INTERNATL MICROBIOL (1999) 2:227–232

RESEARCH ARTICLE

M. Carmen Alonso[1]
Jaime Rodríguez[2]
Juan J. Borrego[1]

[1]Department of Microbiology, Faculty of Sciences, University of Málaga, Spain
[2]Department of Ecology, Faculty of Sciences, University of Málaga, Spain

Enumeration and isolation of viral particles from oligotrophic marine environments by tangential flow filtration

Received 12 March 1999
Accepted 15 June 1999

Correspondence to:
Juan J. Borrego. Department of Microbiology. Faculty of Sciences. University of Málaga. Campus de Teatinos. 29071 Málaga. Spain.
Tel.: +34-952131893. Fax: +34-952132000.
E-mail: jjborrego@uma.es

Summary A method for concentrating, enumerating and isolating viral particles from marine water samples was developed and evaluated. The method consists of a concentration step by a tangential flow filtration (TFF) system, ultrafiltration by centrifugal concentrator, and visualization by transmission electron microscopy (TEM). This procedure allows to reduce volumes of ca. 2 l of seawater to 10–20 µl, which can be dispensed on electron microscopy grids to count total viral particles. This method allows the recovery of small numbers of viral particles from oligotrophic seawater samples, in which viral numbers ranged from 10^5 to 10^6 viral particles/ml. The tangential flow filtration system was evaluated as quantitative technique using suspensions of two different bacteriophages (T6 and ϕX174) in autoclaved seawater. Recovery rates varied depending on both the viral morphology and flow rate; recovery percentages reached 117.4% for T6 and 60.6% for ϕX174 using low flow rate.

Key words Virus enumeration · Tangential flow filtration · Oligotrophic environments · Marine viruses · Transmission electron microscopy

Introduction

Several studies have demonstrated the presence of large numbers of viruses in the marine environment [3, 10] and their potential role in the control of bacterial populations [5, 7, 16]. However, to establish their ecological significance it is necessary to provide an accurate methodology that allows to obtain a detailed distribution of viral concentration in the marine ecosystem.

Classically, studies on viral abundance have been performed with samples from bays and estuarine environments, in which virus numbers exceed 10^7–10^8/ml [3, 5, 21]. Several attempts have been made to count viruses from small volumes of these samples, including sedimentation by ultracentrifugation directly onto transmission electron microscopy (TEM) grids [3, 5, 24], and epifluorescence microscopy applied in conjunction with 4-6-diamidino-2-phenylindole (DAPI) [10, 15] or Yo-Pro-1 [12] stain. However, with oligotrophic samples it is necessary to apply a previous concentration step, which allows to reduce large volumes to a few µl to be dispensed onto TEM grids. Analyses by TEM make it possible to enumerate viruses, and also provide information about the viruses morphologies and sizes.

Molecular filtration by tangential flow had been used previously to concentrate different groups or size categories of phytoplankton and particles [2, 8, 17], or specific types of viruses [4, 18, 22]. It had not been applied, however, to concentrate total viral particles from seawater. We tested the performance of this system in oceanographic studies, and calculated recovery rates for several types of bacteriophages.

The tangential flow filtration (TFF) has important advantages over other procedures currently used to concentrate viruses. In fact, (i) it does not depend on virus adsorption, and consequently it minimizes virus loss resulting from competition for adsorption sites [4]; (ii) it is not based on the net charge of the viral particles, eliminating the need for acidifying or adding polycationic salts [22]; and (iii) it avoids the elution process.

Due to problems related to concentration as a previous step for counting viruses by TEM, we developed and evaluated a procedure to estimate the number of total viral particles from oligotrophic marine samples. This procedure is based on TFF, ultrafiltration and visualization by TEM.

Materials and methods

Sampling and processing Samples were collected from several stations at the Alboran Sea (Southwestern Mediterranean) using a Niskin bottle. They were sampled during summer stratification at depth of the subsurface chlorophyll maximum (40–55 m) in oligotrophic conditions (0.04–0.2 µg of chlorophyll *a* per liter). Surface temperatures ranged from 21.5 to 24.3°C, and the salinity was ca. 37‰. Each sample was split into two subsamples. Those for DAPI-staining were fixed with 2.5% glutaraldehyde. Those for TEM studies, without fixative, were filtered immediately to remove prokaryotic and eukaryotic microorganisms, and were processed in the first 4 h following sampling to prevent changes in the virus concentration. Samples were kept at 4°C in darkness until analyses were performed.

Microorganisms were removed by two systems: (i) gentle negative filtration (<130 mm of Hg) through 1.2 µm and 0.2 µm pore-size filters consecutively, (ii) filtration using a TFF system with a 0.16 µm pore-size and a 50 cm^2 filtration area (Filtron, Mini-Ultrasette). This latter system keeps larger particles in the retentate volume, and only those smaller than 0.16 µm are collected in the filtrate. To prevent virus adsorption on the filters, the TFF system was pretreated with 50 ml of 3% flocculated beef extract solution [13]. The beef extract was flocculated by acid precipitation, centrifuged, and finally the pH was readjusted to pH 7.

Virus concentration by TFF The method developed to concentrate viruses from seawater is given in Fig. 1. The TFF system has an area of 50 cm^2 and an exclusion size of 50 kDa. We used a peristaltic pump (Masterflex) at a flow rate of 280 ml/min to pump the sample (2 l maximum volume). We recirculated the retentate volume until only 5 to 10 ml of sample remained in the original vessel.

After each experiment, we cleaned the filtration unit with a 1 N NaOH solution for 15 min, followed by filtration of 1 l of deionized water. Before each experiment, we stabilized the flow through the TFF system for 10 min. During this time we made the samples recirculate without concentrating them. To determine the concentration efficiency of the TFF system, we performed laboratory assays using stocks of bacteriophages with different characteristics *(Myoviridae*, T6; *Microviridae*, ϕX174). We diluted phage stocks in 100 ml of autoclaved seawater and added bacterial host *(Escherichia coli* C, ATCC 13706) to simulate the loss of viruses by adsorption to host cells during the concentration. We performed several assays to determine whether the flow rate or the suspending medium affected the efficiency of virus recovery. We also carried out a direct plaque assay with modified Scholten agar [11] to calculate phage titers expressed as plaque forming units (PFU)/ml. We used ultrafiltration centrifugal concentrators (Filtron, Microsep) containing an OMEGA membrane with an exclusion size of 10 kDa to concentrate 100-fold the retentante volume resulting from TFF system (Fig. 1). We reduced volumes of 3.5 ml to 10–20 µl in 60 min by centrifuging at 5,000× *g*. Then we placed volume retained on the filter on grids for counting of the viral particles by TEM.

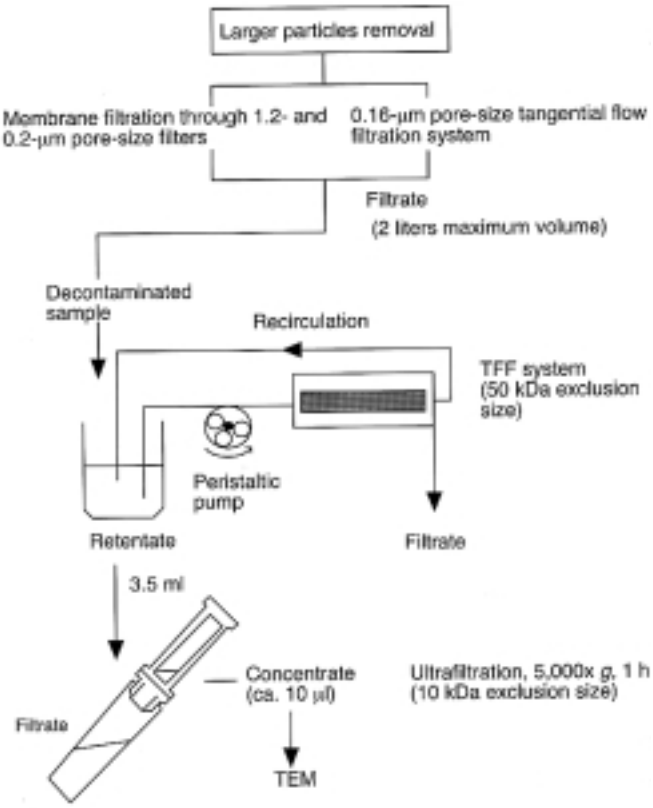

Fig. 1 Schematic representation of the method developed to concentrate and enumerate total viral particles by TEM

Enumeration of viruses from environmental samples by TEM We used electron microscopy to enumerate viruses from natural seawater previously concentrated as above. We placed small volumes (15 µl) on 400 mesh copper grids (Bio-Rad) covered with a Formvar film. Then we rinsed the grids in several drops of deionized water to remove inorganic salts and stained them with 1% (wt/vol) uranyl acetate solution (pH 4.5). After allowing the grids to air dry, we observed them at ×20,000 using a Philips EM100 transmission electron microscope at 80 kV.

We randomly selected a minimum of 20 microscopic fields and counted the viruses by photography using Kodak Eastman film (400 ASA). The morphological types of viruses were recorded from micrographs taken randomly to represent all different types of viruses in a sample. Although we did not use catalase crystals to confirm the size of the viral particles, we calibrated the magnification of the TEM prior to the study.

Epifluorescence microscopy We counted the bacteria by using epifluorescence microscopy of DAPI-stained preparations (1 µg/ml) filtered onto polycarbonate 0.2-µm pore-size filters

Table 1 Percentage of viruses in the filtrate with the tangential flow filtration (TFF) system (0.16 µm nominal pore size) operating at different flow rates and treatments

Phage	Flow rate (ml/min)	No. of samples	Virus in the filtrate (%)[a]	
			TFF without treatment	TFF pretreated with 3% flocculated beef extract
T6	280	6	1.4 ± 1.1	0.4 ± 0.1
T6	550	4	2.0 ± 0.5	ND[b]
ϕX174	280	3	89.6 ± 31.3	43.9 ± 6.1
ϕX174	550	3	54.1 ± 33.7	ND

[a]mean ± standard deviation·
[b]ND: not done.

(Millipore, GTTP) as described by Porter and Feig [15]. We counted at least 100 cells on randomly selected fields, using a Nikon microscope under UV light excitation at ×1,000 magnification. To count DAPI-stained viral particles, we modified the classical method, and used 0.02-µm pore-size aluminium oxide (Anodisc, 25) filters**.**

Results and Discussion

Large particle removal To avoid interferences in the virus enumeration and to prevent virus replication during the storage period, we removed zooplankton, phytoplankton and bacteria before the concentration step. Initially, we tentatively removed particulate material by using a TFF system with a 0.16 µm pore size. The effects of both phage characteristics (size and morphology) and flow rate on the viral recovery from filtrate in this system are given in Table 1. The percentage of viral particles in the filtrate averaged only 1.4% and 2% for T6 viruses, without significant differences between the recovery efficiency using high (550 ml/min) and low (280 ml/min) flow rate. The recovery efficiency of ϕX174 phages was higher (89.6%) with low flow rate than high flow rates (54.1%).

In an attempt to increase virus recovery, we treated the filters with 3% beef extract flocculated solution using low flow rate. Although this method has been previously used to improve the efficiency of poliovirus recovery in distilled water [4], in the present study the percentage of viral particles in the filtrate was not significantly different ($p > 0.05$) when we treated the filter with the organic solution (0.4% T6 phage; 43.9% ϕX174 phage) (Table 1). Since pre-treatment does not increase the percentage of viruses in the filtrate, we can assume that the loss of viral titer is due to inactivation, and not to adsorption to the filter.

Alternatively, we calculated the percentage of T6 and ϕX174 in the filtrate when we employed gentle negative filtration through different filters to remove particulate matter. As some viruses can lose their infectivity depending on the type of filter used, which is not strictly determined by the pore-size [20], we based the count of viruses on viral infectivity. Both phages remained infective after filtration through polycarbonate 0.2-µm pore-size filters (Millipore, GTTP) (Table 2), yielding recoveries of about 100% for both T6 and ϕX174 (93.8% and 100%, respectively). Cellulose-based 1.2-µm pore-size filters (Millipore, GS) seem to cause the loss of infectivity of T6 phages (recovery efficiency of 54.6%) (Table 2). Fiber-glass filters (Whatman, GF/C) allow the recovery of more than 80% of both types of viruses. However, the efficiency of recovery may be lower from natural seawater because of viral adsorption to particulate material.

Based on the results obtained in this study, we recommend the use of two filtration steps with fiber-glass filters, and polycarbonate filters consecutively to remove particles.

Concentration procedure. Efficiency of TFF system We concentrated phages suspended in autoclaved seawater (Fig. 1) by using the TFF system with a 50 kDa nominal molecular weight limit. Table 3 shows that virus recovery after 5 to 20-

Table 2 Virus recovery in the filtrate using different types of filters by gentle negative filtration to remove bacteria

Filter type	Phage	No. of samples	Virus recovery in the filtrate (%)[a]
Polycarbonate (0.2-µm)	T6	4	93.8 ± 4.6
	ϕX174	4	100 ± 6.1
Cellulose-ester (1.2-µm)	T6	2	54.6 ± 13.3
	ϕX174	2	100 ± 47.3
Fiber-glass (GF/C)	T6	2	82.4 ± 8.0
	ϕX174	2	99.8 ± 29.5

a ± SD: plus/minus standard deviation.

Table 3 Concentration of T6 and ϕX174 bacteriophages from seawater by molecular filtration using TFF system with a 50 kDa nominal molecular weight limit

Phage	Flow rate of filtration (ml/min)	Sample		Retentate volume		Virus recovery (%)
		Volume (ml)	Titer (PFU/ml)	Volume (ml)	Titer (PFU/ml)	
T6	550	83	8.5×10^5	15.0	2.8×10^5	6.0
T6	550	82	7.6×10^6	7.5	3.8×10^7	45.2
T6	550	100	6.5×10^6	18.0	6.5×10^6	18.5
x ± SD						23.2 ± 11.8
ϕX174	550	73	3.2×10^6	6.0	8.6×10^6	22.6
T6	280	100	3.4×10^6	12.0	2.7×10^7	94.1
T6	280	100	2.0×10^6	5.0	5.5×10^7	135.0
T6	280	100	5.3×10^6	14.5	5.0×10^7	135.8
T6	280	86	2.5×10^5	14.5	1.5×10^6	104.8
x ± SD						117.4 ± 10.6
ϕX174	280	100	2.9×10^3	7.0	2.3×10^4	55.2
ϕX174	280	82	2.7×10^3	7.0	1.6×10^4	50.0
ϕX174	280	85	4.5×10^3	9.5	2.2×10^4	55.3
ϕX174	280	100	6.1×10^3	7.5	6.7×10^4	82.0
x ± SD						60.6 ± 7.2

x ± SD: mean plus/minus standard deviation.

fold concentration ranged from 22.6% (for ϕX174 phage) to 23.2% (for T6 phage) when a high flow rate was used. There were no significant differences ($p > 0.05$) when we varied the initial number or the concentration factor, but the use of a low flow rate resulted in the increase of the recovery efficiency of both viral particles (117.4% for T6, and 60.6% for ϕX174) (Table 3). The recovery rates we obtained were higher than those calculated by Watanabe et al. [22] and Berman et al. [4] when they concentrated viruses suspended in deionized water without stabilizing agents. However, recovery rates obtained by these authors were higher when the samples were supplemented with protein solution, and fetal bovine serum. The virus recovery in the filtrate was under 1% in all conditions tested.

After determining the optimum flow rate (280 ml/min), we performed several assays using this flow rate to find out if the high concentration of ions in seawater could cause adsorption of viral particles to the filter. For this purpose, we calculated the recovery rates of T6 and ϕX174 phages suspended in 1 M Tris-HCl pH 7.5, supplemented with NaCl (5.8 g/l) and $MgSO_4 \times 7\ H_2O$ (2 g/l) (SM buffer), and compared them with those obtained by using seawater as suspending medium. The use of SM buffer did not improve the efficiency of virus recovery, resulting in virus recovery averaging 38.2% for T6 phages and 35.7% for ϕX174 phages. These findings showed that the concentration of ions contained in seawater did not affect negatively the bacteriophage recovery.

The results indicate that the above described molecular filtration system can effectively concentrate total viruses from seawater, which is especially necessary in oligotrophic environments. However, the removal of particles larger than viruses may be required, and the degree of recovery depends on experimental conditions such as flow rate, which is consistent with the results obtained by Barthel et al. [2] and Rodríguez et al. [17].

This system is useful to isolate and enumerate viruses from medium volumes (ca. 2 l) of seawater. As we performed viral enumeration in this study by plaque assay, we can establish that concentration by TFF does not affect significantly viral infectivity when low flow rate is used.

Analyses of natural seawater samples The concentration of viral particles obtained by epifluorescence microscopy from DAPI-stained seawater samples varied from $<10^3$ to 7.2×10^4 virus-like particles (VLP)/ml (Table 4). These values are smaller than those reported by other authors on different marine environments [3, 16, 19]. For this reason, we assumed that, in those samples, there were viruses which could be identified morphologically by TEM and not detected by epifluorescence microscopy. This would agree with previous studies in which counts by TEM exceeded those by epifluorescence [7, 9, 14].

To use TEM for counting viruses from these oligotrophic samples, we concentrated samples following the procedure described above. Viral concentrations obtained were always below 2×10^6/ml (Table 4), which agree with the results reported by other authors who found the lowest concentrations of viruses in oligotrophic waters [14, 23]. Virus concentration was only slightly higher than that of bacteria,

Table 4 Viral and bacterial abundance in natural seawater samples of an oligotrophic marine environment

Samples	Viral abundance by DAPI stain (VLP[a]/ml)	Viral abundance by TEM (VLP/ml)	Bacterial abundance (bacteria/ml)
1	7.2×10^4 (± 2.6)	2.6×10^5 (± 0.5)	1.0×10^5 (± 0.7)
2	3.3×10^4 (± 1.6)	1.4×10^6 (± 0.3)	1.3×10^5 (± 0.5)
3	1.4×10^4 (± 0.9)	2.2×10^5 (± 1.1)	1.6×10^5 (± 0.2)
4	1.9×10^4 (± 0.9)	6.2×10^5 (± 0.9)	1.4×10^5 (± 0.3)
5	2.3×10^4 (± 1.1)	7.3×10^5 (± 1.9)	6.8×10^4 (± 2.5)
6	$< 10^3$	1.8×10^6 (± 0.3)	9.0×10^4 (± 3.1)
7	3.4×10^4 (± 2.0)	3.0×10^5 (± 0.8)	1.1×10^5 (± 0.2)
8	2.3×10^4 (± 1.2)	4.5×10^5 (± 1.7)	2.0×10^5 (± 0.4)

[a]VLP: virus-like particles.

which was in accordance with Paul et al. [14], who had shown that, in oligotrophic systems, viral and bacterial numbers were similar.

The TEM study demonstrated that the procedure which we developed produced a viral concentrate without bacteria, with most viruses in good conditions. Samples contained a mixture of morphologically different viruses, which suggests that they may be also diverse in terms of the hosts that they infect. It was possible to observe viral particles with long tails (Bradley group A or B), short tails (Bradley group C), and without tails (Bradley group E) [6]. The latter may belong to a wide range of hosts, including eukaryotes.

As a general pattern, the virus population was dominated by small forms (<30 nm), and tails were rarely seen, which is in agreement with the results reported by several authors [1, 3, 10, 24]. Although loss of phage tails may have taken place during the concentration step, the high recovery rates of T6 phage we obtained led us to discard this hypothesis. Neither filamentous nor pleomorphic phages were observed. TEM observations revealed that only a small proportion of the virus was devoid of nucleic acids, indicating that most of them are potentially infective.

To sum up, we have designed a method to count total viral particles from marine waters with low concentrations of microorganisms. In this procedure, we used, consecutively, negative filtration (to remove planktonic microorganisms), a TFF system (using low flow rate) and ultrafiltration by centrifugation to reduce the samples until ca. 10–20 µl which could be observed by TEM. The application of this method although time consuming, is convenient when the number of viral particles is small; it provides a highly efficient recovery of several types of viruses when low flow rates are used. In addition, this method may be applied to isolate marine viruses, because they do not seem to lose their infectivity.

Acknowledgments This work was supported by a grant AM93-0614-CO2 from the Comisión Interministerial de Ciencia y Tecnología (CICYT) from the Spanish Government. We thank M.J. Navarrete for her help in the English revision of the manuscript.

References

1. Ackermann HW (1992) Frequency of morphological phage description. Arch Virol 124:201–209
2. Barthel KG, Schneider G, Gradinger R, Lenz J (1989) Concentration of pico- and nanoplankton by means of tangential flow filtration. J Plankton Res 11:1213–1221
3. Bergh O, Borsheim KY, Bratbak G, Heldal M (1989) High abundance of viruses found in aquatic environments. Nature 340:467–468
4. Berman D, Rohr ME, Safferman RS (1980) Concentration of poliovirus in water by molecular filtration. Appl Environ Microbiol 40:426–428
5. Borsheim KY, Bratbak G, Heldal M (1990) Enumeration and biomass estimation of planktonic bacteria and viruses by transmission electron microscopy. Appl Environ Microbiol 56:352–356
6. Bradley DE (1967) Ultrastructure of bacteriophages and bacteriocins. Bacteriol Rev 31:230–314
7. Fuhrman JA, Suttle CA (1993) Viruses in marine planktonic systems. Oceanography 6:51–63
8. Giovannoni SJ, DeLong EF, Schmidt TM, Pace NR (1990) Tangential flow filtration and preliminary phylogenetic analysis of marine picoplankton. Appl Environ Microbiol 56:2572–2575
9. Guixa-Boixareu N, Calderón-Paz JI, Heldal M, Bratbak G, Pedrós-Alió C (1996) Viral lysis and bacterivory as prokaryotic loss factors along a salinity gradient. Aquat Microb Ecol 11:215–227
10. Hara S, Terauchi K, Koike I (1991) Abundance of viruses in marine waters: assessment by epifluorescence and transmission electron microscopy. Appl Environ Microbiol 57:2731–2734
11. Havelaar AH, Hogeboom WH (1983) Factors affecting the enumeration of coliphages in sewage and sewage-polluted water. Antonie van Leeuwenhoek J Microbiol 49:387–397
12. Hennes KP, Suttle CA (1995) Direct counts of viruses in natural waters and laboratory cultures by epifluorescence microscopy. Limnol Oceanogr 40:1050–1055
13. Katzenelson E, Fattal B, Hostovesky T (1976) Organic flocculation: an efficient second-step concentration of viruses in tap water. Appl Environ Microbiol 32:638–639
14. Paul JH, Jiang SC, Rose JB (1991) Concentration of viruses and dissolved DNA from aquatic environments by vortex flow filtration. Appl Environ Microbiol 57:2197–2204
15. Porter KG, Feig YS (1980) The use of DAPI for identifying and counting aquatic microflora. Limnol Oceanogr 25:943–948
16. Proctor LM, Fuhrman JA (1990) Viral mortality of marine bacteria and cyanobacteria. Nature 343:60–62
17. Rodríguez V, Bautista B, Jiménez-Gómez F, Guerrero F, Blanco JM, Rodríguez J (1998) Loss of pico- and nanoplankton cells during concentration by tangential flow filtration. J Plankton Res 20:1087–1097
18. Shibley GP, Manousos M, Munch M, Zelljadt I, Fisher L, Mayyasi S, Harewood K, Stevens R, Jensen KE (1980) New method for large-scale growth and concentration of the Epstein-Barr viruses. Appl Environ Microbiol 40:1044–1048

19. Suttle CA, Chan AM, Cottrell MT (1990) Infection of phytoplankton by viruses and reduction of primary productivity. Nature 347:467–469
20. Suttle CA, Chan AM, Cottrell MT (1991) Use of ultrafiltration to isolate viruses from seawater which are pathogens of marine phytoplankton. Appl Environ Microbiol 57:721–726
21. Torrella F, Morita RY (1979) Evidence for a high incidence of bacteriophage particles in the waters of Yaquina Bay, Oregon: ecological and taxonomical implications. Appl Environ Microbiol 37:774–778
22. Watanabe RA, Fryer JL, Rohovec JS (1988) Molecular filtration for recovery of waterborne viruses of fish. Appl Environ Microbiol 54:1606–1609
23. Weinbauer MG, Fuks D, Peduzzi P (1993) Distribution of viruses and dissolved DNA along a coastal trophic gradient in the northern Adriatic Sea. Appl Environ Microbiol 59:4047–4084
24. Wommack KE, Hill RT, Kessel M, Russek-Cohen E, Colwell RR (1992) Distribution of viruses in the Chesapeake Bay. Appl Environ Microbiol 58:2965–2970