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Enumerating Viruses by Using Fluorescence and the Nature of the Nonviral Background Fraction

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Bulk fluorescence measurements could be a faster and cheaper way of enumerating viruses than epifluorescence microscopy, flow cytometry, or transmission electron microscopy (TEM). However, since viruses are not imaged, the background fluorescence compromises the signal, and we know little about its nature. In this paper the size ranges of nucleotides that fluoresce in the presence of SYBR gold were determined for wastewater and a range of freshwater samples using a differential filtration method. Fluorescence excitation-emission matrices (FEEMs) showed that >70% of the SYBR fluorescence was in the <10-nm size fraction (background) and was not associated with intact viruses. This was confirmed using TEM. The use of FEEMs to develop a fluorescence-based method for counting viruses is an approach that is fundamentally different from the epifluorescence microscopy technique used for enumerating viruses. This high fluorescence background is currently overlooked, yet it has had a most pervasive influence on the development of a simple fluorescence-based method for quantifying viral abundance in water.

The number and astonishing diversity of aquatic viruses have shaped our current view of them as microbial predators that influence biogeochemical cycles, microbial evolution, and possibly climate change (5, 14, 16, 19). New methods have been one of the keys in advancing our knowledge of microbial processes in aquatic environments. The epifluorescence microscopy technique is most widely used to determine viral abundance in water. It was introduced in 1991 (7) and improved in 1998 (10) with the introduction of the fluorescent dye SYBR to stain viral nucleic material for counting viruses while still using an epifluorescence microscope. The method continues to be routinely used to quantify viral abundance in aquatic ecosystems. The technique has altered our view of viral abundance in water, showing that viruses are in the tens of millions per milliliter in the oceans (15) and hundreds of millions in freshwater (13). Anyone who has peered down an epifluorescence microscope at water samples stained with SYBR will have often noticed a bright fluorescent background. It is this background fluorescence that is under scrutiny here.

SYBR stains bind to DNA or RNA, and when the complex is exposed to blue wavelengths (486 nm) of light they fluoresce to emit a yellow wavelength of light. The SYBR only binds to DNA/RNA. Flow cytometers use the same mechanism, and they have been used successfully for over a decade to quantify bacterial and viral abundance using fluorescence stains (3, 9, 15, 17). SYBR-labeled particles like bacteria and viruses are separated from the sample so that there is no interference from any background fluorescence.

Fluorescence excitation and emission matrices (FEEMs) can differentiate dissolved organic substances in aquatic systems (1, 2, 8) but as yet have not been used to quantitatively determine viral abundance. A scanning fluorescence spectrophotometer has the ability to scan samples stained with SYBR at a range of excitations and emissions, thus allowing for greater accuracy in determining fluorescence peak intensities, positions, and shapes of the spectra. FEEMs have been used to detect bacteria in natural water samples stained with SYBR gold (18) to develop a rapid and inexpensive online and portable fluorimeter for measuring bacterial numbers in water. However, it falls short of measuring viral abundance because of what appears to be high background fluorescence. The aim here was to understand why these new rapid and inexpensive methods have not been applied to the *in situ* measure of viral abundance in water samples using FEEMs. How much of the SYBR stain is related just to the viral-DNA–SYBR complex, and how much is related to background fluorescence?

MATERIALS AND METHODS

Wastewater. Wastewater (50 ml) effluent was sampled from the Yatala Brewery in South East Queensland, Australia, in January 2010, immediately passed through a 0.2-μm sterile polyvinylidene fluoride (PVDF) low-protein-binding Durapore (Millipore, Billerica, MA, USA) filter into a new sterile Falcon centrifuge tube, and finally stored in the dark at 4°C to minimize viral decay. No fixatives were used, as they cause rapid viral decay (20). Stored samples (3 ml of the 0.2-μm-filtered filtrate) were incubated for 15 min with 10 kU of DNase II (Sigma-Aldridge, Sydney, Australia) per ml of sample as described previously (18). Viruses were then stained with SYBR gold (Molecular Probes Inc., Eugene, OR, USA) using the method of Patel et al. (11). This solution was used to create the fluorescence excitation-emission matrices (FEEMs). Each fluorescence intensity data set was adjusted using sterile virus-free water blanks. Fluorescence intensity values for SYBR gold DNA/RNA peaks were calculated for each excitation between 475 and 520 nm for emissions between 504 and 650 nm, and the ranges of these values were examined to determine the influence of other dissolved organic compounds (peaks T_1 and T_2) in the sample on the DNA/RNA SYBR gold intensity peak.

Freshwater. Moggill Creek, freshwater meandering through the western urban environment of Brisbane, Queensland, Australia, was sampled (50 ml). The water was immediately filtered (0.2-μm pore size) and then stored as described for the wastewater in the previous section. Stored filtrates (3 ml) were placed in a test tube (Amicon Ultra-4 centrifugal filter unit) with a 100,000 (100K) nominal-molecular-weight limit (NMWL) and centrifuged at 4,000 × *g* for 15 min at 4°C. Viruses were concentrated in the supernatant (retentate, the solution that did not cross the mem-

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TABLE 1 Relative fluorescence units of freshwater samples in South East Queensland, Australia, for the 0.2-μm filtrate, control (VFW), and the 10-nm to 200-nm size fractions, along with the percentage of the bulk fluorescence that was nonviral fluorescence[a]

Freshwater sample source	Latitude and longitude	No. of RFU (no. of viruses) in: 0.2-μm filtrate (bulk fluorescence)	Control (VFW)	10- to 200-nm size fractions less control (viral DNA)	% of bulk fluorescence not viral DNA	Virus concn ($\times 10^7 \cdot ml^{-1}$) (no. of viruses)
Brook	28°8′23.13″S 152°34′24.39″E	24.14	13.17	6.96	72	5.70 ± 0.37 (10)
Creek	26°20′47.74″S 152°39′19.49″E	27.47 ± 0.36 (3)	10.40 ± 1.40 (3)	3.93 ± 0.36 (3)	86	2.95 ± 0.51 (30)
Creek	26°11′0.70″S 152°31′39.40″E	37.68 ± 1.50 (2)	8.96	9.39 ± 0.02 (2)	75	4.31 ± 0.39 (20)
Creek	28°0′41.37″S 152°33′23.51″E	54.01 ± 10.68 (2)	8.96	16.80 ± 3.44 (2)	69	12.18 ± 2.41 (20)
River	26°30′31.28″S 152°57′24.37″E	54.09	8.96	10.83	80	4.49 (10)

[a] Viral abundance determined using SYBR stain and epifluorescence microscopy is also included. Means and standard errors are shown.

brane) of the Amicon Ultra-4 centrifugal filter unit. The filtrate was then placed into a 10K-NMWL test tube and again centrifuged at 4,000 × *g* for 15 min at 4°C. For each filtrate and supernatant, FEEMs were created or subsampled for the counting of viruses using the epifluorescence microscopy method (see next section). The particle size fractions were defined using the manufacturer's (Millipore, Billerica, MA, USA) molecular-weight-cutoff specifications: 0.2-μm PVDF filtrate, particles of <200 nm; 100K-NMWL filtrate, particles of <10 nm; and 10K-NMWL filtrate, particles of <4 nm.

Water was also sampled from other creeks, a brook, and a river at the locations in South East Queensland shown in Table 1. These samples were treated as described above for Moggill Creek. For the controls, the creek or river water sample was replaced with sterile virus-free water (VFW) that was produced using the Sartorius Arium Pro VF (with an optional TOC monitor) (purchased from Sartorius Stedim Biotech, Goettingen, Germany).

Enumerating viruses using epifluorescence microscopy method. A Leica (Leica, Sydney, Australia) DM 400 epifluorescence microscope (using a magnification of ×1,000) was used to count viruses as described by Patel et al. (11). Brook, creek, and river water samples were filtered (0.2-μm pore size) and stored as described for the wastewater. Stored freshwater filtrates were subsampled (3 ml) and incubated for 15 min with 10 kU of DNase II (Sigma-Aldridge, Sydney, Australia) per ml as described previously (18). Viruses were then stained with SYBR gold (Molecular Probes Inc., Eugene, OR, USA) using the method of Patel et al. (11). The diluted stock SYBR gold solution (diluted 100×) (15 μl) was added to the 3-ml sample containing the DNase. After incubation at room temperature in the dark for 15 min, 1 ml of the sample was filtered through a 0.02-μm-pore-size Anodisc membrane filter (Whatman International, Maidstone, United Kingdom) with a 0.8-μm-pore-size backing membrane filter. After drying, the filter was mounted on a glass slide with a drop of Molecular Probe Slow Fade (Molecular Probes Inc., Eugene, OR, USA) antifade solution. The viruses were counted under blue epifluorescent excitation. For each filter, >200 viruses were counted in 10 to 15 fields of view selected randomly. As a control, a 3-ml sample of sterile virus-free water was treated as per a water sample. This control was subtracted from the viral abundance determined with epifluorescence microscopy and when measuring fluorescence.

Transmission electron microscopy. Brook, creek, and river water samples were filtered through filters of 0.2-μm pore size and stored as described for the wastewater. Viruses in the stored filtrates (3 ml) were concentrated using the 100K-NMWL test tube by centrifugation at 4,000 × *g* for 15 min at 4°C. The viral concentrates in the supernatant were placed onto carbon-coated collodium membranes on copper grids and subjected to negative staining with 1% uranyl acetate for 30 s. Stained grids were examined with a JEOL 1200EX transmission electron microscope (TEM) (Philips CM200 TEM operating at 80 kV) and photographed with a Gatan charge-coupled-device (CCD) camera (Gatan, Pleasanton, CA). All images were recorded at a calibrated magnification of ×100,000 at the CCD. Magnifications were ×100,000 unless stated otherwise. The filtrate was also examined for viruses.

RESULTS AND DISCUSSION

FEEM. Researchers use three-dimensional fluorescence excitation-emission matrices (FEEMs) to characterize the nature of organic matter in water—three-dimensional plots of the excitation and emission wavelengths against intensity (4, 8). Figure 1a shows the FEEMs of a wastewater sample for the 10- to 200-nm particle size range. Neither DNase nor SYBR gold was added to this sample. The two prominent peaks in Fig. 1a have been classified (4) as peak T_1 [tryptophan-like ($\lambda_{ex/em}$ = 275/340 nm)] and peak T_2 [tryptophan-like ($\lambda_{ex/em}$ = 225 to 237 nm/340 to 381 nm)]. These tryptophan peaks are characteristic of wastewater (1, 8). Note that there are no peaks in the $\lambda_{ex/em}$ = 475 to 510 nm/530 to 560 region for the wastewater sample shown in Fig. 1a.

Figure 1b shows the FEEMs for the same wastewater sample, but the sample was treated with DNase and SYBR gold before the FEEM was produced. We now see a new fluorescence peak, V_1 ($\lambda_{ex/em}$ = 475 to 510 nm/530 to 560), that is independent of the

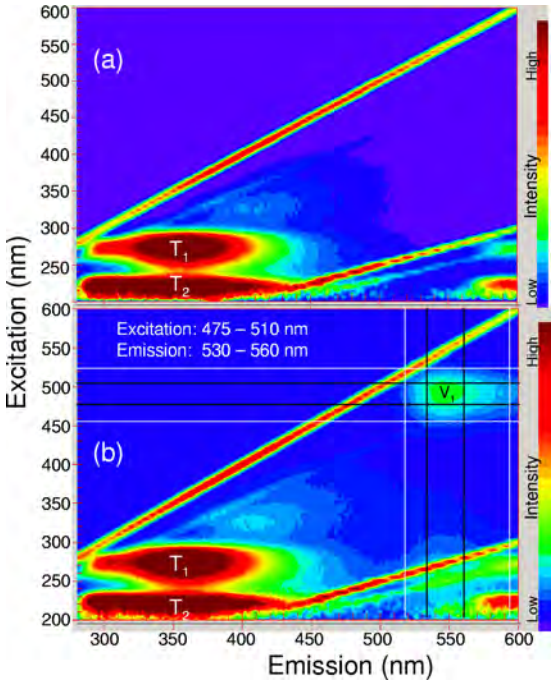

FIG 1 Fluorescence excitation and emission matrices for the 10- to 200-nm particle size range. (a) Wastewater before the addition of SYBR. (b) Wastewater incubated with DNA II and SYBR gold.

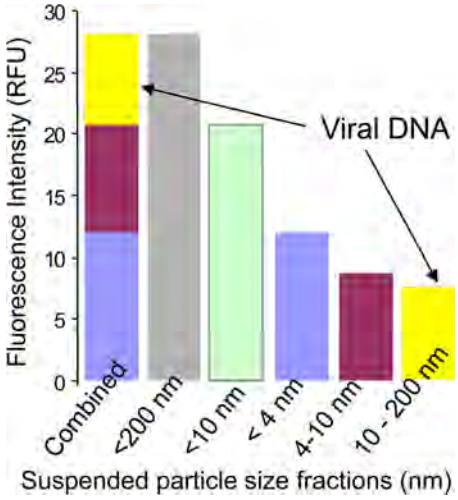

FIG 2 Relative fluorescence intensities ($\lambda_{ex/em}$ = 475 to 510 nm/530 to 560 nm) of different molecular size fractions for a freshwater creek (Moggill Creek, Brisbane, Queensland, Australia) sample after SYBR gold and DNase II were added as described for Fig. 1. Note that virus-sized particles are mostly in the 10- to 200-nm size range and account for less than 30% of the bulk fluorescence in the <200-nm size fraction.

tryptophan-like peak T_1 and peak T_2. Most importantly, this scan shows that the organic compounds in these fractions are not interfering with the fluorescence of the SYBR-DNA complex (peak V_1). Similarly, no interference from the organics was seen in the FEEMs of the freshwater creek of Queensland.

FEEMs of different molecular size fractions. Figure 2 shows the relative fluorescence intensities ($\lambda_{ex/em}$ = 475 to 510 nm/530 to 560 nm) of different molecular size fractions for a freshwater creek (Moggill Creek) sample after SYBR gold was added. The relative fluorescence intensities in the different-size fractions indicate where most of the DNA/RNA resided. Virus-sized particles are mostly in the 10- to 200-nm size range. Here we see that this viral fluorescence accounts for less than 30% of the fluorescence in the <200-nm size range.

The same analysis was conducted on water sampled from a brook and a river in South East Queensland, Australia. The locations and results are presented in Table 1. In every case the results were similar to those from Moggill Creek. The relative fluorescence units (RFU) for the 0.2-μm filtrate (bulk background fluorescence) dominated the FEEM profile; 69% to 80% of the RFU was not associated with viruses. The number of viruses in each water sample was determined using the classic epifluorescence microscopy method (10). These virus numbers are presented along with the RFU associated just with viruses (10- to 200-nm size fraction, less the control) (Table 1). The two sets of results are

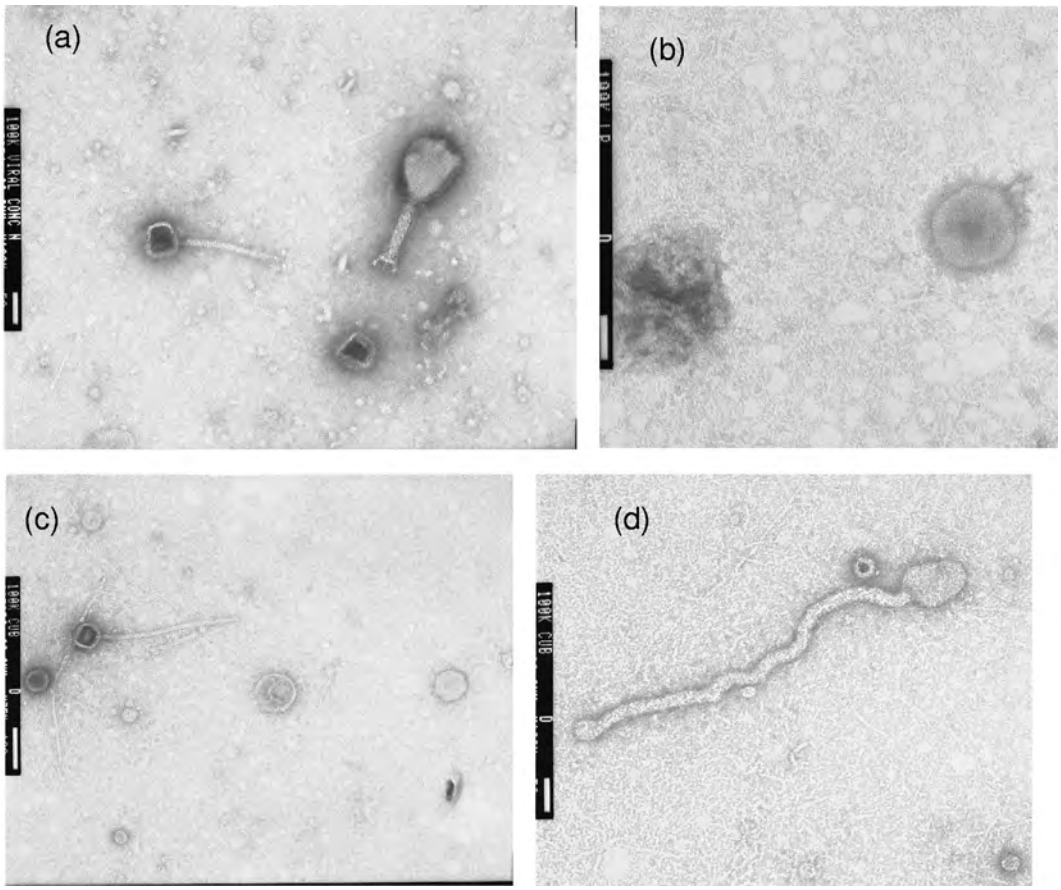

FIG 3 Transmission electron micrographs of the viruses seen in the 10- to 200-nm size range. No viruses were seen in either natural or wastewater samples in the less <10-nm size fraction; the fluorescence seen in this fraction was most likely due to SYBR binding to dissolved DNA. White bars, 50 nm.

well correlated ($r^2 = 0.8$) and show that the fluorescence in the 10- to 200-nm size fraction can be used to determine the viral abundance.

The sensitivity of this fluorescence approach is directly related to the extent that the viruses in the 10- to 200-nm size fraction are concentrated. As more prefiltered water is passed through the 100K-NMWL Centriprep, more viruses are concentrated in the supernatant. We have concentrated 500 ml of a prefiltered water sample down to 500 μl in this supernatant fraction. This represents a 1,000-fold increase in the concentration of viruses, which enables us then to detect viral concentrations down to 10^4 viruses per ml based on the concentrations detected (Table 1).

Epifluorescence microscopy using SYBR stains (10) is currently the accepted approach to determining viral abundances in water. Researchers have not been concerned with, or are unaware of, the background fluorescence as the stained viral particles shine through as bright specks. However, it is this bright background fluorescence that has prevented the development of a rapid, inexpensive direct fluorescence measure of viral abundance in water.

A TEM study of the <10-nm size fraction showed that no viruses were present in this size range ($n = 50$) in either wastewater or freshwater. The TEM pictures in Fig. 3 show the viruses typically observed in the 10- to 100-nm size range, where viral head diameters ranged from 50 to 70 nm. Some of the smallest viruses we see in the literature are the animal picornaviruses that are smooth round viruses ~30 nm in diameter (6). Most viruses in aquatic environments fall into a capsid size range of 30 to 70 nm (21). The proportion of virioplankton that fell into the 30- to 60-nm size class was >65%. This is consistent with the observed TEM capsid size range in this study (Fig. 3).

Others have also reported the presence of large amounts of dissolved DNA in freshwater samples (12). When using SYBR stains to count viruses using epifluorescence microscopy, the background fluorescence hue is obvious. Presumably the SYBR stain is binding to the loose/dissolved DNA. The use of DNase did little to lower this high background fluorescence. As Paul et al. (12) explained, naturally occurring dissolved DNA in freshwater can be inaccessible to nucleases. They suggest that this is because the dissolved DNA is bound to proteins and/or other polymeric material. While this is consistent with the observations in this study, future studies are needed to describe the nature of the compounds binding to the dissolved DNA.

In natural creek water or wastewater samples, the SYBR background fluorescence of the <10-nm size fraction aligned with the excitation and emission fluorescence for the viral-DNA–SYBR complex. Studies to further characterize the SYBR-DNA background along with ways to digest and remove it from a water sample will help provide a very efficient method for quantifying viruses directly using fluorimetry. Use of a mixture of proteases and nucleases may be the solution to the removal of this high background fluorescence and/or simply measuring the fluorescence of the 10- to 200-nm size fraction after staining with SYBR.

Conclusion. This paper shows that when SYBR stains were added to freshwater and wastewater, most of the fluorescence was in the <10-nm size fraction and was not associated with intact viruses. Researchers using viral-DNA–SYBR complexes and fluorescence need to be aware that as much as 70% of the fluorescence in the <200-nm size range of a SYBR-stained water sample is not associated with intact viruses. The study suggests that fluorescence microscopy associated with differential filtration and/or a suite of digestive enzymes could provide the basis for a fast and sensitive method for determining the concentration of viruses in water.

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