A protocol for the determination of total cell concentration of natural microbial communities in drinking water with FCM

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A protocol for the determination of total cell concentration of natural microbial communities in drinking water with FCM.

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Summary

The conventional approach to characterize the general microbial quality of drinking water is the use of heterotrophic plate counts (HPC), which enumerate the bacterial cells which can grow up to form visible colonies on specific semi-solid media at selected incubation temperatures within a selected time period. On average, about 1 % of the total bacteria in drinking water are culturable with standard HPC methods.

A method for the rapid determination of total bacterial cell concentrations in drinking water, using fluorescent staining and flow cytometry, has been established and standardised. The method uses SYBR Green I which binds to microbial nucleic acids, is excited by a laser at 488 nm, and emits fluorescence at 520 nm, which can then be detected with a flow cytometer.

The FCM method is fast (10 minutes required for staining and 3 minutes for the analysis). The calibrated flow cytometer has an overall error of less than 5%, inclusive of instrument and operator error. It is possible to detect cell concentrations as low as 200 cells/mL with this method, but all evidence up to now suggests that this is well below the typical range of drinking water (c.a. 1×10^5 cells/mL).

The value of accurate total cell counting is evident, e.g. when treatment plant operators aim to monitor the affectivity of specific processes such as membrane filtration or ozonation, or when regrowth during treatment (e.g. GAC filtration) is studied.

Contents

1 Introduction

1.1 Background

High concentrations of planktonic bacterial cells (> 1 x 10^4 cells mL^{-1}) of broadly diverse populations are commonly found in both bottled mineral water and tap water upon consumption (Figure 1) (Leclerc and Moreau, 2002; Hoefel *et al.*, 2003; Berry *et al.*, 2006; Hammes *et al.*, 2007). The primary objectives of drinking water treatment (from a microbiological perspective) are to ensure the absence of any pathogenic bacteria in the finished product, and to limit any uncontrolled regrowth during distribution of the water. Irrespective of the different disinfection processes, it is common for bacteria to regrow during treatment and distribution.

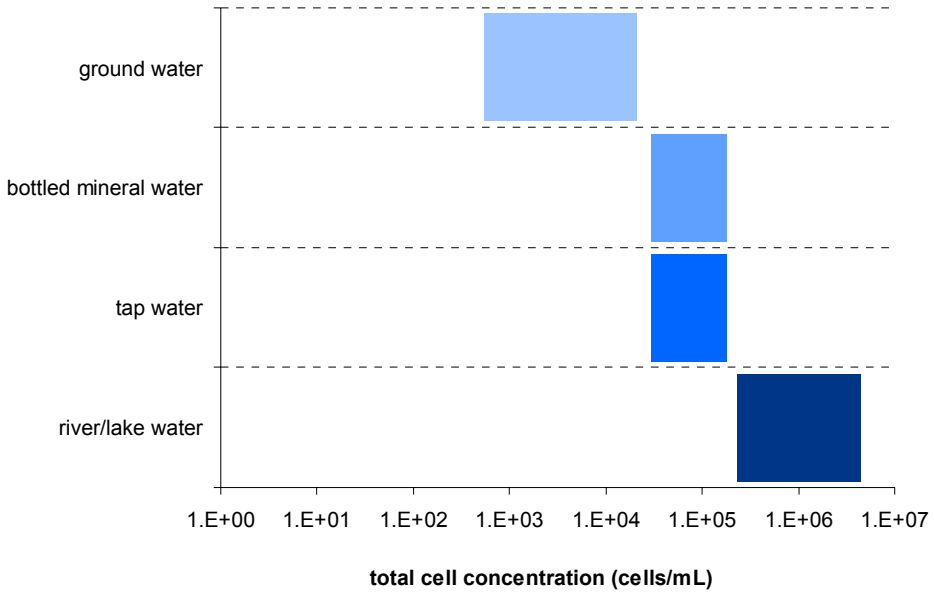

Figure 1. *Typical total cell concentrations for groundwater, bottled mineral water, non-chlorinated tap water and lake water. All data typical for Swiss water sources.*

The cultivation-dependant method of heterotrophic plate counts (HPC) has been in use for more than 100 years (reviewed in e.g., Bartram *et al.* (2003) and Sartory (2004)). HPC still remain the primary parameter for assessment of the general microbiological quality of drinking water, even though it has become dogged by questions about what the method actually measures, how the method should be performed, and how the results should be interpreted (Allen *et al.*, 2004; Sartory, 2004). Most critically, in what became commonly known as "the great plate count anomaly", HPC detect only a small fraction of the total bacterial cells in aquatic environments (Staley and Konopka, 1985; Allen *et al.*, 2004). Typical HPC guidelines for drinking water is in the range

of 100 – 500 cfu/mL, while typical total cell concentrations in non-chlorinated drinking water is in the range of 100,000 cells/mL (Figure 1.1). Even though the immense discrepancy between total bacterial concentrations and cultivable bacterial cell concentrations (HPC) in aquatic samples has been shown numerous times (Staley and Konopka, 1985), the former is still not included in drinking water legislation, guidelines or everyday operational decision-making. The value of accurate total cell counting is evident, e.g. when treatment plant operators aim to monitor specific processes such as membrane filtration or ozonation.

Flow cytometry (FCM) has tremendous potential as a tool for the analysis of bacteria in drinking water. This method can be used for direct enumeration of the total cell concentrations in water (Hammes and Egli, 2005; Lebaron *et al.*, 1998), staining and detection of specific cellular features such as viability (Hoefel *et al.*, 2003; Phe *et al.*, 2005; Berney *et al.*, 2006), or specific detection of targeted cells with antibodies (Vital *et al.*, 2007).

1.2 Goals

The aim of this work was to establish a standard flow cytometry protocol for the determination of total cell concentrations of natural microbial populations in drinking water. The specific target was to establish a method which is rapid (< 30 minutes per analysis), accurate and user friendly.

1.3 Acknowledgement

This report contains the protocol for total cell counting with flow cytometry. An extended version of this work with details of the methodology has been accepted for publication in Water Research (Hammes *et al.*, in press).

2 Total cell concentration

2.1 General overview

The flow cytometric method produces direct total cell counts with high speed (10 – 15 minutes), high accuracy (less than 5 % instrument error), and high sensitivity (lower detection limit of about 200 cells/mL). It does not permit differentiation of bacterial cells on the basis of taxonomy, metabolic activity, culturability or viability, and estimations of biomass or biovolume from light scattering data are complex and subjective and difficult to do. The method requires a flow cytometer operator that can distinguish stained microbial cells from background noise signals on the basis of relevant control samples and experience. The method assumes proper maintenance, standardization and calibration of the flow cytometer used. The method consists of staining an aqueous sample of suspended bacteria with a DNA-targeting fluorochrome and enumeration of the stained cells by counting with a flow cytometer. Fixatives for storage of samples are optional (see Standard Methods 9216 B, Greenberg et al. (1993)) but long-term storage before analysis is not recommended.

2.2 Apparatus

a. *Flow cytometer*, equipped with a 488 nm blue laser with a minimum power of 15 mW[1].
b. *Filters*, a 520 nm bandpass filter for green fluorescence detection, a 630 nm bandpass or longpass filter for red fluorescence detection.
c. *Counting modus*, volumetric counting hardware are inclusive to some instruments[2] and preferable, though this can be compensated for by the use of commercially available bead count standards[3].
d. *Flow cytometry measuring tubes*, comes standard for different brands of instruments.

2.3 Reagents

a. *Fluorochrome*, SYBR® Green I[4], diluted 1:100 from original stock solution in 0.2 µm filtered DMSO, and stored at -20 °C until use.
b. *Dilution liquid*, preferably from the same solution in which the bacteria are suspended, filtered with a 0.1 µm sterile syringe filter prior to use.
c. *EDTA stock solution*, 500 mM EDTA suspended in nanopure water and sterile filtered (0.2 µm) prior to use.
d. *Lysis buffer stock solution*, comprising 10 % v/v Triton X-100, 5 % v/v Tween 20, 10 mM Tris-HCl, and 1 mM EDTA.

[1] Partec GmbH or equivalent
[2] Partec GmbH or equivalent
[3] CountBright (Invitrogen) or equivalent
[4] Or equivalent DNA binding stains e.g. SYBR Green II or SYTO9

2.4 Procedure

- Collect water samples as directed in Section 9060 of Standard Methods (Greenberg *et al*. 1993). Fixation can be performed as described in Section 9216 B of Standard Methods (Greenberg *et al*. 1993), but direct processing of samples is highly recommended.
- Take 1 mL of the sample in a sterile Eppendorf tube and add 10 µL of the fluorochrome and 10 µL of EDTA stock solution, mix thoroughly by vortexing and incubate in the dark at room temperature for at least 10 minutes. If the bacteria concentrations are expected (or found) to exceed 1 x 10^7 cells/mL, it is advised to increase the concentration of fluorochrome accordingly (10 µL of respectively fluorochrome and EDTA for every 1 x 10^7 cells per mL).
- If the flow cytometer is not equipped with volumetric counting hardware, add just before measurement a commercially available bead standard of known concentration as prescribed by the manufacturer[5].
- Run the sample through a flow cytometer using the instrumental settings appropriate for the filter set-up and trigger on the green fluorescence channel.
- If the bacterial concentration in the sample exceeds 2 x 10^5 cells/mL, dilute the sample to a concentration between 0.1 – 2 x 10^5 cells/mL after staining and just before measurement with the dilution liquid.
- If the bacteria are present in clusters (can be controlled with fluorescence microscopy or evidenced through FCM light scatter data), dispersion of clustered cells can be achieved with the addition of 1 % v/v of lysis buffer prior to staining, coupled with vortexing or gentle sonication.

2.5 Calculations

Optimal separation of stained bacteria from background noise is achieved on a two dimensional dotplot of green fluorescence (520 nm) and red fluorescence (630 nm). Background noise and stained bacteria are separated from each other by means of electronic gating (see section 3 of this report), using the flow cytometry software. Depending on the flow cytometer used, the software usually performs the counting and calculations automatically. Any sample dilution should be accounted for by the operator.

[5] CountBright (Invitrogen) or equivalent

3 Discussion

Flow cytometry is able to clearly discern stained bacterial cells from background. Figure 3.1a shows a typical FCM dot-plot of green fluorescence (FL1) and red fluorescence (FL3) in which each individual dot represents the corresponding green and red fluorescence signals for each particle that was detected. The bacterial populations are separated from the instrument noise and background particles (e.g. inorganic crystals) by means of electronic gates which are constructed manually using the supplied software of the flow cytometer. The distinction between background noise signals and bacteria is based on experience with the instruments and relevant controls (e.g. a water sample before and after filtration with 0.1 µm).

We have found that the best separation is achieved using the combination of green and red fluorescence (Figure 3.1a), although some authors use green fluorescence alone, or green fluorescence in combination with scattered light (particle size; Figure 3.1b) (Hammes and Egli, 2005; Hammes *et al.*, 2007). The scatter signals (SSC) shown in Figure 3.1b provides additional information of the relative size of the particles (reference), and in the example displayed below, one can clearly recognise the tow distinct populations, different in both fluorescent intensity and size. These represent the so-called high nucleic acid (HNA) and low nucleic aid (LNA) bacteria (Lebaron *et al.*, 2001).

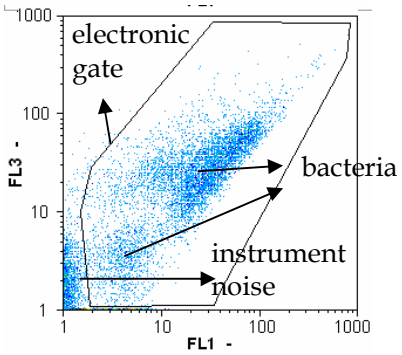

Fig. 3.1a. *Flow cytometry* dot-plot *of bacteria from the effluent of a granular active carbon reactor. Each dot represents corresponding green fluorescence (FL1) and red fluorescence (FL3) of each particle which was analysed.*

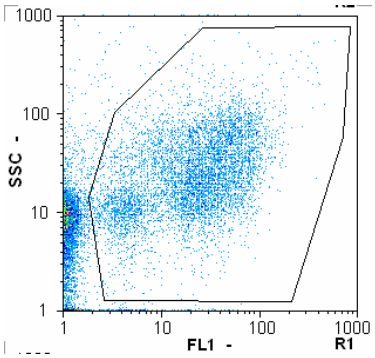

Fig. 3.1b. *Flow cytometry* dot-plot *of bacteria from the effluent of a granular active carbon reactor. Each dot represents corresponding green fluorescence (FL1) and scattered light signals (SSC) of each particle which was analysed.*

Note that the red fluorescence (FL3) in Figure 3.1a also originates from the SYBR® Green I fluorochrome which is used for staining (Figure 3.2). Even though the emission of SYBR® Green I is 10-fold higher at 520 nm (FL1) than at 615 nm (FL3). This difference, however, is compensated for by using higher voltage settings for the FL3 detectors (section 2, this report).

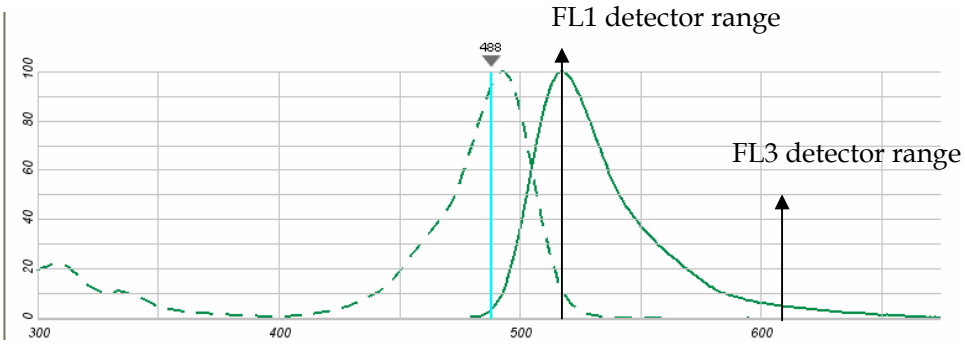

Figure 3.2. *The excitation and emission spectrum of SYBR Green I used for the staining of bacteria. The optimum emission is at 520 nm, similar to the filters used in the flow cytometer for detection. A tail of the stain is also visible above 600 nm (range of the red FCM filters).*

The method is rapid – it requires 10 minutes for staining and 3 minutes per analysis. The result is that a large volume of samples can easily be processed and the quick results allow treatment plant operators to take immediate action in case the results suggest a problem in the system. The method is also very accurate. The standard deviation on triplicate FCM measurement is in the range of 5 %, of which half can be ascribed to user error and half to instrument error. The method is sensitive (Figure 3.3). It is possible to measure accurately in a concentration range of 200 cells/mL, although the optimal range of measurement lies between 10 000 – 100 000 cells/mL.

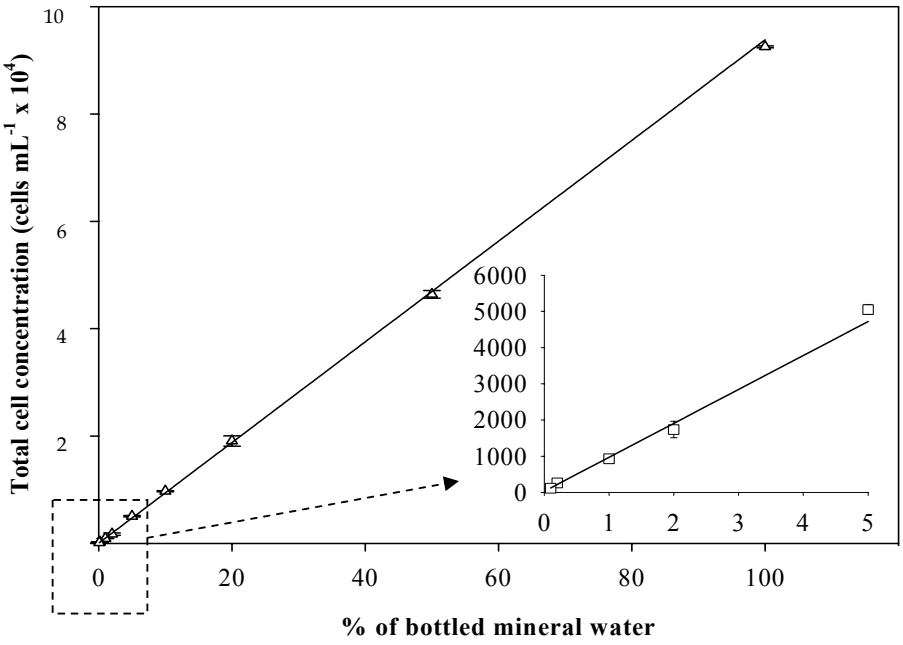

Figure 3.3. *Sensitivity and reproducibility of FCM measurements of bacteria from bottled mineral water diluted with cell-free water (n = 9). All data points are average values of triplicate samples (data from Hammes et al. (2007)).*

The method is statistically sound. In the analysis of one sample, 200 uL of solution is actually measured. This means that in a sample with a cell concentration of 100 000 cells/mL, and effective amount of 20 000 events would be recorded (Hammes and Egli, 2005).

4 Conclusion

The total cell count method with flow cytometry has been standardised and tested to a level where it is deemed usable for the analysis of total bacteria cell concentrations in drinking water. The method can be used directly for samples with bacterial concentrations in the range of 200 – 200,000 cells/mL, which is also the typical range for drinking water bacteria. In case of higher cell concentrations, I dilution step would be required. The FCM method represents a fast and accurate way to measure total microbial cell concentrations in drinking water. The values obtained this way will be particularly useful for the accurate description of the microbiology in drinking water treatment trains (Hammes *et al.*, 2007), and for the correct assessment of the microbial stability of drinking water. Given enough experience and data gathered with this manner, it is foreseen that it might bring about a paradigm shift in the legislative guidelines for assessing the general microbial quality of drinking water. The method will be tested on pilot scale and full scale treatment and distribution systems for final validation and interpretation of the results.

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