4 Quantification of Algal Viruses in Marine Samples

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CONTENTS

Introduction and background
Concentration of viruses in water samples by ultrafiltration
Most probable number (MPN) assays
Plaque assays
Conclusions

INTRODUCTION AND BACKGROUND

Phycoviruses (viruses that infect either cyanobacteria or eukaryotic algae) impart significant mortality on their hosts in aquatic environments. Microorganisms (both eukaryotic and prokaryotic) in marine systems are thought to be responsible for as much as 50% of the photosynthetic carbon fixation on the planet (Field *et al.*, 1988). It is therefore apparent that agents of mortality that act directly to reduce primary production in marine environments will alter carbon and energy flux through these systems (Wilhelm and Suttle, 1999; Fuhrman, 1999). This has, in part, led to the increased interest in the ecology of marine viruses that has occurred through the last decade.

Studies concerning the distribution and activity of viruses at the community level commonly rely on direct counts to monitor changes in the natural viral community. While this information is pertinent to many studies, it does not address the issue of the infectivity of these viruses or the range of organisms that may be directly influenced by viral activity. Outside molecular techniques (see below), the identification and enumeration of phycoviruses requires the observation of interactions between virus and their hosts. It is therefore pertinent to many studies to be able to quantify the abundance of infective viruses that may impart mortality on specific phytoplankton. However, as these measurements require that virus–host interactions be observed, it is necessary from the onset that the host phytoplankton be cultivable. Therefore, the techniques highlighted in this paper require that the host organisms can be cultured in the lab in order to enumerate potential viruses.

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The identification of viruses in the sea that infect specific cyanobacteria and bacteria is still in its relative infancy compared to studies on viruses infecting marine heterotrophic prokaryotes (Suttle, 1996). While the total abundance of virus-like particles ranges from 10^5 to 10^8 ml^{-1} seawater (Wilhelm and Suttle, 1999), viruses infecting and lysing phytoplankton only represent a subset of this population. However, viruses infecting the marine *Synechococcus* spp. commonly occur at concentrations > 10^3 ml^{-1} in coastal waters, and have estimated at concentrations as high as 2.5×10^5 ml^{-1} (Suttle and Chan, 1993; 1994; Waterbury and Valois, 1993). Concentrations of viruses infecting eukaryotic phytoplankton can be equally as high; Cottrell and Suttle (1995) measured abundances of lytic viruses infecting *Micromonas pusilla* at > 10^5 ml^{-1}. Viruses infecting other phytoplankton, including *Aureococcus anophagefferens* (Milligan and Cosper, 1994), *Chrysochromulina* spp. (Suttle and Chan, 1995), *Emiliania huxleyi* (Bratbak *et al.*, 1993), *Heterosigma akashiwo* (Nagasaki and Yamaguchi, 1997; Lawrence *et al.*, 2000) and *Phaeocystis pouchetii* (Jacobsen *et al.*, 1996) have also been isolated from pelagic marine systems in recent years.

In recent years it has also been demonstrated that infectious phycoviruses can also be isolated from marine sediments. In the Western Gulf of Mexico, Rodda *et al.* (1996) found cyanophages in concentrations ranging from 9.4×10^4 ml^{-1} at the sediment/water interface of a 47 m water column, to 3.0×10^2 ml^{-1} at 30 cm below the sediment surface. As the water over the sediment contained an order of magnitude less virus, this suggests that the vertical transport and subsequent burial of infectious cyanophage or infected cyanobacteria was occurring (as the production of cyanophage in the absence of light is unlikely).

As molecular techniques for the enumeration of phycoviruses are currently under development, it remains premature to include them as protocols in this chapter. Using the polymerase chain reaction (PCR) and virus specific primers, Suttle and co-workers have been able to establish a baseline of information on the genetic diversity of one group of algal viruses, the Phycodnaviridae (Chen and Suttle, 1996; Short and Suttle, 1999). Recently they have been able to estimate the diversity of at least a portion of the Phycodnaviridae using degenerate primers for the segments of the DNA polymerase genes of these algal viruses and denaturing gradient gel electrophoresis.

Similarly, Fuller *et al.* (1998) have described the genetic diversity of cyanophage isolates infecting *Synechococcus* spp. using PCR techniques targeted at the DNA region encoding a capsid assembly protein. However, as with studies involving viruses infecting eukaryotic phytoplankton, these results remain qualitative. The advent of new techniques (e.g. quantitative PCR, *in situ* PCR) will hopefully soon provide qualitative values for the distributions of these algal viruses.

This review describes the current methods available for the enumeration of specific viruses infecting phytoplankton in aquatic environments. It represents a compilation of methods that have been employed for many years in classic virology and those that have been adapted for use by 'viral ecologists' working in natural systems. Two approaches, the plaque assay

and MPN assay, are described here which allow researchers to both enumerate and isolate viruses that lytically infect marine photoautotrophs.

◆◆◆◆◆◆ CONCENTRATION OF VIRUSES IN WATER SAMPLES BY ULTRAFILTRATION

Principle

In many situations the abundance of lytic phycoviruses in a natural water sample is too low to accurately quantify. In these cases, the use of ultrafiltration techniques may be required to increase the concentration of viruses in the sample. Ultrafiltration involves the removal of bacterial and algal components (> 0.2 μm) of the microbial community followed by the concentration of the 'viral size fraction' (typically 30 kDa to 0.2 μm). Small scale (0.5–20 ml) ultrafiltrations can be carried out with a commercially available centrifugation systems ('spin-columns') such as Centriprep or Centriplus units (Millipore). While these often will work well with laboratory virus–host systems, these sample sizes are often too small to properly examine environmental samples. In these cases, techniques such as tangential flow filtration or vortex flow filtration can be used to handle larger volumes (1–200 l). With these techniques, concentration of the viral particles is achieved by successive circulation of the sample across a 30 kDa membrane surface. This allows the water to be removed from the sample (ultrafiltrate) while the viruses are concentrated into the retained volume. The resulting viral concentrate can then be used as the 'sample' to screen (as described below). In this protocol, we describe the use of the Amicon M12 ultrafiltration system, as this is the system currently in use in our laboratory (adapted from Chen *et al.*, 1996). Similar systems are provided by other suppliers, and it is suggested that the reader consider these other alternatives prior to making any investment in a system.

Equipment and reagents

- Submersible pump with pressure gauge.
- Two containers for water samples (20–200 l each, depending on volume to be concentrated).
- 142 mm diameter glass fiber filters (MSF GC50; nominal pore size, 1.2 μm) with holder(s) and appropriate tubing (non-toxic).
- 142 mm diameter, 0.2 μm nominal pore-size filters (polycarbonate or low protein binding) with holder(s) and appropriate tubing.
- Amicon ProFlux M-12 ultrafiltration system with non-toxic tubing designed for use in peristaltic pumps (such as Masterflex from PharMed).
- Millipore S10Y30 spiral wound membrane cartridges (30 kDa molecular weight cutoff).
- Header kits for S10 cartridges.

Application

Collect the water sample (20–200 l) into one of the holding containers. Using the submersible pump, prefilter (at < 17 kPa) the sample first through the 142 mm diameter glass fiber filters (MSF GC50; nominal pore-size, 1.2 μm). Two or more of these may be set up in parallel for larger sample volumes. Follow this by filtering the sample through a 0.2 μm filter into the second container. These filters will remove large particulates, algae, bacteria, etc. but will allow most viruses to pass. Throughout these steps, subsamples of water should be collected so that the recovery efficiency of this process can be calculated (see below).

After filtration, use an Amicon ProFlux M-12 ultrafiltration system to concentrate the filtrate containing the viruses. Set the M-12 up for concentration mode, with a Millipore S10Y30 (S10) spiral wound membrane cartridge (30 kDa cutoff). This cartridge, with a total membrane area of 0.93 m^2, will allow water to pass through but retain virus particles. Connect tubing from the container with the filtrate into the pump inlet of the M-12. Standard operating procedures involve running the pump at 40 to 50% of the maximum speed, with 50 to 60 kPa of backpressure.

From the pump, run the tubing to the inlet header of the S10 cartridge and from the outlet header back to the container holding the sample to return the retenate. Connect tubing from the permeate connector to remove the ultrafiltrate, which can be discarded. As the system runs, the permeate (without virus particles) is removed, thereby concentrating the viruses in the remaining retenate. With this setup, a volume of 200 l of seawater can be concentrated to 500 ml in about 1 hour. Take care not to attempt to reduce the volume of the retentate below the summed volume of the cartridge and the tubing. Measure the final volume of viral concentrate so that the concentration factor (CF) can be estimated as follows:

$$CF = \textit{volume of sample} / \textit{volume of retentate}$$

If required, the retenate can be further concentrated to a smaller volume (100 to 200 ml) using a smaller system (e.g. an S1Y30 cartridge, Chen and Suttle, 1996). Alternatively, other low-cost methods are available to concentrate samples. Centrifuge-based concentrators (e.g. Centriprep, Centriplus) can be used to concentrate viruses in small volumes of sample.

The recovery efficiency (*a.k.a.* concentration efficiency) of this process must be determined when using viral concentrates to estimate the abundance of infectious phycoviruses. This can be most easily determined by direct counts of the total viral abundance pre- and post-concentration (see chapter by Noble for direct counting techniques). The recovery efficiency (RF, as a %) is determined as follows:

$$RF = 100\ (A_{con} / A_{sam}) / CF$$

where A_{con} is the abundance of virus particles in the viral concentrate, A_{sam} is the abundance of viruses in the original sample, and CF is the concen-

tration factor (determined above). Typical recovery efficiencies vary, but are generally greater than 50% and commonly approach 100% (Suttle *et al.*, 1991; Wommack *et al.*, 1995).

Troubleshooting

A series of problems can occur when making and using viral concentrates. Most problems are associated with the concentration of the viruses. The problems include leakage from old tubing, loss of viruses during prefiltration, and incorrect estimates of the viral concentration factor. Establishing familiarity with the concentration equipment and procedure(s) is a sure cure for many of these problems.

The other considerations to be made with ultrafiltration are problems associated with filter integrity and cleaning. Breakthrough of viruses in damaged filters can seriously hinder concentration efficiency. All manufacturers, however, provide instruction on testing the integrity of their filters. As filters are often costly, cleaning procedures are important and designed to maximize the life of the filter cartridge. In the case of the system described, we recirculate 0.1 M NaOH to remove residual organics from the filter after each concentrate is made. The NaOH is subsequently removed using ddH_2O, dilute H_3PO_4 and then ddH_2O. Again, all manufacturers provide information on the chemical compatibility of their filters, and this should be checked in each case. This is especially important as some sterilizing agents (hydrogen peroxide, bleach, strong NaOH) can damage certain membranes and thus compromise their integrity.

◆◆◆◆◆◆ MOST PROBABLE NUMBER (MPN) ASSAYS

Principle

Assessment of lytic viral activity requires that the virus particle destroys a host cell. Using a dilution approach, we can estimate the abundance of viruses in a sample. This process is based on the theoretical assumption that a single infectious virus can destroy a population of sensitive host cells (given time). The MPN approach to quantifying infectious viruses involves the exposure of a series of log-based dilutions of the sample containing the viruses to a liquid culture of host cells. Given an appropriate range of dilution (crossing the range where the mean viruses per aliquot sample is approximately 1.0) then the abundance of infectious viruses can be estimated. While the individual MPN exposures are scored '±', comparison of these scores to MPN tables (or analysis by computer software) allows for an estimate of infective units. Replication can be achieved in several ways, with many labs now commonly using multi-well plates to enhance this (see Alternative technique). Ultimately, the desired levels of sensitivity and accuracy will dictate the volumes, number of replicates and scale of cultures to be used.

Equipment and reagents

- Culture medium for marine phytoplankton. ESAW (Harrison *et al.*, 1980) and its derivations commonly work well as a general growth medium.
- Liquid culture of host organism in exponential growth phase.
- 7 ml glass culture tubes (13 × 100 mm) with polypropylene screw caps.
- Fluorometer with filter set for chlorophyll determinations (λs, 420_{ex}; $>640_{em}$).
- 25 mm, 0.22 µm nominal pore-size low protein binding filters (e.g. *Durapores*®).
- Filtration funnel and receiver or Swinnex® filter holder (and 10 cm^3 syringe) for 25 mm filters.
- Pipette (1–5 ml) and tips for liquid dispensing.
- Culture facilities for phytoplankton.

Alternative technique (requires the following materials)

- 96-well microtiter plates with lids.
- Multichannel pipette and tips.
- Fluorescent plate reader with filters for chlorophyll (λs, 420_{ex}; $> 640_{em}$).

Application (using 7 ml culture tubes)

Collect water samples for screening into sterile polypropylene or polycarbonate containers and maintain them at 4°C in the dark until they are screened. Screen samples as soon as possible. Prior to screening, filter 25 ml of sample through a 0.22 µm nominal pore-size filter to remove bacteria, algae, protists, etc. In some cases other pore-size filters can be used (see Troubleshooting). From this, make a set of serial dilutions (10-fold dilutions with sterile culture medium) with the sample to provide a dilution range of 1 to 10^{-4} of the sample. Add 1 ml of each of these dilutions to the exponentially growing host cultures (below) to screen for lytic activity.

To prepare hosts for screening, transfer an aliquot of the host to fresh culture medium. For example, transfer 50 ml of an exponentially growing batch culture into 450 ml of medium. Monitor growth in this culture so that cells can be used as soon as the exponential phase of growth begins. As exponential growth begins, transfer 5 ml to each of fifty-five 7 ml culture tubes, assuming that ten replicates (and five controls with no sample added) is the desired number for the experiment, and that five dilutions are being used (Suttle, 1993). Gently mix the tubes, record the fluorescence and place the tubes in culture facilities. Remove the tubes daily (for up to seven days) and repeat the measurement of fluorescence. Cultures not clearing in seven days are assumed to not contain virus.

For each dilution, record the number of tubes that have cleared and use this data to calculate the MPN for the concentration of viruses in the sample. The MPN can be determined from published values in tables

(Koch, 1981) or by using computer programs which can also provide confidence intervals and standard estimates of error (Hurley and Roscoe 1983). Sample results are shown in Figure 4.1.

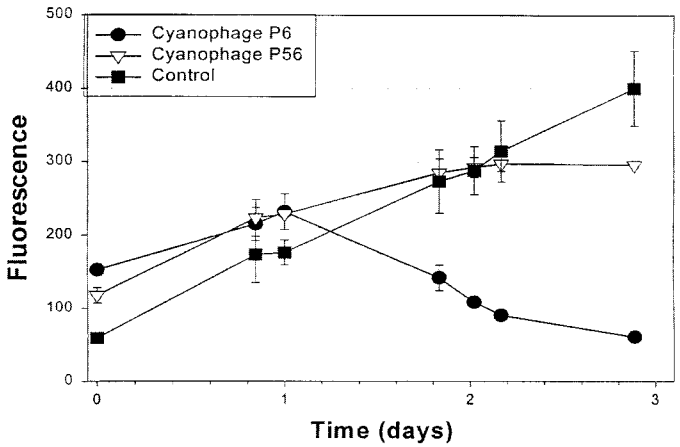

Figure 4.1. Typical chlorophyll fluorescence from the cyanobacteria *Synechococcus* sp. WH7803 in culture with and without added viruses. The addition of cyanophage P6 demonstrates the typical clearing seen in tubes during MPN assays. Both the control and cyanophage P56 (which does not infect this *Synechococcus*) demonstrate no clearing, and this was consistent up to 7 days (not shown).

Alternative technique

As described by Suttle and Chan (1993) and Bratbak *et al.* (1998), microtiter plates can be substituted for culture tubes to screen cultures that will grow in these systems. Repeat the process as above, but substitute a 96-well microtiter plate for the 7 ml culture tubes and adjust volumes of host (100 µl) and virus (50 µl). Maintain cultures under standard growth conditions and screen them daily, either visually or with a fluorescence plate reader equipped to monitor chlorophyll fluorescence. When using microtiter plates, it is easy to expand the dilutions from 5 to 7 (or more) tenfold steps. However, in the microtiter assay less sample is screened, so the minimum detection limits (sensitivity) of the assay is decreased.

Troubleshooting

One problem commonly associated with the screening of natural samples is the breakthrough of unwanted organisms (e.g. bacteria and protozoans) through the filter into the sample to be screened. This problem is often difficult to diagnose until after the experiment has been carried out, but samples can be examined by epifluorescence microscopy to determine the

presence of unwanted organisms if this problem becomes of concern. Another problem that occurs is the destruction or removal of viruses in the sample during storage, often by bacteria or protozoan grazers. To avoid this problem, filter samples upon collection (as described) and store in the dark at 4°C until use. It should be pointed out that viral infectivity will decay, even under these conditions. However, in at least one case infective viruses have been found in these concentrates after storage for 7 years under the above conditions (Wilhelm and Suttle, unpublished data).

Another problem to consider is the removal of infectious viral particles by filtration. Different size filters are commonly suggested in different protocols (e.g. 1.0 μm, Suttle, 1993; 0.45 μm, Garza and Suttle, 1998). While we have suggested the use of a 0.22 μm filter in this protocol, it should be considered that decreasing the pore-size of the prefilter increases the possibility of viral retention during that step. In any case, all filters will retain some degree of viruses during this step, so consistency in pore-size, filter matrix and technique (e.g. pressure) is critical in providing reproducible results.

Growth in the controls must also be closely monitored. Should all or a subset of the controls not grow, then it is not possible to determine if clearing in the test cultures is due to viral activity or non-experimental effects. It is therefore important to have established the ability to consistently grow the host culture in the lab prior to attempting to quantify viral particles.

Finally, while the choice of using culture tubes relative to microtiter plates is left to the investigator, we would like to point out that the use of microtiter plates decreases the detection limit for infective viruses in the samples. As described above, the tube method will increase the detection limit 20-fold relative to the microtiter method (as 20 times more sample is screened).

◆◆◆◆◆ PLAQUE ASSAYS

Principle

Plaque assays are commonly used in bacteriophage studies in order to enumerate the abundance of infectious phage in a sample. These same techniques may be applied to the enumeration of phycoviruses. Plaque assays have the advantage over MPN assays of providing increased accuracy, but have the disadvantage of requiring that hosts cells must be culturable and provide a confluent lawn on agar solidified growth medium. The principle of the plaque assay is simple: it assumes that, within a complete lawn of organisms on a Petri plate, each individual virus will produce a clearing or 'plaque' where it has lysed the localized host cell population. The plaque assay also provides the added advantage of allowing individual plaques to be isolated directly from the plate, providing a clonal copy of each virus. Moreover, in some cases the presence of turbid plaques can be taken as an indication of a potential lysogenic virus (although significant testing is required to confirm this).

Equipment and reagents

- Culture medium for marine phytoplankton. ESAW (Harrison *et al.*, 1980) and its derivations commonly work well as a general growth medium.
- Agar for the solidification of culture medium (e.g. BactoAgar from Difco).
- Liquid culture of host organism in exponential growth phase.
- Autoclave/microwave.
- Temperature controlled water bath or dry block.
- Microfuge.
- Vortex mixer.
- 25 mm, 0.22 µm nominal pore-size low protein binding filters (e.g. *Durapores*).
- Petri plates (plastic, 15 × 100 mm).
- 1 and 5 ml pipetters and tips.
- 1.5 ml microfuge tubes.
- 13 × 100 mm disposable borosilicate glass tubes and rack(s).
- Erlenmeyer culture flask (250 ml).
- Culture facilities for algae.

Application

Prior to screening cultures, plates for the establishment of confluent lawns must be created. Bottom agar for these plates is created by adding 1% (w/v) agar to the appropriate culture medium and autoclave sterilizing the sample. After the medium is allowed to cool to 60°C, pour plates (15 to 20 ml) under sterile conditions and allow them to solidify. It is important that plates are only poured to ≈ 50% capacity. Once dried, invert the plates and store them as other cultures plates (4°C, dark). They are usually good for up to a week or more.

Top agar is also required for plaque assays. To prepare it, add 0.6% (w/v) agar to 100 ml of growth medium in a 250 ml flask or media bottle. If sealed after sterilization, this can be stored at room temperature. When required, the agar is remelted in a microwave oven and 2.5 ml aliquots placed into three 7 ml disposable culture tubes per sample to be screened. Maintain these tubes at a temperature between 45 and 47°C in the water bath/hot block until use. Fill three tubes with top agar to use as controls.

To prepare water samples for screenings, filter 25 ml of sample through a 0.22 µm pore-size filter to remove bacteria, algae, protists, etc. as these organisms may cause false plaques to form. It might be necessary with some samples to carry out a series of dilutions prior to undertaking the plaque assay, as it is desirable to have only 20–200 infectious viruses per aliquot. These dilutions can be carried out as described above, using sterile marine medium and a series of culture tubes.

To begin the plaque assay, start cultures of phototrophs to allow for yields of around 10^7 ml^{-1} of exponentially growing cells. Harvest the cells by gentle centrifugation (3000–5000g) and then resuspend them to around 10^9 cells ml^{-1}. When working with heterotrophic bacteria this

concentration step is generally not required. Transfer cells (500 µl) to three sterile microfuge tubes, and 500 µl of sample to each tube, then close the tubes and mix by vortexing. For each set of experiments add 500 µl of sterile culture medium to hosts in microfuge tubes to act as a control. After samples are combined in microfuge tubes, a brief spin in a microfuge will remove any liquid from the interior lid of the tubes. Allow samples to sit so that the virus can adsorb to the host cells. Adsorption times for heterotrophic bacteria commonly range from 5–25 minutes, but 30–45 minutes is sufficient when the kinetics of adsorption are unknown (Suttle, 1993).

After the adsorption period, mix the contents of each microfuge tube with a tube of top agar by vortexing. Quickly pour this mixture onto the bottom agar in the Petri plate, and 'swirl' the sample on a flat surface to evenly distribute the top agar mixture. After the plates dry and solidify (≈ 60 minutes), invert them and move them to appropriate culture facilities.

Enumeration of plaques on the plates occurs once confluent host lawns have established (2–6 days). Individual plaques are enumerated and assumed to represent the presence of one lytic virus in the samples. For statistical relevance, it is desirable to enumerate plates from dilutions with plaque abundances ranging from 20–200 per plate.

Once plaques are enumerated, the abundance of viruses infecting the host can be determined as follows:

$$A = (p \,/\, d) \times (1 \,/\, v)$$

where A is the abundance of infectious viruses (ml^{-1}), p is the number of plaques on the plate, d the dilution factor for that plate and v the volume (ml) of sample added (as described above, 0.5).

A comparison of the two assays (most probable number and plaque) is given in Table 4.1.

Table 4.1 Comparison of the most probable number assay with the plaque assay for the enumeration of phycoviruses

Method	Advantages	Disadvantages
MPN assay	• Flexible with culture requirements • Amenable to high replication • Does not require growth on solidified medium • Enumerates only infective viruses	• Less precision than plaque assay • Low probability of detecting lysogens
Plaque assay	• Higher accuracy than MPN assay • Provides indication of potential lysogens • Provides for easy purification of viruses • Enumerates only infective viruses	• Must be cultured on solidified medium • Natural bacteria can cause plaque-like clearings

Troubleshooting

The most significant problem associated with variations in plaque assay results is inconsistency of technique. Ensuring that cells are in the same phase of growth in each experiment is vital to providing reproducible results. As well, culture conditions (including temperature) should be held constant to allow for an intercomparison of samples. To account for variation, positive controls consisting of samples with a known abundance of infectious viruses can be included in every experiment. However, it should be remember that stored samples of viruses slowly lose infectivity over time, so samples that are examined 6 months apart may not be comparable using the same positive control.

It should be stressed that the plaque assay method is often difficult to use with eukaryotic plankton (the exception being strains of *Chlorella* spp.). As well, certain cyanobacteria will not grow on standard agar, as it usually contains too many impurities. Better growth of cyanobacteria on agar plates can be achieved by first removing these impurities (Waterbury and Wiley, 1988).

◆◆◆◆◆ CONCLUSIONS

Two methods for the enumeration of infectious phycoviruses as well as a method to increase the concentration of viruses in a water sample are discussed here. Each method for viral enumeration has its particular advantages and disadvantages (Table 4.1). The choice of the particular method will ultimately depend on the culturability of the host cells in question.

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