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Assessing the feasibility of total virus detection with flow cytometry in drinking water

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Contents

1 Introduction

Human pathogenic viruses which are transmitted via drinking water are normally summed up as human enteric viruses, because they preferably multiply in the intestine (except hepatitis and poliovirus). The transmission route is faecal-oral. Viruses transmitted via drinking water are usually of humans origin, but for rotavirus (Cook et al., 2004) and for hepatitis E virus (Clemente-Casares et al., 2003) animal reservoirs have also been identified.

As a result of the low concentration of human pathogenic viruses in drinking water it is essential to concentrate big sample volumes before detection is possible. This is done using filtration techniques, flocculation or affinity chromatography and is usually associated with virus loss and sometimes inactivation due to the treatment (Auckenthaler, 2003). The most common technique for detection after concentration is to grow viruses on cell cultures and subsequent analysis of the plaques, which are formed on the cell monolayer. Unfortunately not all viruses are able to grow in culture. Furthermore, the technique takes a lot of time (one week), is very expensive and needs skilled laboratory workers.
New molecular methods like reverse transcriptase (realtime) PCR seem to be a good alternative and circumvent problems connected with the cell culture approach (Beuret, 2004). PCR is very sensitive, but also associated with several disadvantages. Detected viruses are not essentially infective and substances present in the water matrix, like humic acids, can inhibit the amplification reaction (Walter, 2000). Furthermore, RNA is very unstable and the capsid is often very well structured and therefore not easy to break up for isolation of nucleic acids.

Studies have been carried out using (entero)bacteriophages as indicator organisms for investigation of processes connected to viral behaviour in the field of drinking water. Most of these studies are based on single selected bacteriophage species and have shown not to give good results. Another approach is to consider all bacteriophages as indicators to investigate virus related issues in drinking water research. A study detecting total bacteriophages during a drinking water treatment train based on microscopic analysis has been published (Rinta-Kanto, 2004). Furthermore, enumeration of total viral abundance in natural samples with the flow cytometer has been reported (Marie et al., 1999) and an optimization protocol is available (Brussaard, 2004). The combination of total viral counts and flow cytometry for drinking water analysis is presently not available, but it could provide a fast screening tool.

1.1 Goals

The aim of this study was to implement and test a published protocol (Brussaard, 2004) for rapid, quantitative, unspecific detection of viruses in natural water samples by flow cytometry. Based on the detection of the model

organism bacteriophage T4, the feasibility of this method for drinking water related applications was assessed.

2 Methods

2.1 Growing of host cells and propagation of T4 bacteriophages

E. coli K12 cells were grown overnight in LB medium at 37 °C, containing 5 mM $CaCl_2$. 10 mL of the medium have been inoculated with 100 µL of over night culture. The suspension was put in a water bath for ~2 h at 37 °C (precitherm® PFV, Boehriger Mannheim) and 100 µL of T4 phage suspension (Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH (DSMZ)) was added. The propagation was carried out for 3 h at 37 °C in the incubation oven (Lab-Shaker, Adolf Kühner AG). Three drops of chloroform were added and the suspension was centrifuged for 10 min at 4000 rpm (Megafuge 1.0R, Sepatech) to spin cell fragments down. The supernatant contained the T4 bacteriophages and subsequently titration of the lysate was performed. Phage solutions were stored at 4 °C.

2.2 Plaque-forming unit assay

100 µL of host culture grown over night in LB containing 5 mM $CaCl_2$ and 100 µL of phage solution of different dilution (10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}) were added. One tube did not contain any phages to allow a comparison of the bacterial lawn with and without T4 plaques. Pre-adsorbing of the phage to the host was done by incubating the mixed solution at 37 °C in a water bath for 10 min. Then 2.5 mL of R-top agar was added to each tube and the suspension plated on R agar plates. Three series in parallel have been carried out to ensure a reliable result.

2.3 Staining of viruses

The staining procedure was performed according to the protocol described (Brussaard 2004). First, fixation of viruses was achieved by putting 1 mL of T4 phage solution in an Eppendorf tube, adding of 125 µL of 4 % PFA (final concentration 0,5 %) and incubation for 30 min at 4 °C. For permeabilisation samples were put in liquid N_2 (-180 °C) for 1 min, thawed again, and finally diluted in Tris-EDTA (TE) buffer down to 10^{-8}. Subsequently 5 µL of 100x diluted (DMSO) SYBR® Green I was added to the different solutions. Staining was performed for 10 min at 80 °C in the dark, followed by sonication for 30 sec before analysis by flow cytometry.

2.4 Analysis using different flow cytometer models

From the virus suspension a dilution series to 10^{-8} was made (see above) and subsequently the -5, -6 and -7 dilutions have been measured on three different flow cytometers; PASIII (Partec), CyFlow® Space (Partec), Cytomics FC 500 MPL (Beckman Coulter). Instrument settings where optimized for highest sensitivity using 0.5 µm fluorescence beads. The trigger was always set on the green fluorescence channel.

3 Results

3.1 Validation of the staining procedure using epifluorescence microscopy

The reported protocol was used to stain T4 bacteriophages (Brussaard, 2004). The stained viruses are visible as small green fluorescent spots with epifluorescence microscopy (Figure 1). Note that the T4 bacteriophages were specifically chosen for initial testing of the method since detection using flow cytometry has already been reported and due to the large size (capsid ~ 100 nm) and high DNA content of these particles (Brussaard, 2004).

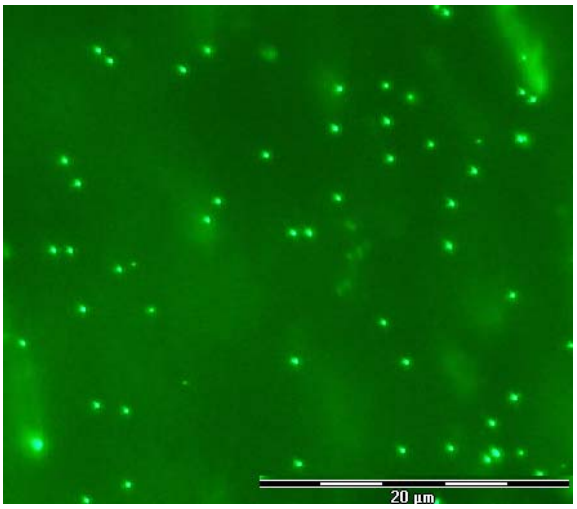


Figure 1. SYBR® Green stained T4 phage suspension imaged with an epifluorescence microscope.

3.2 Detection of T4 bacteriophages by flow cytometry

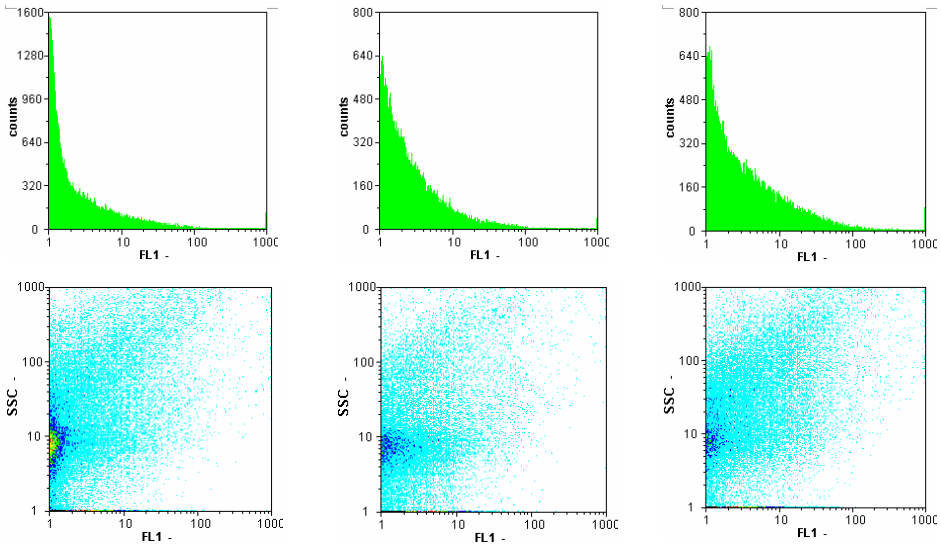
A dilution series of the stained virus suspension (above) was prepared and subsequently analyzed on three different flow cytometers (Table 1). Additionally, a plaque count assay was performed as a reference. Corresponding flow cytometric data is shown in Figure 2.

Table 1. Measuring T4 bacteriophages, stained with SYBR Green on different flow cytometers. A plaque count assay was performed as reference.

Flow cytometer	**counts per mL**		
plaque counts	**1.3 x 10^5**	**1.3 x 10^4**	**1.3 x 10^3**
PASIII (Partec)	background	background	background
CyFlow® Space (Partec)	3.75 x 10^5	5.07 x 10^4	background
Cytomics FC 500 MPL (B C)	4.67 x 10^5	7.09 x 10^4	background

PASIII (Partec)

10^{-5} diluted	10^{-6} diluted	10^{-7} diluted



CyFlow® Space (Partec)

10^{-5} diluted	10^{-6} diluted	10^{-7} diluted

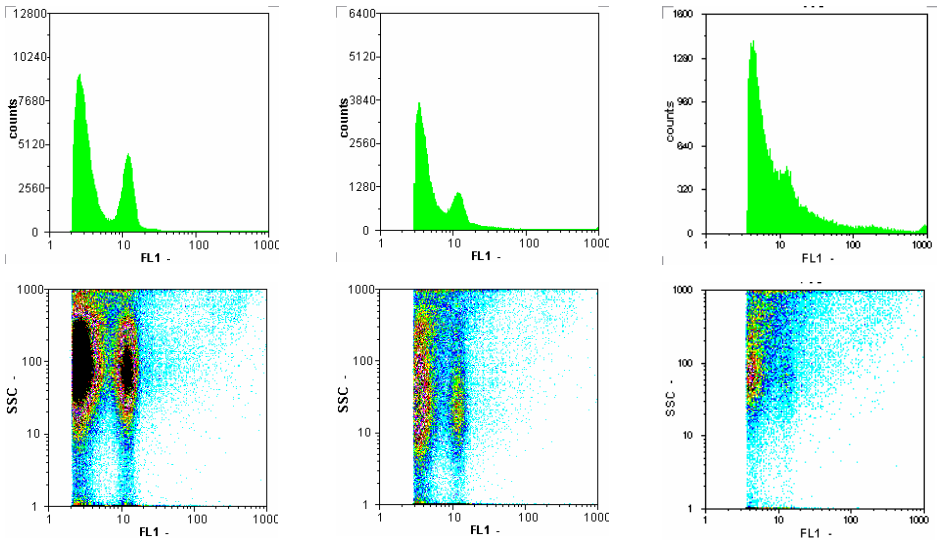


Figure 2. Measuring a dilution series of stained bacteriophages T4 using different flow cytometers. The upper row shows histograms illustrating green fluorescence intensities versus counts. In the bottom row, dot plots indicating green fluorescence intensities versus sideward scatter signals are imaged. The trigger was always on FL1 (green fluorescence). Counts (Table 1) were acquired on the FL1-SSC dot blot.

Cytomics FC 500 MPL (Beckman Coulter)

5x diluted	6x diluted	7x diluted

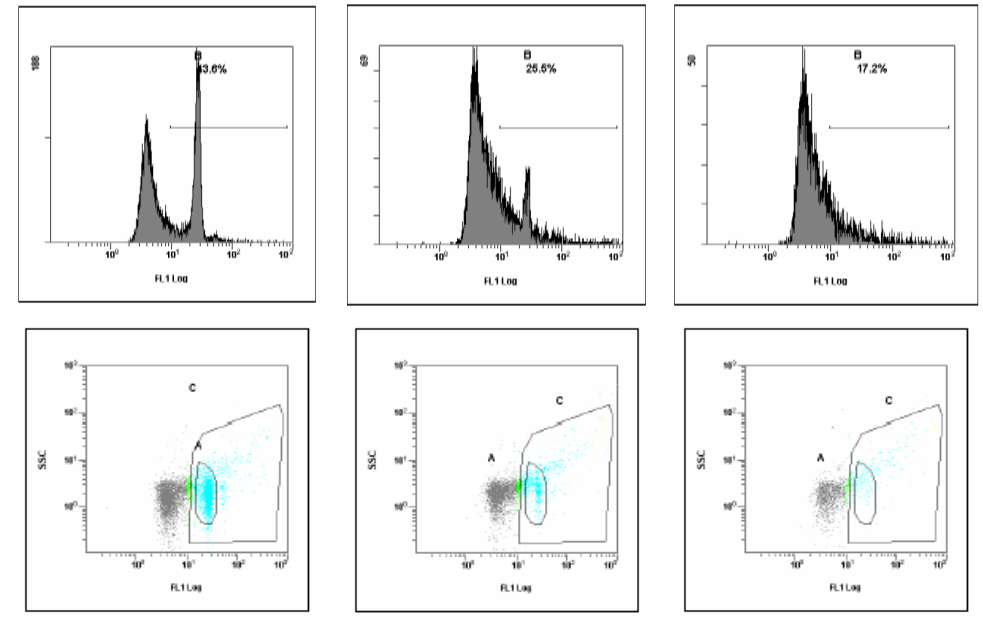


Figure 2 (continued). Measuring a dilution series of stained bacteriophages T4 using different flow cytometers. The upper row shows histograms illustrating green fluorescence intensities versus counts. In the bottom row, dot plots indicating green fluorescence intensities versus sideward scatter signals are imaged. The trigger was always on FL1 (green fluorescence). Counts (Table 1) were acquired on the FL1-SSC dot blot.

4 Discussion

T4 bacteriophages could be detected using flow cytometry (Figure 2). A separated peak was visible on two of the instruments that were tested, and enumeration down to a concentration of about 5 x 10^4 viral particles/mL was possible. No viral counts could be detected using the PASIII instrument. This result is due to lower power of the laser of the machine and less improved optical equipment in comparison to the other instruments. It has to be noted that extreme cleaning and calibration of the instruments were required in order to detect the weak fluorescent signals arising from the stained viral particles above the instrumental background. Moreover, due to the small size of viral particles, it was not possible to use size as an additional discriminating parameter, as is often the case with bacterial analysis with flow cytometry. The discrepancy between viral counts and obtained plaque forming units (Table 1) could be explained by loss of infectivity of the phages. Only infective particles are detected using the plaque count assay, whereas all intact particles, containing DNA, are stained with SYBR® Green and therefore counted by flow cytometry and/or microscopy.

The experiments were performed under laboratory conditions using an optimal model organism (large T4 bacteriophages) for analysis and by additional minimizing instrumental background signals. This virus was chosen since it is relatively large (~100 nm capsid) and because it contains dsDNA, where SYBR® Green preferably binds to and yields the highest fluorescence signal. Nevertheless the detection was on the limit of sensitivity for the flow cytometers (Figure 2). The green fluorescence peak was very near to the background signal. Applications for natural samples face even more difficulties. Naturally abundant particles are interfering with the measurement by their auto-fluorescence increasing the background signal significantly. Additionally the dye SYBR® Green is reported to bind to abiotic particles giving false positive counts near the background (Kerner et al., 2003). Furthermore, even though it is considered, that the majority of natural abundant viruses in nature are belonging to the order C*audovirales,* which have a dsDNA genome, also other viral genome structures (ssDNA or RNA) have been characterised, which are not readily detectable when using the SYBR Green dye (Weinbauer, 2004).

In our opinion, the application of this method for the rapid and quantitative detection of total viral counts down to the required concentration levels (10^4 to 10^5 per mL) for drinking water analysis is therefore not yet feasible using the tested instruments and protocol. The main limiting factor is interference from background signals in natural water samples. Results would be very vague and could therefore easily lead to false interpretations and reactions of drinking water facilities. However, improvements in optics and sensitivity of flow cytometers, together with advances in staining protocols, could yield better total virus detection and thus enable the method applicable for drinking water research in future.

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