size 8 μm, diameter 25 mm, Schleicher & Schuell, Germany). Samples were filtered using a standard filtration apparatus (Millipore, Germany) with an absolute surface area ($A_{surface}$) of 283.5 mm^2 for each filter. Filters were air-dried and stored in 6-well cell culture plates (Sarsted, Germany), darkness and at room temperature until analysis.

Cyanobacterial filaments were observed with an epifluorescence microscope (ECLIPSE TS100, Nikon, Germany) using 100-, 200- and 400-fold magnification. Image analysis of the filters was performed using an epifluorescence microscope (Zeiss Standard 25 including an HBO $50/_{AC}$-lamp, Germany) with a ×10 objective (Zeiss A-Plan ×10/0.25). Samples were illuminated through a filter block allowing blue light excitation (λ = 450–490 nm).

Several fields-of-view from each membrane-filter were transferred to a Pentium II PC (450 MHz, 384 MB RAM, NVIDIA 128/128ZX graphics card, Windows 2000) with a monochrome CCD-camera (KAM02E, EHD, Germany, resolution 752 × 582 pixel, 1/2 in. CCD) in combination with an IDS Falcon frame grabber and were digitalised using the image processing system Visiometrics *IPS* 1.119 (Visiometrics GbR, Germany). Brightness and contrast were adjusted until filaments appeared as white lines on a dark background (Fig. 1). Length scales were calibrated using the gridlines of a Neubauer haemocytometer (Brand, Germany) as reference (the calibration factor was $p_x = 0.86$ μm/pixel and $p_y = 0.87$ μm/pixel; resulting in an area per field-of-view (A_{view}) of 0.32 mm^2 with a ×10 objective).

Filters containing the filaments were focussed and image analysis was started via seven automatic steps using the image processing system as follows:

1. For each field-of-view 50 digitized video frames were averaged in order to achive an image with high signal-to-noise ratio.
2. A 5 × 5 median filter was applied, to remove small bright features in the background while preserving the outline of the filamtents.

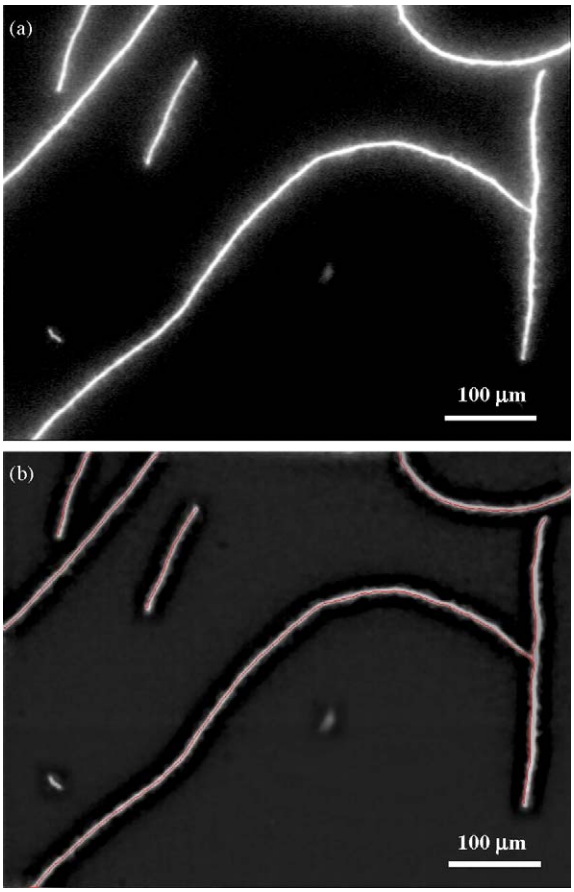

Fig. 1. (a) Image of a filter membrane containing *P. rubescens* filaments enumerated by fluorescence microscopy and (b) skeletons of the filaments calculated using the image processing system Visiometrics *IPS*.

3. Spatial intensity gradients in the image background arising from the inhomogeneity of the illumination and due to the inherent and spatially variable fluorescence of the membrane were corrected using the local adaptive background correction of Visiometrics *IPS*.
4. The image was automatically segmented into filaments and background by binarization with a single intensity threshold.
5. The resulting image was thinned using the skeleton routine of Visiometrics *IPS* (Fig. 1), which employs the skeletonising algorithm published by Arcelli et al. (1975).
6. Skeletons, with a length <30 pixels (user-defined threshold) were omitted from the analysis to minimise unspecific contributions to the total filament length (Fig. 1).
7. To determine the total length of the remaining filaments, the region of each filament pixel was analysed: For each of the left, right, top and bottom neighbouring pixels, one interpixel distance was added to the total filament length. For the top-left, top-right, bottom-left and bottom-right neighbours, $\sqrt{2}$ times the interpixel distance was added. Since during the analysis each distance is counted twice, the final result L_{raw} is divided by two. The lengths of the remaining filaments were determined by calculation from the number of skeleton pixels and summed to yield the total length of all filaments per field-of-view. Measuring length by means of interpixel distances has the disadvantage of overestimating the actual length in certain directions (Walsby and Avery, 1996). This error can be corrected by application of a statistical correction factor of 0.948 to L_{raw}. Hence, the total corrected filament length in the view is given as: $L_{view} = L_{raw} \times 0.948$.

The total length of filaments per filter (L_{filter}) was calculated using the following equation:

$$L_{filter} = A_{surface} \times L_{view} \times A_{view}^{-1} \text{ for the } \times 10 \text{ objective}$$
$$L_{filter} = 886.6 \times L_{view}$$

To obtain an approximation for the total cell count (C_{total}) per filter the total length of filaments per filter (L_{filter}) was divided by the average cell length ($L_{average}$) of *P. rubescens*.

$$C_{total} = L_{filter} \times L_{average}^{-1}$$

$L_{average}$ was determined by measuring the cell length of 29 randomly selected cells of different filaments. However, cells aligned into a filament were only barely distinguishable (Anagnostidis and Komárek, 1988). Average cell length of *P. rubescens* isolated from Lake Ammersee was determined to be 2.8 ± 0.44 μm. This is in accordance to the description of Geitler (1932), who described cell lengths to range from 2 to 4 μm. For calculation of total cell counts, average cell length was assumed to be 3 μm/cell for *P. rubescens*.

Finally, cell densities were calculated by dividing the determined cell count C_{total} through the filtered sample volume.

2.3. Method validation

To investigate if filaments were homogeneously distributed on the filters, the results of differently oriented picture grids were compared. For this purpose, 20 ml of the environmental samples (samples 1–3) were collected by filteration onto a membrane as described above. Each filter was counted four times, analysing 20 different fields-of-view per filter selected along varying grids (Fig. 2).

To determine how many fields-of-view per filter must be analysed in order to obtain a representative cell count, 10, 20, 40, 60 and 80 randomly selected fields-of-view were analysed for each filter of the environmental *Planktothrix* samples (samples 1–3). Analyses were then compared to determine, if the results were dependent on the number of fields-of-view analysed per filter.

The filament capacity of the method was determined by analysing filters with various sample volumes. Thus, 0.1–100 ml of a culture sample (sample 4) were filtered onto different membrane-filters. Each filter was analysed by counting 10 fields-of-view. Analyses were then compared to investigate, whether or not the resulting counts per field-of-view correlated to the filtered sample volumes.

For comparison of cell counts and chlorophyll a measurements, eight different volumes of the culture sample (sample 4) were filled with tap water to give a final volume of 1 l. Various volumes (10–30 ml) of these samples were filtered onto nitrocellulose filters and stored for cell count analyses as described above.

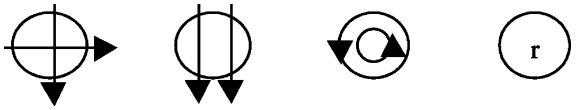

Fig. 2. Scheme of picture grids analysed in order to screen the filament distribution on the membrane-filters (10 fields-of-view were analysed along each direction; *r* means analysis of 20 randomly distributed fields-of-view per filter).

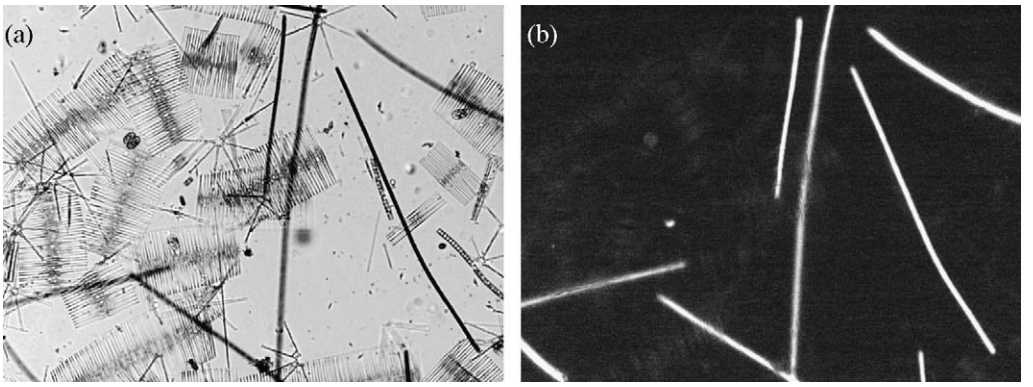

Fig. 3. (a) Light microscopy of a Lake Ammersee seston sample containing diatoms, other eukaryotic algae and cyanobacteria predominantly filamentous *P. rubescens* and (b) differentiation of cyanobacteria in the same field-of-view from eukaryotic algae using epifluorescence microscopy (magnification ×100).

Between 250 and 750 ml of the remaining volumes were filtered onto GF/C filters (Whatmann, UK) and chlorophyll a concentrations were determined according to the standard protocol DIN 38412 L16. Chlorophyll a measurements and cell count analyses were finally compared to determine correlation of the data.

2.4. Statistics

Data analyses were carried out using JMP® 4 (USA) Software. Values represent the mean ± standard error of the mean (S.E.M.). Results were analysed for statistical differences using analysis of variance (ANOVA) and the Tukey–Kramer Multiple Comparisons Test ($p \geq 0.05$). Regression analyses were performed using Microsoft Excel.

3. Results

In Lake Ammersee field samples, cyanobacterial filaments could be automatically distinguished from other planktonic organisms by both their structure and fluorescence properties. Using blue light excitation, the *P. rubescens* filaments fluoresced orange while eukaryotic algae did not fluoresce at all (Fig. 3). After filtration and video transfer, filaments appeared as white lines on a dark background (Fig. 1).

Analysis of the filtered environmental samples investigated for method validation yielded filament densities of 25,601 μm filament per mm^2 filter (sample 1), 12,052 μm filament per mm^2 filter (sample 2) and 3345 μm filament per mm^2 filter (sample 3) corresponding to approximately 60,490; 28,475 and 7904 cells/ml, respectively. Neither significant differences nor tendencies of differences could be observed analysing the three filters along diverse picture grids thus a systematic error due to gradients in filament distribution on the filters can be excluded (Fig. 4). Additionally, there were no significant differences between the cell counts resulting from variable number of fields-of-view per filter. Although, the standard error of the mean decreased with an

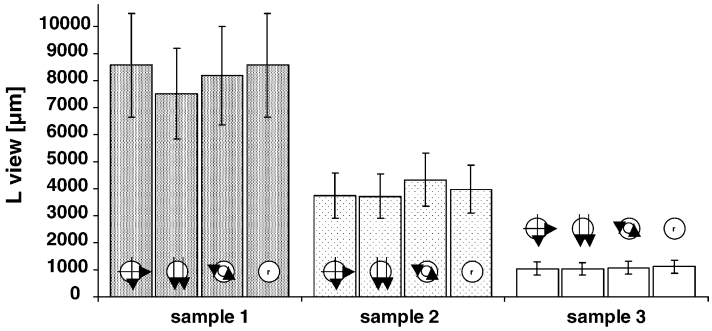

Fig. 4. Comparison of *P. rubescens* cell counts analysed by varying picture grids to test for homogeneous filament distribution on the filter membrane. Three environmental samples of various filament densities were filtered. There were no significant differences ($n = 20$; error bars = S.E.M.).

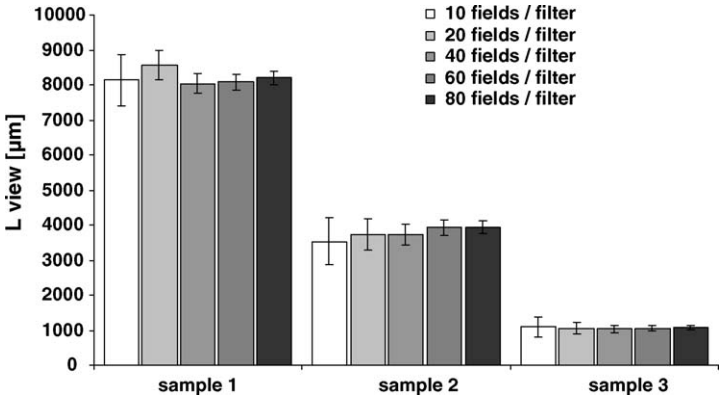

Fig. 5. Cell counts of three environmental *P. rubescens* samples of various filament densities. Each filter was counted analysing 10, 20, 40, 60 and 80 fields-of-view per filter. There were no significant differences between the cell counts resulting from variable number of fields-of-view per filter (error bars = S.E.M.).

Table 1
Capacity of the method: filters holding various volumes of a *P. rubescens* culture sample were analysed for µm filament per field-of-view (L_{view}), µm filament per mm^2 filter and *P. rubescens* cell density

filtered volume [ml]	0.1	0.2	0.4	0.6	0.8	1	2	4	6	8	10	20	40	60	80	100
Lview [µm]	25	35	170	220	251	383	606	1,190	1,641	1,977	3,055	6,020	9,114	12,739	16,970	18,256
[µm filament mm^{-2} filter]	78	110	530	687	785	1,197	1,895	3,723	5,131	6,182	9,552	18,826	28,499	39,835	53,064	57,087
[cells x 10^3 ml^{-1}]	73	52	125	108	93	113	90	88	81	73	90	89	67	63	63	54

Analyses of filters containing between 2000 and 20,000 µm filaments mm^{-2} filter resulted in comparable cell counts (highlighted). In comparison, analyses of filters with <2000 µm mm^{-2} tended to result in an overestimation of cell counts while analyses of filters with >20,000 µm mm^{-2} resulted in lower cell counts.

increasing number of fields-of-view analysed per filter (Fig. 5).

In order to determine the measurement precision and the ideal range of filament density on the filter, several filters from an identical culture sample, but with various sample volumes were analysed. For most samples double volume corresponded to double counts. There was a significant correlation between filtered volumes and the analysed counts per field-of-view ($R^2 = 0.98$). Analyses of filters containing a filament density of 80–60,000 µm filament per mm^2 filter resulted in a mean cell count of 81,790 with a standard error of the mean ± 5132 cells/ml (Table 1). Measurement precision increased (85,701 ± 2629 cells/ml), if cell counts corresponded to filament densities ranging between 2000 and 20,000 µm filament per mm^2 filter (Table 1), indicating the error of measurements to depend on the filament density on the filter. Finally, there was a significant correlation between cell counts and chlorophyll a measurements resulting in a ratio µg chlorophyll a l^{-1}:cells/ml of 1/2000 (Fig. 6).

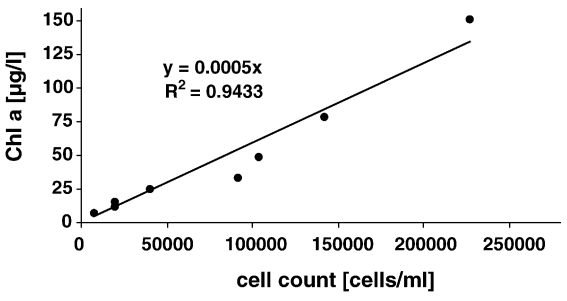

Fig. 6. Comparison of chlorophyll a measurements and cell counts analysing a *P. rubescens* culture sample (sample 4).

4. Discussion

The analyses of the field samples confirm former findings that cyanobacteria can be distinguished from other algae using epifluorescence microscopy as these

other planktonic species (diatoms, green algae, etc.) have a much weaker fluoresence than cyanobacteria (Walsby and Avery, 1996; Sieracki and Wah Wong, 1999). Discrimination can be achieved by setting the brightness level of the Visiometrics *IPS* system to a value at which cyanobacterial filaments can be recognised, but no organisms with weaker fluorescence. Thus, the determination of cyanobacterial cell densities by image analysis can be applied to cultured as well as natural samples. This conclusion also corroborates the earlier findings of Leboulanger et al. (2002), who determined vertical *P. rubescens* distribution in Lake Bourget, France, via the fluorescence properties using a submersible spectrofluorometer.

The counting of samples on membrane-filters has several advantages: samples must only be focussed in one focal plane, the filaments do not move on the microscope slide, fluorescence properties increase if filaments are dried, samples can be stored on the filters for several months without loss of quality and storage space requirements are reduced. Thus, the samples may be reanalysed if necessary (Walsby and Avery, 1996).

The data obtained with the image processing system presented here demonstrate, that fully automated cell quantification of filamentous cyanobacteria can be carried out in a robust and highly reproducible manner. This is largely due to the following improvements in image processing:

1. Filaments were separated from background automatically, by binarization with a single intensity threshold, followed by thinning of the resulting image using the skeleton routine of Visiometrics *IPS*, which employs the skeletonising algorithm published by Arcelli et al. (1975). A prerequisite for this automatic measurement is a clear, sharp image and low noise.
2. These images can be obtained by generating an average image of 50 individual video frames.
3. Image quality was additionally improved using a median filter and the automatic locally adaptive background correction of Visiometrics *IPS*.

Thus, the image processing described here, represents a valuable improvement over the initial automation protocol suggested by Walsby and Avery (1996).

To obtain significant cell counts by analysis of the lowest possible number of randomly distributed fields-of-view, filaments must be distributed on the filter homogeneously. This condition was proven by examination of different modes of image acquisition. Comparison of cell counts determined along varying picture grids showed no differences demonstrating cell counts to be independent of the mode of image acquisition. This indicates that, indeed, filament distribution is homogeneous.

Measurement precision increased with an increasing number of fields-of-view used for filter analysis, as shown by a decreasing standard error of the mean for measurements resulting from variable number of fields-of-view per filter. However, for practical purposes, cell counts were shown, to be independent of the number of fields-of-view used for analysis. Consequently, the analysis of 10 randomly taken fields-of-view is sufficient to obtain representative cell counts per filter. Walsby and Avery (1996) even suggested that the analysis of five randomly taken fields-of-view would suffice in representative cell counts. In contrast to our investigations, Walsby and Avery used a $\times 4$ objective for their image analyses. Consequently, these authors analysed larger fields-of-view. Whereas, despite that in the present study 10 fields-of-view are considered necessary, using a $\times 10$ objective is an improvement, as it results in better filament resolution and pronounced differences in fluorescence properties. In addition, higher magnification reduces the number of filament intersections and filament touching per field-of-view. This simplifies *Planktothrix* filament discrimination from other structures (eukaryotic algae and zooplankton) and increases the image processing accuracy.

The measurement precision depended on the total filament length on the filter. This is a function of the filament concentration, the filtered volume and the filter area analysed, which in turn depends on the number of fields-of-view analysed and their magnification. In order to determine the measurement precision of the method and the ideal range of filament density on the filter, filters with various sample volumes of an identical culture sample were analysed. Cell counts correlated significantly with the filtered volume analysing the culture sample. However, cell counts tended to be higher when analysing filters containing <2000 μm filaments per mm^2 filter and tended to be lower when analysing filters containing $>20{,}000$ μm filaments per mm^2 filter demonstrating discrepancies for filters containing high and low filament densities, respectively. This observation is also supported by earlier studies (Walsby and Avery, 1996; Embleton et al., 2003). The underestimation resulting from the analysis of filters with high filament densities is most likely due to superimposition of filaments on the filter, while inaccuracies analysing filters of low filament densities are probably caused by a statistical error due to increasing standard errors with decreasing filament

densities. This is confirmed by the findings of Walsby and Avery (1996) who demonstrated the standard errors tend to decrease as a hyperbolic function of filament concentration. Using the described method, analyses of highest precision could be expected for analyses of filters in a range of 2000–20,000 μm filaments per mm^2 filter. This could easily be achieved by sample dilution or an increase of the volume filtered.

The WHO has provided recommendations for a framework of risk assessment starting at a first alert level of 20,000 cells/ml (Chorus and Bartram, 1999; Falconer, 2001). The Brazilian regulation for drinking water envisions an expansion in cyanobacterial monitoring at a density of 10,000 cells/ml (Azevedo, 2001). Filtration of 20 ml of a sample containing 10,000 cells/ml would result in a cell density of 200,000 cells/filter corresponding to approximately 2000 μm filament per mm^2 filter. A sample containing 150,000 cells/ml would correspond to the highest documented *P. rubescens* cell density demonstrated for Lake Zürich, Switzerland (Hoeger et al., 2005). Ten millilitres of this corresponds to 16,000 $\mu m/mm^2$. Therefore using suitable filtration volumes, the detection limits of the described method are sufficient for an effective risk assessment as recommended by the above named authorities.

Furthermore, the results demonstrate a significant correlation between cell counts and chlorophyll a measurements resulting in a ratio of 1 μg chlorophyll a l^{-1} per 2000 cells/ml. This is in agreement with Chorus et al. (2000), who assumed the same chlorophyll a to cell ratio relating to alert levels for risk assessment regarding cyanobacterial contamination.

This highlights the accuracy of the described method, which is additionally, in contrast to pigment, biomass and protein analyses, not susceptible to disturbances, e.g. effects caused by sample turbidity and/or the presence of other organisms (algae and zooplankton) in the samples. This method thus provides more accuracy than commonly used methods, especially in the analysis of environmental samples.

In addition, this method, requiring only a few minutes per filter, is much faster than other methods normally used for quantification of *P. rubescens*, e.g. most notably microscopic counting. Therefore, a higher number of samples can be counted in a given time frame allowing for continuous and precise observations of *Planktothrix* cell densities, an essential prerequisite for effective risk assessment and management on toxic cyanobacteria.

If an appropriate epifluorescence microscope and sufficient computer capacity are present, costs for the described method are limited to camera equipment, frame grabber and the image processing software. The method described here is therefore a reasonable alternative to the relatively expensive acquirement of a submersible spectrofluorometer and/or other technical laborious measurement procedures.

Acknowledgements

We would like to thank the Arthur and Aenne Feindt Foundation (Germany) as well as the European Union (PEPCY QLRT-2001-02634) for kindly funding parts of this study.[SES]

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