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Quantification of aquatic viruses by flow cytometry

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Abstract

For many laboratories, flow cytometry is becoming the routine method for quantifying viruses in aquatic systems because of its high reproducibility, high sample throughput, and ability to distinguish several subpopulations of viruses. Comparison of viral counts between flow cytometry and epifluorescence microscopy typically shows slopes that are statistically not distinguishable from 1, thus confirming the usefulness of flow cytometry. Here we describe in detail all steps in the procedure, discuss potential problems, and offer solutions.

Introduction

Viruses are the most numerous biological entities in aquatic ecosystems, typically on the order of 10^7 mL^{-1} (Suttle 2007). Many studies have contributed to the acknowledgment that viruses are active and diverse players in freshwater and marine ecosystems (e.g., Brussaard et al. 2008b; Suttle 2007). Viral activity profoundly impacts ecosystem function and structure by affecting host population dynamics, species succession, biodiversity, and global biogeochemical cycles.

To detect specific viruses or virus subpopulations, the use of antibodies, plaque assay, or dilution to extinction in the presence of the appropriate host and approaches based on molecular markers have proven very useful (Larsen et al. 2007; Mühling et al. 2005; Schroeder et al. 2003; Short and Suttle 2002). To count total free viruses in aqueous samples, transmission electron microscopy (TEM), epifluorescence microscopy (EFM), and flow cytometry (FCM) are most often used. TEM has the advantage of providing specific information about the morphology and size of the virus particles, but TEM is time-consuming and costly and, although information-rich, has inherently low throughput. Over the past two decades, the introduction of highly sensitive fluorescent nucleic acid–specific dyes (e.g., SYBR Green) in combination with affordable EFM have greatly facilitated the detection and quantification of viruses in a broad range of aquatic ecosystems. Although the same sensitive nucleic acid–specific stains can be used in combination with FCM, its powerful analytical capabilities allow sensitive detection, accurate quantification, and rapid analysis of viruses relative to other conventional techniques such as TEM and EFM. FCM is a high-throughput method that, in addition, permits the discrimination of various virus populations based on their fluorescence and scatter signal after staining (Brussaard et al. 2000; Jacquet and Bratbak 2003). This is of benefit for spatial and seasonal analysis of viral dynamics and structure in a large number of natural samples (Brussaard et al. 2008a; Li and Dickie 2001; Payet and Suttle 2008).

This article describes critically and in detail the protocol to enumerate aquatic viruses by FCM based on the methodology previously developed by Marie and co-workers (1999a) and optimized by Brussaard (2004). Its application for samples from different environments is discussed and compared to results based on EFM.

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Materials and procedures

An outline of the FCM assay for detection and quantification of aquatic viruses is presented in Fig. 1.

Reagents and solutions—FCM detection and enumeration of viruses requires high-quality reagents. Water samples are preserved using 25% electron microscopy (EM)-grade glutaraldehyde (Sigma; storage at 4°C). The EM-grade glutaraldehyde is free of polymers and other contaminants and, hence, is optimal for fixing the samples. To avoid cross-contamination of samples in pipetting, it is important to prepare small aliquots of the fixative solution.

The use of ultrapure sheath fluid is essential, since one works close to the limits of detection of the instrument. An improved FCM signal is obtained when using Milli-Q water (ultrapure deionized water with resistivity of 18.2 MΩ cm^{-1}) instead of the commercially available sheath fluids (e.g., FACSFlow). Working stain solution of SYBR® green I (10,000× concentrate in DMSO; Invitrogen, Molecular Probes; storage at –20°C) is usually prepared by diluting the commercial stock (1:200) in either autoclaved Milli-Q or grade molecular water. Working solution stain can be reused but it is best to limit the number of freeze–thaw cycles (two or three) to prevent the loss of staining efficiency over time. Thus, small aliquots (1 mL) of working stain solution should be prepared. The commercial stock is supplied in DMSO, but further dilution in DMSO typically increases noise levels upon addition to the samples. Occasionally, the fluorescent dye seems responsible for generating noise, and a brief spin (~20,000*g*) of the stock solution in a microfuge generally reduces the noise levels.

Samples are diluted in sterile TE-buffer, pH 8.0 (10 mM Tris-hydroxymethyl-aminomethane, Roche Diagnostics; 1 mM ethylenediaminetetraacetic acid, Sigma-Aldrich) to avoid electronic coincidence (e.g., *see* below). The use of any diluents (e.g., phosphate-buffered saline, Milli-Q water, etc.) other than TE-buffer was found to negatively affect flow cytometric signatures of stained viruses (Brussaard 2004).

TE-buffer should be autoclaved directly after preparation to maintain low background fluorescence (check pH before use and adjust if needed using HCl). The quality of Tris may differ depending on the supplier, and thus, it is likely to affect the quality of TE-buffer (Brussaard unpubl. data). In principle, filtration before first-time use of the TE-buffer should not be necessary. But, once opened, small batches (i.e., ~50 mL) of the TE-buffer should be prefiltered (sterile FP30/0.2 µm; Schleicher & Schnell) just before use. Change the filter for each batch of the TE-buffer. Filtration may result in enhanced noise level, depending on the filter type. Filtration of the TE-buffer through 30-kDa molecular weight cutoff filters would be ideal but time-consuming. Instead, use a new batch of sterile TE-buffer and carefully check the noise level by running a stained blank (*see* "Blank and reference") before use.

Sampling and storage—Proper storage and preservation of aquatic samples is crucial to prevent loss of virus particles. Typically, there is no need to filter or treat the natural water samples before fixation. Filtration of the samples before fixation may result in substantial loss of viruses (data not shown).

Step	Process	Method	Notes
1	Fixation	Fix sample with 0.5% glutaraldehyde, 30 min 4 °C	Prevent prolonged fixation as it will result in reduced virus counts
2	Storage	Flash freeze of sample critical; storage can be at -80 °C	Thaw once (NO refreezing) and keep cool before analysis
3	Prestart	Clean flow cytometer, check optimal settings, determine flow rate	Verify noise level FCM is low: check with MilliQ and TE blank
4	Sample preparation	Dilute sample, stain with SYBR Green I, 10 min 80 °C in dark	Dilution factor >10, use TE-buffer; cool down for 5 min
5	Counting	Count sample for 1 min at flow rate of 30-50 µL min^{-1}	Event rate <1000 sec^{-1} to avoid coincidence; but >100 sec^{-1}
6	Data analysis	Gate virus populations; subtract blank from samples	Be consistent in gating, include baselines

Fig. 1. Different processes, accompanying methodology, and critical notes for flow cytometric enumeration of aquatic viruses.

Samples of 1 mL are usually taken (replicate sampling is advised), transferred into 2-mL cryovials, and fixed at a final concentration of 0.5% glutaraldehyde for 15–30 min at 4°C in the dark. After fixation, the samples are flash frozen in liquid nitrogen. Flash-freezing is very important, as fixed samples stored at 4°C show significant and rapid reductions in virus counts (Brussaard 2004; Wen et al. 2004). For the same reason, it is important to minimize the fixation period to less than an hour (15–30 min is optimal). Once frozen in liquid nitrogen, the samples should be stored at –80°C (testing storage for 6 months showed no detectable virus decay [Brussaard 2004]). Note that after a field expedition, frozen samples should be sent either in dry ice or in a liquid nitrogen dry-shipper to keep samples deep-frozen during transport.

FCM setup—Not all FCMs have equal sensitivity to detect and enumerate aquatic viruses. Whereas some FCMs will detect only the higher green fluorescent virus subpopulations, others may not be sensitive enough to detect viruses at all. The 488-nm argon laser benchtop FCMs of Becton Dickinson (e.g., BD-FACScalibur) provide high sensitivity for virus detection. The advantage of benchtop FCMs is that the machines can be easily taken on board ship.

Virus particles are too small to scatter light of standard benchtop FCMs. The use of nucleic acid–specific stains, such as SYBR Green I, is thus essential for virus detection. FCM signatures of stained viruses from natural samples can partially overlap with background fluorescence generated by FCM. Ultimately, it is important to work with a clean FCM with low background noise to obtain high-quality, reproducible data. Moreover, several blanks should be run before analysis to check whether the FCM and the reagents are clean or not (*see* "Blank and reference").

Use maximum voltage for the green fluorescence photomultiplier tube (PMT) at which no electronic or laser noise is detected. This can be obtained by running freshly prepared Milli-Q water as sample and increasing the voltage for the green PMT until noise is detected; the maximum voltage that can be used is just below this. In some instances, the machine may seem to be clean but after running a stained blank high levels of noise are observed. Try running TE-buffer for some time, followed by another stained blank to check. If still dirty, a useful remedy can be to purge (prime) a few times and clean once more. Once the FCM is clean and ready for use, try to analyze the samples in one series, not interrupted by analysis of other organisms and use of other dyes. Bacterial enumeration can also be done from the same sample using a slightly different setting and staining protocol (Marie et al. 1999b) with no interference for virus counts. In case of a high-event-rate sample (i.e., >1000 events s^{-1}), rinse shortly with TE-buffer or Milli-Q before the next sample.

Sample dilution—Typically, samples need to be diluted before analysis to minimize electronic coincidence of the virus particles (i.e., two or more virus particles pass the analysis window of the laser simultaneously, reducing accuracy of the analysis). For virus samples, this coincidence is minimized at event rates <1000 events s^{-1} (Marie et al. 1999b). Salts present in the water samples can strongly interfere with the efficiency of the stain SYBR Green I, resulting in inaccurate quantification of the viruses. Consequently, the final dilution factor should be greater than 10-fold, with a sample volume ≥50 µL used for the dilution. For each sample, a serial dilution of three to four different dilutions of 500 µL final volume is usually optimal to obtain an event rate within 200–800 events s^{-1}). Subsequently, the rest of the samples can be analyzed using this optimal dilution factor.

Sample staining—The samples are stained with SYBR Green I at a final concentration of 0.5×10^{-4} of the commercial stock (i.e., add 5 µL working stain solution to 500 µL sample). The samples are then incubated at 80°C for 10 min in the dark, followed by a cooling period at room temperature in the dark for 5 min before analysis. Heating of fixed samples significantly enhances the staining efficiency of viruses (Brussaard 2004; Marie et al. 1999a).

Blanks and references—Control blanks, consisting of TE-buffer with autoclaved 0.2-µm-filtered (or 30-kDa ultrafiltered) sample at the same dilution factor as the natural samples, should be used before FCM analysis of the samples. Filtering natural sample through a 0.02-µm pore-size filter instead of autoclaving is not advised, as this may generate substantial background noise.

Blanks are diluted, stained, and processed identically to the samples. Very low coincidence (0–15 events s^{-1}) and background fluorescence levels should be detected before proceeding with sample analysis. Blanks ideally show a total amount of 400–1100 events in 1 min of acquisition at a flow rate of ca. 40 µL min^{-1}. During the analysis, always add one to two blanks to every batch of samples to monitor whether the noise level stays low.

An internal reference can be used not only to normalize the fluorescent signal of the stained virus populations, but more importantly to detect deviations of the FCM from standard behavior. Highly diluted and well-mixed fluorescent microspheres (FluoSpheres carboxylate modified yellow-green fluorescent microspheres; 1.0 µm diameter; Invitrogen, Molecular Probes; F8823; stored at 4°C) may be used as reference. An initial brief sonication of the primary stock (1% vol/vol, storage at 4˚C) is recommended to disrupt the aggregates. Working bead solutions are then prepared by diluting the primary stock in sterile Milli-Q water (i.e., add 10 µL stock in 2.5 mL Milli-Q water) every day.

Acquisition and data analysis—The appropriate settings for detection of stained virus particles are specific for each FCM. Fluorescence and scatter signals are collected on a logarithmic scale (4-decade dynamic range) for best results. The trigger for detection is set on green fluorescence, and data are acquired on a dot plot displaying green fluorescence versus side scatter signal (Fig. 2). Commercial benchtop FCMs come with a certain minimum threshold. This standard instrument threshold level (typically 52 for BD-FACScalibur) should be used during acquisition of the data.

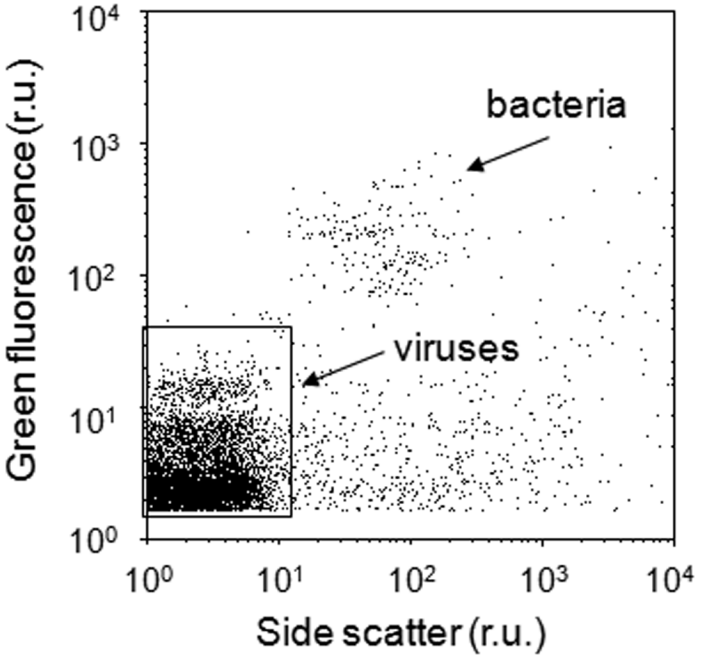

Fig. 2. Cytogram of SYBR Green I–stained viruses in typical natural aquatic sample according to protocol described herein (10,000 events plotted). For optimal reproducibility and to include the very low green fluorescent virus particles in the data analysis, the gating should always be set to include all the particles. r.u., relative units.

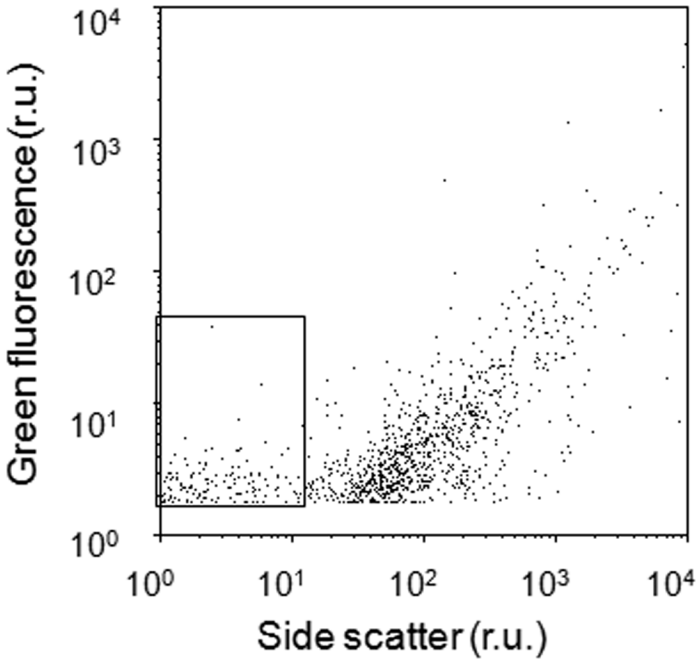

Fig. 3. Cytogram of SYBR Green I–stained blank (using autoclaved 0.2-µm pore-size or 30-kDa prefiltered seawater instead of natural sample) according to protocol described herein (all events obtained plotted, i.e., a total of 840, of which 222 were in the window used to discriminate viruses). The diagonal streak of dots outside and on the right side of the virus window is due to the TE-buffer in combination with the fluorescent dye (SYBR Green I). r.u., relative units.

A medium flow rate between 30 and 50 µL min^{-1} is adequate to detect viruses. FCMs with a sample injection port (e.g., BD-FACScalibur) should have the outer sleeve cleaned between samples to prevent cross-contamination (wipe with Kimwipes® tissue). Samples should be mixed by hand before analysis, as vortexing may result in decay of viruses (reduction of 15% for natural coastal seawater, data not shown). Allow the flow rate to stabilize before analyzing the sample. Acquisition time is typically 1 min.

Data analysis of the raw data collected in list-mode files can be performed using a wide array of software (either supplied with the FCM or freeware from the internet; e.g., CytoWin or WinMDI). For optimal reproducibility and to include the very low green fluorescent virus particles in the data analysis, the gating should always be set to include all the particles (Fig. 2). Importantly, virus counts in the sample should be corrected for particles counted in the blanks (Fig. 3) before calculating virus concentrations.

Assessment

Staining—FCM analysis of the stained aquatic viruses generally discriminates two or three viral subpopulations (V1–V3) with different green fluorescence properties (Fig. 4). A fourth viral subpopulation (V4) may be observed (Fig. 5), commonly representing large dsDNA algal viruses (Brussaard et al. 2000; Jacquet and Bratbak 2003). Although most of the bacteriophages (i.e., viruses infecting bacteria) are thought to be included in the lower fluorescent viral subpopulations (V1 and V2 windows, Fig. 4), it was recently found that some eukaryotic algal viruses displayed similar low fluorescence upon staining (Brussaard and Martínez Martínez 2008). Similarly, some pro- and eukaryotic algal viruses were also found in the V3 window (Brussaard et al. 2000). Furthermore, the level of nucleic acid–specific fluorescence is not indicative of the viral genome size. There was no linear relationship between the viral genome size and green fluorescence properties upon staining with a nucleic acid–specific stain (Brussaard et al. 2000).

SYBR Green I has a strong affinity for dsDNA but can also stain ssDNA and RNA, according to the manufacturer (Invitrogen). Several tests using various types of viruses indicated that these ssDNA and RNA viruses can be stained with SYBR Green I (Brussaard et al. 2000). Nevertheless, some RNA-virus populations may not be fully separated from the background noise fluorescence; using other acid-specific dyes such as SYBR Green II (higher quantum yield when bound to RNA than to dsDNA) or SYBR Gold did not improve the detection of these viruses (Brussaard et al. 2000; Brussaard 2004).

SYBR Gold, a fluorescent dye, detects DNA and RNA and is more sensitive than SYBR Green I and can also be used as an alternative of SYBR Green I for FCM detection of viruses (Chen et al. 2001). However, FCM data revealed significantly higher counts of viruses stained with SYBR Green I than with SYBR Gold (Brussaard 2004). Thus, SYBR Green I seems best for opti-

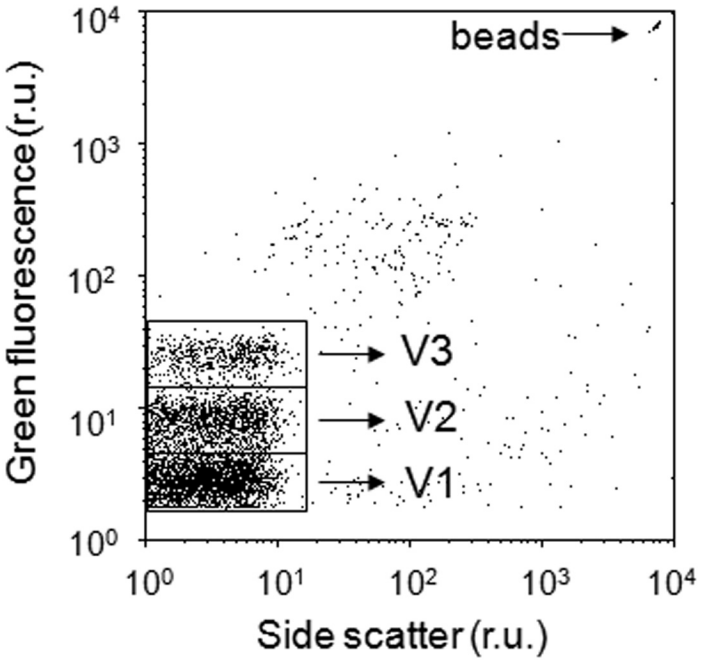

Fig. 4. Cytogram of SYBR Green I–stained viruses in typical natural aquatic sample according to protocol described herein (10,000 events plotted). Virus subpopulation with lowest green fluorescence is named V1, with midlevel fluorescence V2 and highest fluorescence V3. r.u., relative units.

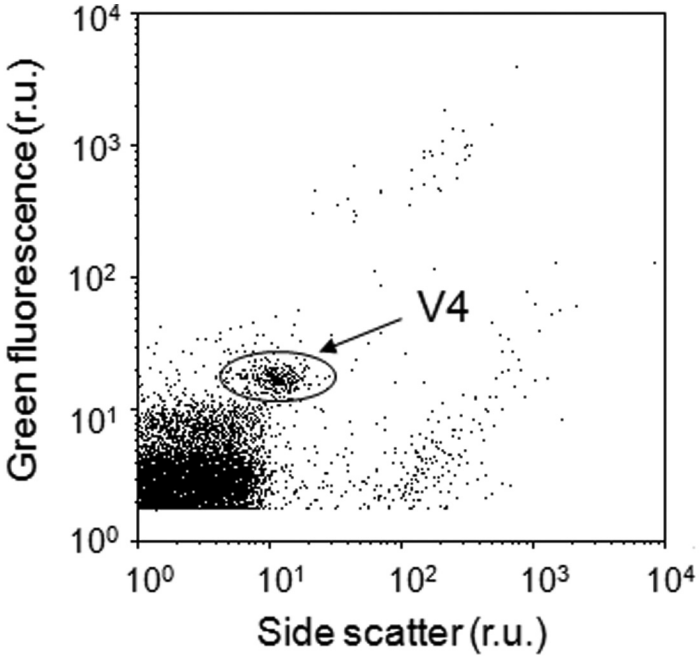

Fig. 5. Cytogram of SYBR Green I–stained viruses in natural aquatic sample according to protocol described herein (10,000 events plotted). A fourth subpopulation with enhanced side-scatter signal may be observed. This subpopulation, V4, commonly represents large dsDNA algal viruses. r.u., relative units.

mal staining and detection of viruses in aquatic environments. It might be useful, however, to test whether other fluorescent stains or combination of stains can improve detection when working with specific viruses.

Reproducibility—A critical question for the FCM user is how reproducible the analysis is and how representative of the "correct" concentration. Usual practice is to include replicate counts in a random order. Standard deviations should be smaller than 5%. Samples should possible be run in small batches (i.e., 6–10 samples) to prevent poor reproducibility due to virus decay in thawed samples. Once thawed, the samples can be stored at 4°C for at most a few hours. Refreezing and reanalysis of samples must be avoided due to extensive loss in virus counts.

Accuracy is improved by regular calibrations of the sample flow rate. Weighing the sample before and after a known time period of running at one of the flow rates provides good estimates of the flow rate. However, this cannot be achieved when on board a ship. Instead, preweighed and sealed tubes containing Milli-Q water can be used as an alternative, and the flow rate can be determined once the tubes are weighed back in the laboratory. Another rough estimate of the flow rate while on board can be obtained using back-pipetting: a known volume is dispensed in the tube, the remaining volume is back-pipetted after the run, and the actual volume taken up by the FCM can be estimated by dividing change in volume over time. Running a sample of fluorescent beads of known concentration for determination of flow rate is not advised, since this may be unreliable due to clumping of beads.

Comparison of FCM versus EFM counts—A large data set (n = 259, Table 1) from distinct marine environments was used to compare viral counts obtained by FCM with EFM (using the protocol of Hennes and Suttle 1995). Overall, total virus counts ranged from <1 to 200 × 10^6 mL^{-1}, with highest counts in Southern North Sea (e.g., 10^7–10^8 viruses mL^{-1}). Linear least-squares regression analysis indicated a strong correlation between FCM and EFM counts (FCM = 1.08 × EFM + 0.65, r^2 = 0.80, n = 259). Regression slopes and intercept values were not significantly different from a 1:1 regression line with a slope of 1 and an intercept of 0 (slope: t-test = 0.143, P = 0.886; intercept: t-test = 0.069, P = 0.945).

Additionally, regression slopes ranged from 0.97 to 1.70 and were not significantly different between the environments (analysis of variance [ANOVA] on ranks, $P > 0.05$). Highest slope values were found for North Atlantic and Curaçao samples. The deep samples (>500 m, n = 8) are likely to explain this result for North Atlantic; ratio of FCM to EFM of those samples are high and ranged from 4 to 6 (2500–4350 m, n = 4). The high slope value for Curaçao samples is likely due to the small number of samples leading to a nonsignificant regression (r^2 = 0.36, $P < 0.28$, n = 5). Coastal and offshore marine samples displayed similar regression slope values (Table 1). Moreover, the depth of sampling did not influence regression slopes (Table 1). In the Arctic, samples were collected over a seasonal cycle at different stations, but no seasonal and/or spatial trends were observed in the FCM versus EFM regressions (Table 2).

Table 1. Linear least-squares regression analysis for viral abundance determined by flow cytometry and epifluorescence microscopy (FCM = slope × EFM + intercept).

Origin	Slope	Intercept (× 10^6 mL^{-1})	r^2	^{P} value	n	Depth range, m
Mackenzie shelf	1.00	1.29	0.89	<0.0001	59	0–526
Mackenzie river plume	0.97	1.25	0.94	<0.0001	26	0–240
Amundsen Gulf	1.06	0.07	0.92	<0.0001	51	0–530
Franklin Bay	0.95	0.79	0.90	<0.0001	94	0–223
Curaçao coral reef	1.50[a]	1.48[a]	0.36[a]	0.2821[a]	5	3–40
Southern North Sea	0.97	13.49	0.66	0.0013	12	5
North Atlantic	1.70	0.42	0.77	0.0002	12	5–4350
Offshore	1.11	0.76	0.84	<0.0001	98	0–4350
Coastal	1.08	0.45	0.80	<0.0001	161	0–526
Marine	1.08	0.65	0.80	<0.0001	259	0–4350

[a]Not relevant ($r^2 < 0.5$) regression.

Discussion and recommendations

FCM versus EFM—For bacterial samples, FCM counts are generally identical to EFM counts (Monfort and Baleux 1992, Payet and Suttle 2008). Furthermore, quantitative intercomparison between FCM and EFM counts of large dsDNA algal viruses also showed a strong correlation (Marie et al. 1999a). We show here (Fig. 6) that also for natural marine virus samples, typically dominated by lower fluorescent bacteriophages, counting viruses by FCM and EFM gave similar results (FCM = 1.08 × EFM + 0.65, n = 259). FCM allows high-speed detection and enumeration of viruses and may represent a better alternative than EFM.

The method presented here should be taken into account for FCM detection and enumeration of aquatic marine viruses. Importantly, nonfrozen samples and low dilution factors will result in unreliable virus counts. The potentially higher total virus count obtained by flash-freezing compared to nonfrozen samples (Brussaard 2004) is not an artifact caused by lysis of infected organisms and subsequent release of viruses. Tests with pure virus cultures and also with 0.2-µm-filtered natural samples (to remove all organisms) systematically showed higher total virus counts upon flash-freezing (data not shown).

In contrast to EFM, FCM is more sensitive and has the advantage of discriminating different virus populations, thus providing more information about the community structure of viral communities in a broad range of aquatic ecosystems. Furthermore, the high throughput of FCM and the ability to discriminate particular large dsDNA algal viruses has permitted the execution of detailed experiments enhancing our insight of virus–host interactions and the impact of viruses on algal bloom population dynamics (Brussaard et al. 2005; Jacobsen et al. 2007; Jacquet et al. 2002; Larsen et al. 2001, 2007).

Application in different environments—Although FCM detection of viruses was initially developed for marine samples (Marie et al. 1999a), it was further applied to other environments such as freshwater and sediments (Chen et al. 2001; Danovaro et al. 2001; Duhamel et al. 2006; Duhamel and Jacquet 2006; Goddard et al. 2005; Lymer et al. 2008). Virus counts obtained from 13 lake sediment samples were 2.5-fold higher using FCM than EFM (Duhamel and Jacquet 2006); however, the FCM signatures of these virus samples are distinctly different from aquatic samples. The extraction of viruses from the sediment is a critical step. Although Danovaro and Middelboe (2010, this volume) present an optimized protocol for enumeration of viruses in sediments using EFM, they also highlight the importance of improving methods for dislodging viruses from particles in different types of sediments. Preliminary tests using different types of sediment samples in combination with FCM (Brussaard unpubl. data) showed that high background levels of very low fluorescent particles, probably colloids, can occasionally interfere with the detection of viruses. We believe that further assessment of the FCM assay for (diverse) sediment samples is needed.

Virus detection—The term virus-like particles (VLPs) is typically used for virus detection by TEM, where the morphology of the viruses is used as the discriminator. The detection of particles by FCM, however, is not based on morphology but on nucleic acid–specific fluorescence and side-scatter signal. Comparison to EFM, TEM, and end-point dilution has shown that the green fluorescent particles counted by FCM (Fig. 2) are indeed viruses. Particles such as gene transfer agents (GTAs) (Lang and Beatty 2007) may be confused with virus particles after staining with a nucleic acid–specific dye, but they seem to represent only a very small portion of the total viral particle pool (Lang pers. comm.). Only in axenic algal cultures have we observed nonvirus contaminants with the same signature on a scatter and fluorescence plot. Addition of bacteria (natural seawater) to the axenic algal cultures (*Chaetoceros calcitrans* and *Micromonas pusilla*) resulted in a steep decrease of these interfering particles to undetectable levels, most likely due to decomposition of the organic matter released upon lysis of infected algal host cells. Thus, under natural conditions (with heterotrophic bacteria present), the abundance of these contaminants will be insignificant and of no consequence for virus enumeration.

FCM detection of viruses requires the use of fluorescent dye; however, not all viruses are readily stainable by currently available fluorescent dyes (Brussaard 2004). Dye penetration and effi-

Table 2. Linear least-squares regression analysis for viral abundance in Arctic waters determined by FCM and EFM (FCM = slope × EFM + intercept).

Origin	Season	Slope	Intercept (× 10^6 mL^{-1})	r^2	n
Coastal arctic	Fall	0.95	0.78	0.91	34
	Winter	0.85	1.04	0.88	52
	Spring	0.98	0.51	0.92	24
	Summer	0.95	2.06	0.89	46
	All	1.00	0.67	0.93	156
Offshore arctic	Fall	0.90	0.95	0.84	33
	Spring	1.30	–0.02	0.97	12
	Summer	1.03	0.91	0.91	29
	All	1.00	0.79	0.89	74
Arctic	All	1.00	0.71	0.92	230

For all regression analyses, the *P* value was <0.0001.

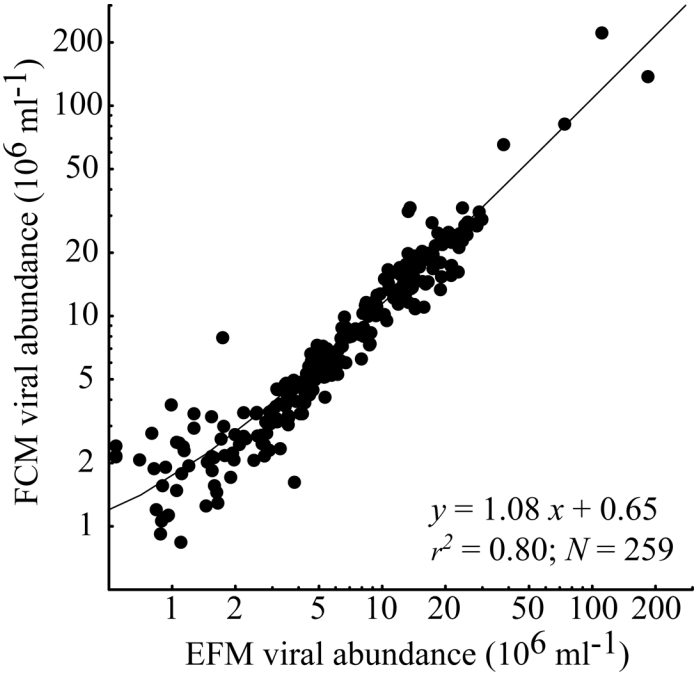

Fig. 6. Linear least-square regression of total viral abundance of aquatic samples determined by FCM and EFM. The line represents the linear least-squares regression. Note that a double-logarithmic scale is used.

ciency of staining can greatly change depending on the type of virus; in some instances, some viruses may have genomes too small for optimal detection (Brussaard et al. 2000). In turn, this may cause underestimation of total virus counts in aquatic environments. Advances in stain sensitivity and FCM technology in coming years will likely allow a better evaluation of these very low fluorescent virus particles in aquatic virus communities.

The dsRNA virus MpRV exemplifies that the optimized assay presented here is not necessarily most favorable for all viruses (Brussaard et al. 2004). Currently, FCM cannot properly distinguish MpRV viruses because their low fluorescent intensities interfere with the background fluorescence. The reason for the poor detection is not related just to genome size (25.6 kb, Attoui et al. 2006), as other viruses with similar genome size were clearly detected on the FCM (Brussaard et al. 2000). MpRV belongs to the nonturreted reoviruses, containing several concentric protein layers (inner and outer capsid layers), which may prevent proper penetration of the fluorescent dye. Using the BD FACSaria (newer FCM), a better detection of MpRV virus was found, but not for the entire population (Brussaard unpubl. data). Furthermore, detection of MpRV was significantly improved by dilution of the virus with Milli-Q water before fixation and flash-freezing; however, predilution of a natural marine sample in Milli-Q water resulted in lower detection of the major virus populations.

Standards—The use of true standards adds to consistency in methodology and allows optimal comparison of results. So far, green fluorescent 1-µm microspheres have been used as internal reference to normalize fluorescence and track instrument performance. These beads have a relatively high fluorescence and are not effective standards for detection efficiency, staining characteristics, and quantitative evaluation. Smaller beads with fluorescence and side-scatter signals comparable to low fluorescent viruses may be useful for quantitative standards, but particles with staining properties similar to those of viruses are preferred to check for the efficiency of the staining procedure. The use of a known (natural) virus sample as standard is not ideal in the long run, as we do not know yet if aquatic samples can be safely stored for prolonged times without virus decay.

On the whole, FCM is an accurate and highly reproducible method for virus enumeration and discrimination of main virus subpopulations in aquatic environments. A major advantage is the high throughput, a key issue for analysis of a large number of samples. There is an increasing interest in aquatic viral ecology, and with benchtop FCMs becoming more affordable, soon many laboratories will be able to routinely perform virus enumerations by FCM.

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