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Virus à la sauce Yo-Pro: Microwave-enhanced staining for counting viruses by epifluorescence microscopy

Abstract—A recently developed method for enumerating aquatic viruses (Yo-Pro stained) was modified to reduce staining time and allow counting of fixed samples. Marine and freshwater virus samples to be enumerated were stained with the cyanine-based dye Yo-Pro-1 by using microwave irradiation, which reduced incubation time from 48 h to 4 min. The modified method and the original protocol yielded similar viral estimates. Easier sample handling and the addition of fixed-sample capability should allow epifluorescence counting of viruses to become a routine part of field studies of viral ecology.

Viruses are now considered to be an important component of aquatic microbial communities. Their extremely small size, however, makes them very difficult to study by direct enumeration. Nevertheless, reliable, uncomplicated estimation of viral abundance will be necessary for rapid progress in understanding the role of viral infection in the dynamics of protozoa, algae and bacteria in aquatic environments.

Viruses currently enumerated with transmission electron microscopy (TEM) are either sedimented without preconcentration onto electron-microscopy grids by ultracentrifugation (Bergh et al. 1989; Borsheim et al. 1990; Maranger et al. 1994) or, in oligotrophic environments, they are preconcentrated by ultrafiltration before the sedimentation step (Proctor and Fuhrman 1990; Paul et al. 1991). These approaches are time-consuming, require expensive equipment, and yet may underestimate true viral abundance (Borsheim et al. 1990; Hennes and Suttle 1995; Maranger and Bird 1995).

Recently, Hennes and Suttle (1995) established a method for counting viruses by epifluorescence microscopy using the cyanine-based nucleic dye Yo-Pro-1 (4-[3-methyl-2,3-dihydro-(benzo-1,3-oxazole)-2-methylmethylenedene]-1-(3'-trimethyl-amonium-propyl)-quinolinium diiodide, Molecular Probes, Oregon). Use of the Yo-Pro method offers numerous advantages such as moderately priced equipment, simplicity, and greater precision. Unfortunately, the Yo-Pro method requires long incubation times (24–48 h) for adequate staining. Also in our experience, bacteria can multiply during the long incubation period, hence making viral enumeration difficult. Furthermore, water samples fixed with formalin or glutaraldehyde resist staining with Yo-Pro so that only fresh samples can be counted with this method. Because certain cir-

cumstances, including field sampling, require fixation of water samples, conserving them with glutaraldehyde or formaldehyde is crucial for an accurate determination of viral abundance.

We found a modification of the Yo-Pro method that reduces incubation time to minutes. The new method also permits staining of fixed samples. These improvements result from the enhanced diffusion that is produced by brief treatment with microwave irradiation.

Microwave techniques are common in histopathology and histochemistry for staining and fixing samples (Moorlag et al. 1987; Suurmeijer et al. 1990). The use of microwaves is advantageous because it reduces staining and fixation time of biological tissues, and it can improve the quality of the microscopic preparation.

In the present case, microwave irradiation seems to facilitate the diffusion of stain across the viral capsid, greatly increasing the rate of its bonding with DNA and RNA. Microwave irradiation increases internal temperature, thus enhancing diffusion and chemical reaction rates (Kok and Boon 1990).

The modified Yo-Pro method was performed as follows. All manipulations were carried out in subdued light. The stain was prepared as described in Hennes and Suttle (1995). Yo-Pro was diluted to 50 μM in an aqueous solution of 2 mM NaCN. Prior to filtering samples, 80- μl drops of the stain were placed in the bottom of a pyrex Petri dish. Water samples were fixed with unbuffered formaldehyde or glutaraldehyde at a final concentration of 2%. Prior to filtration, fixed or unfixed freshwater samples (100 μl) were diluted with 700 μl of prefiltered (pore size 0.02 μm) deionized distilled water (DDW). Marine water samples were diluted at a ratio of 1:4. We found it was more convenient to dilute 200 μl of marine sample with 800 μl of DDW because viral abundance is generally lower in marine environments (Maranger and Bird 1995). In ultra-oligotrophic environments, we dilute 1 ml of sample with 4 ml of DDW.

Samples were filtered (400 mm Hg) on a 0.02- μm pore size Al_2O_3 Anodisc membrane filter (Whatman) with a premoistened backing filter (pore size 0.45 μm). Marine samples were additionally rinsed three times by filtering with 500 μl of prefiltered DDW. These rinses are critical to the final quality of the preparation and cannot be skipped.

Table 1. Comparison of viral abundances estimated by using the Hennes and Suttle (1995) method, the microwave enhancement, and by transmission electron microscopy (TEM). Values are means \pm SD of duplicate samples (ND—not determined).

Site	Sampling date	Viral abundance ($\times 10^7$ ml $^{-1}$)				TEM
		48 h*	MW†	MW-GLUT‡	MW-FORM‡	
Lac Lusignan	9 Jun 95	0.74 \pm 0.24	1.09 \pm 0.13	ND	ND	ND
Lac Croche	3 Aug 96	0.57 \pm 0.21	0.80 \pm 0.12	0.54 \pm 0.30	0.70 \pm 0.12	0.87 \pm 0.14
Lac Brome						
Pelagic	14 Jun 95	2.93 \pm 0.26	ND	3.42 \pm 0.57	3.12 \pm 1.00	1.96 \pm 0.69
Littoral	14 Jun 95	2.81 \pm 0.49	ND	3.84 \pm 0.98	4.87 \pm 0.46	ND
Inlet	31 Jun 95	1.28 \pm 0.62	ND	1.75 \pm 0.54	ND	ND
Outlet	31 Aug 95	4.84 \pm 0.89	ND	5.91 \pm 0.76	ND	ND
Lac Cromwell						
Pelagic	21 Aug 95	2.41 \pm 0.18	ND	2.05 \pm 0.01	ND	ND
Pelagic	3 Aug 96	1.25 \pm 0.14	1.44 \pm 0.49	1.53 \pm 0.04	1.19 \pm 0.12	1.20 \pm 0.26
Littoral	21 Aug 95	1.61 \pm 0.18	ND	1.89 \pm 0.04	ND	ND
Lac Heney	30 Oct 96	0.65 \pm 0.08	0.73 \pm 0.06	0.80 \pm 0.04	0.70 \pm 0.15	ND
T5	20 Jul 95	ND	670 \pm 50	790 \pm 108	ND	684 \pm 13
St. Lawrence estuary	18 Oct 95	1.96 \pm 0.12	2.22 \pm 0.02	2.00 \pm 0.02	2.08 \pm ND	ND

* Sample stained by incubating for 48 h as outlined in Hennes and Suttle (1995).

† Fresh sample stained by microwave irradiation.

‡ Samples fixed with glutaraldehyde (MW-GLUT) and formaldehyde 2% (MW-FORM) stained with microwave irradiation.

Filters were put sample side up on the drops of Yo-Pro. The Petri dish was placed in a cardboard container to protect it from light and was irradiated for no longer than 3.5–4.0 min in a domestic microwave oven equipped with a turntable and set at low-intermediate power level (level 3, Sanyo EM-606T, 0.6 ft 3 , 600 W). When stained longer in the microwave oven, viruses do not fluoresce as brightly, possibly because high temperature causes decomposition of the dye (Suurmeijer et al. 1990). Background staining of the filter also increases to unacceptable levels with excessive irradiation. Maximum power level in microwaves vary from model to model. Staining times may therefore also vary according to the microwave model. The maximum energy output of our microwave model is 600 W for any power level (1 to HI). When it is operated at a low level (e.g. level 3), microwave irradiation occurs intermittently for 30% of the selected time, being interspersed with cooling periods.

The heated Petri dish was left \sim 10 min to cool off. The filter was replaced on the filtering support and rinsed three times with aliquots of 800 μ l of prefiltered DDW. It is critical not to let the filter dry out during the final rinse step.

Filters were placed with a drop of immersion oil or glycerol (spectrophotometry grade) on a slide, covered with a cover slip, and enumerated as outlined by Hennes and Suttle (1995).

Fixed samples must be processed within a week of collection. We found a reduction of viral abundance with time (up to 75% loss within 4 weeks of collection; data not shown). For each sample, 20 fields were selected randomly and $>$ 200 viruses were counted with an epifluorescence microscope (Leica Leitz DMR) at a magnification of 1,000 \times and with excitation with blue light. Small particles that were

regularly shaped and fluoresced bright green were counted as viruses. Bacteria could easily be distinguished from viruses because of their relative size and brightness.

We compared the original Yo-Pro protocol with our microwave-modified method (fixed and fresh samples). Water samples (20 ml) from the surface were collected in scintillation vials. Fresh and fixed samples were kept at 4 $^{\circ}$ C in the dark until processed, always within a few hours after collection for fresh samples and within a week for fixed samples. Study locations include five freshwater lakes that vary in productivity and a saline sample 25‰ from the St. Lawrence estuary. We also stained and enumerated a culture of T5 phage. Samples were fixed upon collection, except for the saline and T5 sample, which were fixed in the laboratory a few hours before filtering. The freshwater sites included oligotrophic Lac Lusignan (46 $^{\circ}$ 41'N, 74 $^{\circ}$ 08'W), Lac Heney (42 $^{\circ}$ 02'N, 75 $^{\circ}$ 55'W), and Lac Croche (45 $^{\circ}$ 30'N, 74 $^{\circ}$ 30'W); two sites (pelagic and littoral) in Lac Cromwell (45 $^{\circ}$ 30'N, 74 $^{\circ}$ 30'W), a humic-mesotrophic lake in the Canadian Shield; and four sites in Lac Brome (45 $^{\circ}$ 15'N, 72 $^{\circ}$ 31'W), a meso-eutrophic lake in the Eastern Townships of Quebec, including its inlet (oligotrophic) and outlet (eutrophic).

Statistical analyses were done with SAS (SAS Institute 1987). All counts presented are means of two or three replicates. Differences between treatments were detected using a nonparametric two-way analysis of variance where the replicates of treatments (e.g. 48 h, microwave) were ranked within lakes. Probabilities \leq 0.05 were considered significant. The nonparametric tests were used because of the small sample sizes and heterogeneity of variance between lakes; a significant result would indicate only that one of the methods tended to give higher counts than the other. We found no

significant differences in viral abundance when we compared microwave staining with Hennes and Suttle's Yo-Pro protocol (Table 1). The ratio of microwave-staining counts to counts using the original method varied between 0.85 and 1.55 among sites. This discrepancy might be explained by viral decay or multiplication in unfixed samples from the time of collection until samples were filtered, which was avoided by fixation. DDW, glutaraldehyde, and formaldehyde blanks prepared with the method are free of any apparent viral particles, so that it is not likely that these are staining artifacts. Another possibility is that microwave treatment resulted in lysis of cells and release of viruses in the latent period. To verify this we microwaved a small freshwater sample (1 ml) from Lac Heney and compared viral counts before and after irradiation using the original Yo-Pro method. A *t*-test showed that the viral abundance of the non-irradiated samples (8.21 ± 0.57 [SD] $\times 10^6$ viruses ml⁻¹, *n* = 3) was not significantly different from the microwaved samples (8.32 ± 0.33 [SD] $\times 10^6$ viruses ml⁻¹, *n* = 3; *P* = 0.85).

Various samples were also enumerated using TEM. Electron microscopy grids were prepared as described in Maranger et al. (1994). Briefly, an aliquot (50 μ l) was centrifuged in a Beckman airfuge ultracentrifuge equipped with an EM-90 rotor at 100,000 $\times g$ for 30 min so that viruses were pelleted directly onto formvar-coated grids. Grids were stained with 2% uranyl acetate for 5 min and 50 fields were enumerated using electron microscopy at a magnification of 90,000 \times . To test for the loss of viruses during centrifugation, an aliquot (3 μ l) was air dried directly on the grid and stained as described above. We obtained similar estimates with both TEM approaches (the means of both techniques are in Table 1). The ratio of Yo-Pro counts (average of all preparations) and TEM for Lac Brome is 1.6, which is within the range found by Hennes and Suttle (1995) for freshwater systems.

Hara et al. (1991) stained small marine particles with fluorescent dye 4',6-diamidino-2-phenylindole (DAPI) and compared DAPI counts with TEM viral counts. They found DAPI:TEM ratios of 1.0–1.6, which still underestimates viral abundance compared to the Yo-Pro:TEM ratio of 1.2–7.1 found by Hennes and Suttle. Paul et al. (1991) found considerably lower counts of DAPI-stained particles than those obtained by TEM. The problem with staining samples with DAPI is that particles are close to the limit of detection and DAPI will not stain RNA viruses. We were successful in staining ssDNA (ϕ X 174) and ssRNA (serological group ss) bacteriophage isolates. We were also capable of staining fixed samples from saline lakes ranging in salinity up to 270‰ (Great Salt Lake; Bird and Robarts unpubl.) by diluting the sample 10-fold before using the microwave protocol for marine samples. Yo-Pro staining seems to be the most suitable method at this time for estimating viral abundances in aquatic ecosystems. Nevertheless, the variability between samples is such that duplicate, triplicate, or even greater numbers of replicate preparations are desirable. Despite a suitable and more affordable alternative for enumeration of VLP, TEM remains useful for sizing and determination of viral morphology. The modifications described here to the original method should allow rapid, accurate counting of viruses from natural samples to become routine.

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