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Isolation and life cycle characterization of lytic viruses infecting heterotrophic bacteria and cyanobacteria

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Abstract

Basic knowledge on viruses infecting heterotrophic bacteria and cyanobacteria is key to future progress in understanding the role of viruses in aquatic systems and the influence of virus–host interactions on microbial mortality, biogeochemical cycles, and genetic exchange. Such studies require the isolation, propagation, and purification of host–virus systems. This contribution presents some of the most widely used methodological approaches for isolation and purification of bacteriophages and cyanophages, the first step in detailed studies of virus–host interactions and viral genetic composition, and discusses the applications and limitations of different isolation procedures. Most work on phage isolation has been carried out with aerobic heterotrophic bacteria and cyanobacteria, culturable both on agar plates and in enriched liquid cultures. The procedures presented here are limited to lytic viruses infecting such hosts. In addition to the isolation procedures, methods for life cycle characterization (one-step growth experiments) of bacteriophages and cyanophages are described. Finally, limitations and drawbacks of the proposed methods are assessed and discussed.

Introduction

It was not until the late 1980s when the use of electron microscopy had revealed the presence of very large abundances (10^6-10^8 mL^{-1}) of viruses in marine pelagic environments that it was realized that viruses were quantitatively important players in natural ecosystems (e.g., Bergh et al. 1989; Proctor and Fuhrman 1990; Suttle et al. 1990). Until then, studies of viruses in marine environments had been limited to examination and quantification of specific bacteria-virus systems, based on relatively few culturable bacterial hosts and co-occurring bacteriophages (e.g., Moebus 1991, 1992). These studies, which were based on numbers of infective units, generally showed that specific viruses were present in low densities (hundreds to a few thousands per milliliter) and did not suggest the significant role of viruses in the marine ecosystem, which was demonstrated a few years later.

The realization that marine viruses were significant agents of mortality for both heterotrophic bacteria and cyanobacteria with large impact also on microbial diversity, population dynamics, and nutrient cycling has accelerated the scientific effort in aquatic viral ecology over the past 15 years (e.g., Weinbauer 2004; Suttle 2007; Brussaard et al. 2008; Middelboe 2008). Since the development and refinement of techniques to quantify total viral abundance (e.g., Hennes and Suttle 1995; Noble and Fuhrman 1998) and production (e.g., Steward et al. 1992; Wilhelm et al. 2002; Winget et al. 2005), a large research effort has been allocated to describe the dynamics and impacts of the total viral community, based mainly on enumeration of total viral abundance, total viral production, and frequency of infected cells. However, little is still known about the dynamics, specificity, evolution, and ecological impact of the most basic property of the aquatic viral community: the interaction

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between a specific virus and its hosts. As the overall viral activity represents the sum of all the virus–host systems at any given time, the role of viruses is therefore a property of the total collection of virus–host interactions.

Viral infection of specific hosts affects the composition, and therefore the biogeochemical properties of the microbial community, as well as the fluxes of carbon and nutrients in the ecosystem. Viral infection also acts as a driving force in microbial evolution by selecting for virus resistant mutants and by mediating genetic exchange between their hosts. Consequently, understanding virus–host interactions at the level of individual cells and populations is a prerequisite for obtaining fundamental insight on the role of viruses for essential biogeochemical and evolutionary processes.

Assessing the impact of viruses on an ecosystem scale requires the inclusion of viruses in models of food web interactions and biogeochemical cycling. To constrain values of viral parameters in models of virus–host interactions, it is essential to obtain detailed information about the fundamental properties of the virus–host interaction, e.g., adsorption rate, life cycle, host range, resistance development, etc, and how these properties are influenced by the environmental conditions. Such models again provide a frame for understanding and predicting the behavior, dynamics, and evolution of specific virus–host systems in more complex natural environments.

More recently, genomic characterization of specific viruses (e.g., Fuller et al. 1998; Mann et al. 2003) has opened a new approach to identify and track genes shared among groups of viruses and explore the genetic diversity and distribution of viruses in the oceans. For instance, specific molecular probes, arising from genomic studies, are now being employed to track populations of both hosts and viruses within environmental samples (e.g., Short and Short 2008) and thus determine how these interactions influence the dynamics of viruses and their hosts.

Consequently, despite the fact that individual viral strains/types and their bacterial or cyanobacterial hosts probably constitute a very small fraction of the total microbial community, there is a lot to learn from studying this particular level of microbial processes. Also, it links very well with the current genomic studies of viral diversity, dynamics, and distribution in a research area that allows the combination of viral ecology, biodiversity, biogeochemistry, and genomics. Common for these types of studies is that they depend on the isolation of specific virus–host systems for the further characterization of their properties.

Isolation of viruses is thus the first step in detailed studies of virus–host interactions, and the present article describes basic methodological approaches for isolation of two groups of viruses, bacteriophages and cyanophages, and discusses the applications and limitations of different isolation procedures. It should be emphasized that viruses targeted by the presented protocols are restricted to (1) lytic viruses and (2) viruses infecting culturable hosts, thus at the same time excluding probably the majority of marine viruses, i.e., viruses with unculturable hosts and temperate or chronic lifestyles.

Materials and procedures

Natural virus communities from the aquatic environment (e.g., seawater, rivers, lakes, ponds, sediments, water surrounding cyanobacterial mats, etc.) can provide the source of potential virus isolates. For the isolation of bacteriophages or cyanophages, samples for screening should be prefiltered or centrifuged before using. For example, use 0.8 µm to 1.2 µm pore-size glass fiber or PCTE filters to remove larger particles and organisms, followed by 0.2 µm or 0.45 µm pore-size low protein-binding PVDF filters (Millipore Durapore) to remove the remaining bacteria and phytoplankton that could interfere with the isolation assays. The drawback of such prescreening, however, is that filtration and centrifugation may also remove a fraction of the phages in the sample, and thus reduce the chance of finding lytic phages against the target bacteria.

Store the filtered samples at 4°C, in the dark until use. If the overall virus abundance is suspected to be low, such as one would expect from oligotrophic environments (e.g., polar regions), virus concentrations in the sample can be increased several hundred fold using ultrafiltration/tangential flow methodologies (e.g., Suttle et al. 1991; Wommack et al. 2010, this volume). For sediment samples, procedures to extract viruses from particles (e.g., Danovaro and Middelboe 2010, this volume) should be applied prior to phage isolation. Much of the following procedures were adapted from earlier techniques described for the isolation of bacteriophages and cyanophages from environmental samples (e.g., Adams 1959; Safferman and Morris 1963; Eisenstark 1967; Berg 1987).

Described below are methods that are used routinely to isolate, purify, and characterize bacteriophages and cyanophages from aquatic environments. Although there are a number of similarities between the methods used, the specific practical protocols for bacteriophages and cyanophages differ from one another. We have therefore chosen a full step-by-step presentation of the proposed protocols for each group of phages, rather than combining the two, and focus on the specific parts where the procedures differ from each other. Our approach results in some overlap between the two sections, but on the other hand, increases the clarity of the individual protocols. It is assumed that culturing methodology has been determined for the target hosts of interest, and if host culture conditions are not optimized, this should be the first step in the isolation procedure for any type of phage.

Isolation and host range characterization of bacteriophages—Bacteriophage isolation by spotting on target host cells: Phage lysis of host bacteria can be visualized by plaque formation on lawns of host cells in soft agar overlayed on agar plates (Adams, 1959; Sambrook et al. 1989). This principle can be used for the detection and subsequent isolation of specific

lytic phages in environmental samples (e.g., Carlson 2005). Spotting environmental samples on lawns of a target host cell would thus reveal the presence of a lytic phage for that particular host cell in the given sample:

1. The host cells are grown overnight in liquid cultures containing an appropriate growth medium for the organism (i.e., a medium that will yield a visible lawn of bacteria in soft agar when plated on an agar plate). Rich media such as Luria Broth (10 g tryptone, 5 g yeast extract, 10 g NaCl in 1 L distilled water) or MLB (0.5 g Casamino acids, 0.5 g peptone, 0.5 g yeast extract, 3 mL glycerol in 800 mL prefiltered [GF/C] seawater and 200 mL distilled water) can be used to culture many varieties of marine bacteria.
2. Measure optical density spectrophotometrically at 525 nm (OD_{525}) and adjust OD to 0.3–0.5 with growth medium. This ensures sufficient bacterial density as well as the capacity for further bacterial growth during the following plate incubation.
3. Soft agar (0.5 to 0.6% agar in media of choice) is melted in a water bath or a microwave oven, then distributed in aliquots of 4 mL to sterile culture tubes and kept just above solidification temperature until use. The solidification temperature of soft agar depends on the agar or agarose used and is about 45°C for common agar. If the host bacteria cannot survive exposure to 45°C soft agar, low-melting-point agaroses are available for a range of lower temperatures (*see* "Cyanophage" section).
4. 200–300 µL bacterial culture is added to the 4 mL tubes with melted soft agar. The bacteria-soft agar mixture is then vortexed and immediately poured onto an agar plate with an agar that supports growth of the host bacterium (e.g., Zobell agar [5 g tryptone, 1 g yeast extract, 15 g agar in 800 mL GF/C filtered sea water and 200 mL distilled water]), and distributed evenly on the plate, which is placed on a flat surface.
5. When the soft agar containing the target bacteria has solidified, triplicate aliquots of 5–10 µL of each of the environmental water samples from which phages should be isolated are spotted on top of the soft agar. Before spotting, the samples should be filtered (e.g., 0.2 µm or 0.45 µm syringe filters) or centrifuged (e.g., 10,000*g*, 10 min) to remove bacteria. These procedures would minimize bacterial contamination in the spotting zone, which may hide clearing zones. As mentioned above, filtration and centrifugation may also remove a fraction of the phages in the sample, and thus reduce the chance of finding lytic phages against the target bacteria. As a negative control 5–10 µL phage buffer (e.g., SM buffer: 450 mM NaCl, 50 mM $MgSO_4$, 50 mM Tris, 0.01 % Gelatin, pH = 8) or 0.02 µm filtered sample water is spotted in triplicate on the soft agar. If the abundance of specific phages is expected to be low, the phages can be concentrated by various procedures prior to spotting on the target bacteria. Concentrating procedures are described elsewhere in this special issue (Wommack et al. 2010, this volume).
6. The plates are incubated for 1–3 d depending on the growth rate of the bacteria, and the presence of lytic phages in the sample is detected as a clearing zone (plaque) in the spotted area of the lawn of bacteria that develops over time on the plate. A single or a few phages added will result in only small plaques in the zone, whereas many phages in the spotted sample will yield a large clearing zone.
7. If clearing zones appear in the spotted area, this indicates the presence of lytic phages, which can be isolated and purified (*see* below). To confirm that the clearing is due to phage lysis and not some other growth-inhibiting factor in the original sample, a dilution series of the sample can be performed and spotted on the target bacteria. Diluted sufficiently, phages would appear as single plaques in the spotted zone rather than a gradual reduction in growth inhibition as would be the case if the clearing was caused by some chemical factor. Alternatively, heat-killed (e.g., 90°C for 5 min) or 0.02 µm filtered controls can be used to verify that a clearing zone is caused by a biological component and not a chemical. Presence of phages can also be verified by SYBR staining and subsequent detection by epifluorescence microscopy (Noble and Fuhrman 1998; Suttle and Fuhrman 2010, this volume).
8. Once detected as clearings in the spotting zone, phages are further isolated and purified from the plates as described below.

The main advantage of this procedure is that presence of lytic viruses is visually apparent as clearing zones on host bacterial lawns, and that subsequent isolation of phages is therefore fast, as the phages can be isolated directly from the plaques.

Bacteriophage isolation using enrichment cultures: A generally more efficient way of isolating lytic phages from marine environments is by the use of enrichment cultures. In this approach, the prefiltered water sample that is to be screened for phages against a given target bacterium, is enriched with a bacterial growth medium and amended with that target bacteria (Eisenstark 1967; Carlson 2005). This allows any lytic phages present in the sample to infect the target bacteria and propagate in the cultures, and subsequently, be isolated and purified. The two main advantages of the enrichment approach are 1) that it allows for screening for phages in a much larger volume of sample (typically 25–50 mL, rather than 5–10 µL), thus increasing the probability of isolating rare phages, and 2) that it allows the combination of different target hosts (e.g., different strains of a specific bacteria of interest) in the same incubation, again increasing the possibility of phage isolation.

The following is the standard procedure in our lab when searching for phages against specific target bacteria in environmental samples. The sample volume and number of host strains used may be varied according to the sample investigated and the purpose of the phage isolation.

1. As for the spot test procedure, the potential host cells are

grown overnight in liquid cultures containing a rich growth medium (e.g., MLB) and adjusted to an optical density measured at 525 nm (OD_{525}) of 0.3–0.5.
2. Approximately 25 mL water sample is filtered (0.2 µm or 0.45 µm syringe filters) to minimize the risk of bacterial contamination of the enrichment cultures (note also here that the filtration may result in loss of a fraction of the phages present in the original sample).
3. Transfer filtered samples to triplicate sterile 100 mL culture flasks and add 3 mL 10× growth medium (i.e., 10 times concentrated medium).
4. The enrichment cultures are now started by adding 1 mL of each of the target host strains of interest (e.g., 1–6 different strains for each incubation) to the culture flask.
5. Incubate the cultures on a shaking table at a temperature and period that is appropriate for the host bacteria (typically 1–5 d). A control culture is established where the environmental sample is replaced by 25 mL artificial seawater (or 0.02 µm filtered water sample) to verify bacterial growth in absence of phages.

We recommend that bacterial growth during incubation is examined by OD_{525} measurements, which can give an indication of whether bacterial growth is inhibited by phage lysis. If only a single target bacterial strain is inoculated in the enrichment culture, the presence of a lytic phage against that particular strain will often result in clearing of the culture. However, if the lytic potential of the phage is limited, and/or if several strains are used, phage lysis may be difficult to detect by visual inspection of the culture, as some strains may be resistant to infection by the present phages. In that case, even a small decrease in OD relative to the control culture may indicate the presence of lytic phages.

1. If phage production is detected (in fact, phage production may have occurred even if lysis is not detectable by reduced OD values [*See* "Assessment."]), the culture is transferred to a 50-mL centrifuge tube and the bacteria are pelleted (10,000*g*, 10 min).
2. The supernatant is then sterile filtered (0.2 µm or 0.45 µm filtered) and kept at 4°C until further analysis. A few drops of chloroform will preserve the sample, however, it also introduces the risk of eliminating lipid-containing phages.
3. To verify the presence of lytic phages in the enrichment culture filtrates, 5–10 µL aliquots of the filtrates are spotted on lawns of host bacteria as described above.

Cleared liquid cultures and clearing zones on plates of immobilized host bacteria may potentially contain several types of infective phages that were present in the original sample and which propagated in the enrichment culture. Subsequent steps of isolation and purification are therefore necessary to obtain stocks of specific phages (*see* below).

A more general search for phages, which are lytic to co-occurring bacteria in the water sample, requires a different procedure. In that case, 25 mL unfiltered water sample is amended with 1 mL 10× growth medium and incubated overnight (or longer depending on incubation temperature and type of target bacteria). During this incubation, lytic phages will potentially propagate by infecting indigenous bacteria that are favored by the given substrate and incubation conditions. As for the incubations above, phages produced during incubation are obtained after centrifugation and sterile filtration.

Concomitant with the enrichment culture incubations, potential bacterial host cells are then isolated after spreading 100 µL subsamples of the original water sample on agar plates containing a growth medium that is similar to the medium in the enrichment cultures. Single colonies are picked and restreaked on new agar plates, and subsequently transferred to liquid medium. New phage–host systems can then be obtained by spotting aliquots of filtered enrichment culture on lawns of bacterial isolates in soft agar, as above, and inspected for clearing zones.

Obtaining pure phage stock: As mentioned earlier, clearing zones on lawns of host bacteria potentially contain all lytic phages against the given host that were present in the original water sample. Little is still known about the occurrence and diversity of phages infecting specific hosts, but generally isolation procedures as those described above may reveal multiple phages against specific target bacteria (e.g., Comeau et al. 2006; Holmfeldt et al. 2007; Stenholm et al. 2008). It is therefore necessary to further isolate the phages to obtain specific stocks of single phages:

1. Transfer phages from the clearing zone on the plate to 1 mL phage buffer or sterile sea water in a sterile tube by scraping off the surface layer of the soft agar containing the phages using a sterile loop. Alternatively, use a Pasteur pipette to harvest a plug of the soft agar.
2. Allow the phages to diffuse into the medium overnight at 4°C.
3. Vortex the tube and centrifuge the sample (10,000*g*, 10 min) to remove bacteria and agar.
4. Transfer the supernatant to a new tube. This sample will typically contain 10^6–10^8 phages mL^{-1}.
5. To isolate single phages, dilutions of this concentrate should be done, followed by plaque assay, and subsequent isolation of phages from single plaques. Different plaque morphologies may be selected as an indication of the presence of different phages. Again, the phages are transferred to 1 mL phage buffer, vortexed, and centrifuged, and subsequently, the supernatant containing the phages are transferred to a new tube.
6. Usually, this procedure is repeated 3 times to dilute out any contaminant phage associated with the phage of interest and increase the probability that only one specific phage is present in the final phage stock.
7. In the end, the phage concentrate is 0.2 µm filtered and kept in the fridge. If the phage is insensitive to chloroform, preservation with a few drops of chloroform will prolong the life span of the phage stock. A viability test should,

however, be carried out before adding chloroform to the sample. Stocks of specific phages in a buffer can remain infective for years.

If the relative abundance of individual phages in a phage assemblage obtained from a single clearing zone vary significantly (more than 10–100-fold), it will be very difficult to isolate the least abundant types as they would be diluted out in the attempt to obtain single plaques on the plates. Consequently, the method selects for isolation of the dominant fraction of lytic phages against a certain host bacterium at the time of sampling.

Life cycle characterization of bacteriophages—

One-step growth experiments: The life cycle of phages can be characterized by one-step growth experiments, which are designed in a way that allows only a single infection cycle to take place (i.e., no re-infections occurring by phages produced during the experiment). Originally developed by Ellis and Delbrück (1939), the one-step growth experiment measures the latent period and the burst size of a given phage on a given host (e.g., Adams 1959; Carlson 2005). Latent period and burst size are essential parameters in a description of phage properties and varies between phages and hosts and also with host growth conditions. The latent period is the minimum length of time it takes from adsorption of the phages to a host cell to lysis of the host with release of progeny viruses (Fig. 1). The burst size is the average number of phages released per infected host cell. The one-step experiment can be adapted to test the effects of different environmental factors on the infection process. For example, the burst size can be affected by the growth rate of the host (e.g., Middelboe 2000); they are expected to be higher when the host cell is nutrient replete and growing exponentially while one might expect a decrease in burst size if the host cells are under nutrient limitation.

To limit the phage–host interaction in the experiment to a single infection cycle, phages and hosts have to be mixed in the right ratio. Prior to experiment, it is therefore necessary to determine the titer of the phage stock and to know the relation between cell density and optical density (i.e., obtain corresponding numbers of cells mL^{-1} and OD) of the host. Infection should be done at low MOI (multiplicity of infection = ratio of phage to host) e.g., between 0.1 and 0.01. At higher MOI, the probability of cells infected by more than one phage would increase and the total estimate of infected cells becomes less than the phage input.

1. 200 µL overnight culture is inoculated in 100 mL culture flask with 50 mL growth medium (e.g., LB), and incubated on a shaking table until the density in the culture has reached cell density of ~5 × 10^8 CFU mL^{-1} (corresponding to an OD_{525} of ~0.3). This may take from a few hours to a day.
2. 1 mL aliquots of the bacterial culture are mixed with subsamples of the phage stock in triplicate microfuge tubes at an multiplicity of infection (MOI) of approximately 0.01 (i.e., ~ 5 × 10^8 CFU mL^{-1} and 5 × 10^6 PFU mL^{-1} (final concentrations), and incubated for 10 min to allow the phages to adsorb to the host cells. At this point, the infection cycle of the adsorbed phages is assumed to begin, which marks the initiation of the experiment (T = 0)
3. Centrifuge the cells (6000*g*, 10 min).
4. Remove the supernatant (removes unadsorbed viruses) and resuspend the pellet in 1 mL growth medium (e.g., LB).
5. Repeat step 3 and 4 to wash out any further unadsorbed phages.
6. Transfer 50 µL of the resuspended culture (bacteria and adsorbed phages) to 50 mL growth medium in a 100 mL culture flask and mix well. Assuming that most of the phages have adsorbed to host cells during step 2, the concentration of adsorbed phages in the 50-mL flask is ~5 × 10^3 PFU mL^{-1}.
7. Transfer 1 mL to a microfuge tube (note the time) and incubate the triplicate 50 mL cultures on a shaking table.
8. Determine the number of PFU (total infectious centers) by plaque assay in the collected sample (*see* below).
9. Continue to collect samples for PFU over time for 6–8 hours.

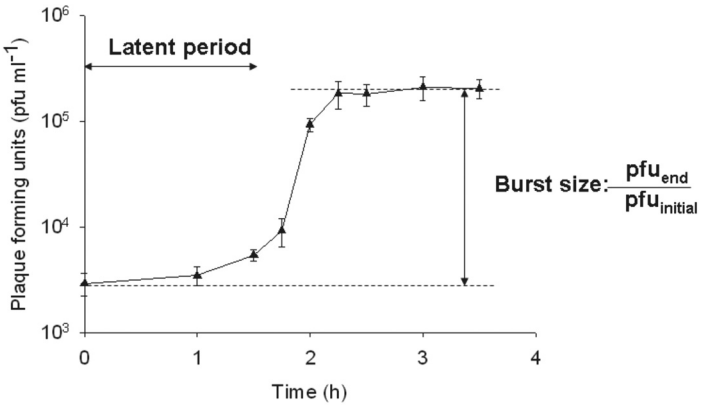

Fig. 1. An example of the development in the number of plaque-forming units during a one-step growth experiment with a bacteriophage, and the definition of the parameters "Latent period" and "Burst size."

It is recommended to carry out a preliminary experiment with just a few time points over a large time span (6–8 h), to get a first idea of time between adsorption and burst. This experiment should then be followed by a more detailed experiment with more frequent samplings (every 10–20 min) around the time when the burst is expected. A successful one-step growth experiment shows a period of constant virus abundance, which reflect the period from when the cell is infected and until mature phages are released. The latent period (Fig. 1) is followed by a single burst of phages from which the burst size can be calculated as the ratio between the number of phages before and after the burst (Fig. 1). The highly dilute bacterial culture reduces contact rate between the virus and host so that no re-infection will occur during the one-step experiment.

One step growth experiments are often difficult to get to work for new phage host systems and adjustments to the standard procedure (e.g., the number of added phages, length of the experiment, sampling frequency, etc.) are often required depending on host growth rate, phage adsorption rate, infection efficiency, etc.

Isolation of cyanophages—

Isolation of cyanophages by liquid bioassays: As mentioned earlier, there are some basic similarities between the isolation procedures for bacteriophages and cyanophages. However, the specific protocols for isolation of cyanophages differ substantially from the procedures presented in the previous section and are described in detail in the following sections. Figure 2 shows possible strategies one could follow, depending on the suspected titer of cyanophages in the sample of interest. These assays assume that the target cells are unialgal and clonal. If cultures are not unialgal or clonal, complete lysis of the culture may not occur or plaques could be obscured by contaminating bacteria. Axenic cultures of the host are preferred for the plaque assay, but not necessary for liquid assays. Many strains of marine cyanobacteria can be purchased from culture collections. Alternatively, new hosts can be isolated from the natural environment of interest. However, it can take a lot of time and effort to produce clonal cultures. As well, many cyanobacteria do not grow well on solid substrate. Unless the target host of interest is already cultured on solid substrate, the simplest method for isolation of novel cyanophages would be via the liquid bioassay. It is simple, inexpensive, and the host need not be axenic.

In principle, a small volume of a water sample is added to the host culture and monitored over time for signs of infection. The treated cultures are compared with control cultures by eye for obvious signs of viral infection such as total lysis (clearing) of the culture, decrease or change in overall pigmentation of the culture, or clumping and settling of cells to the bottom of the culture vessel. This approach has been used to isolate and detect cyanophages from seawater as well as marine sediment samples (Suttle and Chan 1993; Waterbury and Valois 1993; Wilson et al. 1993; Suttle 2000). Multiwell plates (e.g., Corning brand 24- or 96-well polystyrene plates with lids) are the culture vessel of choice. They are conducive to the screening of many samples, require minimal culture volumes, and take up little incubator space. Glass culture tubes with screw caps (e.g., 13-mm or 25-mm diameter) may be substituted and are useful for screening larger sample volumes. These glass tubes allow nondestructive monitoring of the in vivo chlorophyll fluorescence of the cultures using a fluorometer (e.g., Turner Designs TD700) or similar. Any lysis of the cells would result in a decrease in relative fluorescence (rf) compared with the control cultures.

Liquid assays can be used for all aquatic cyanobacteria and for screening all types of samples, including sediments, and host cells need not be axenic. Samples to be tested are not subjected to the possibility of elevated temperatures encountered when using plaque assays.

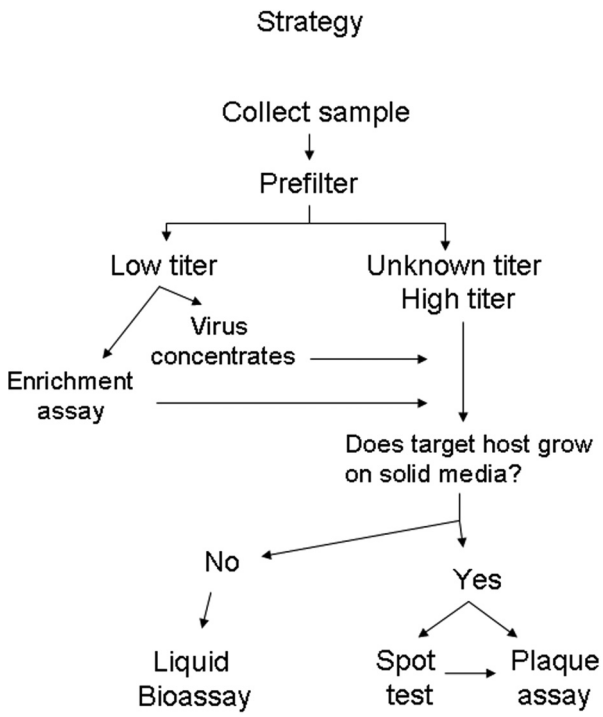

Fig. 2. A flow chart suggesting various strategies of cyanophage isolation depending on type of sample and host characteristics.

Multiwell plates are convenient for isolating cyanophages from environmental samples using the liquid bioassay approach (Table 1). Below is a typical procedure (96-well assay) used to detect and isolate cyanophages from marine samples that lyses *Synechococcus* sp. strain DC2 (also known as CCMP1334 or WH7803). Using this method, greater than 10^5 lytic phages per milliliter of seawater have been detected in the Gulf of Mexico (Suttle and Chan 1994) that lyses this permissive target host:

1. Collect ca. 50 mL seawater sample in a clean, acid-washed plastic (HDPE, PP, or PC) container or sterile Falcon tube.
2. Rinse container 3 times with the sample before filling (if not filtering right away, keep sample cold and in the dark).
3. Remove phytoplankton and bacteria from the water sample by filtration.
   i. Glass fiber filter (e.g., GC50, Advantec); this step may help to reduce premature clogging of the next filter.
   ii. Followed by 0.2 µm or 0.45 µm PVDF filter (e.g., Millipore Durapore filters). As for the bacteriophage isolation (above), this filtration step may also remove some of the larger viral particles.
4. Store filtered seawater sample in the dark at 4°C (or on ice) until use.
5. Have ready, a culture of host cells in exponential growth (approximately 10^6 cells/mL).

Table 1. Suggested volumes of target cells and sample to use for the liquid bioassay.

Culture vessel	Cell volume	Sample volume	Max. total volume
Plate, 96-wells	200–250 µL	50–100 µL	300 µL
Plate, 24-wells	2.5–3 mL	0.2–0.5 mL	3.5 mL
Tube, 13 × 100 mm	3.5–4 mL	0.5–1 mL	4.5 mL
Tube, 25 × 150 mm	30–35 mL	2–10 mL	40 mL

6. Dilute cells ca. 10-fold with sterile media such as F/2 media (Guillard 1975) (to about 1×10^5 cells/mL).
7. Allow a minimum of 30 mL host cells for every 96-well plate.
8. Using a multichannel pipette, aliquot cells into wells, cover plate with the lid and set aside in the incubator (*see* Table 1 for suggested volumes).
9. *Warning*: make sure that the total volume of host and sample does not exceed the maximum capacity of the wells. There should be ca. 1–2 mm clearance from the top; excessive volume would cause overflow of the contents and subsequent cross-contamination of the wells.
10. Prepare 10-fold serial dilutions of the seawater sample (e.g., 0.5 mL sample added to 4.5 mL media in a 15-mL Falcon tube, up to 5 dilution levels) using sterile media as the diluents. Note: triplicate dilutions series are recommended for determining the titer of lytic cyanophages in the sample.
11. Add diluted samples to wells: for example, 50 µL to 16 wells (2 rows of 8 wells each) for each dilution level; with 5 dilution levels, that would leave 2 rows for the negative controls if using 96-well plates.
12. Replace the seawater sample with same volume of media for negative controls.
13. Cover plate with lid; carefully seal the lid to the plate using either parafilm or thin strips of plastic film.
14. Incubate plates at ca. 25°C, between 10 to 25 µmol quanta $m^{-2}s^{-1}$
15. Compare color development in the wells with the control wells; clear wells can be discerned from pigmented wells in 4 to 7 d.
16. Monitor wells daily for signs of lysis for 10 to 14 d (could be longer for slow growing host cells).
17. Choose a clear well from the highest dilution.
18. Transfer the lysate to a microtube, centrifuge the lysate for 5 min ca. 12,000*g* to pellet cell debris.
19. Store the supernatant (about 250 µL) at 4°C and use it for further rounds of purification (via liquid or plaque assay).

Tips and Tricks:

1. Use neutral density screens (gray or black window screening) to attenuate the light. Low light levels enhance development of pigments, which allows for easier discrimination of lysed versus unlysed cultures in the wells.
2. Condensation forming on lids can occur due to temperature changes in drafty incubators. Excessive condensation can make it difficult to visualize the wells. Sandwich the full plates between a layer of empty plates to insulate the cultures from temperature shifts.
3. Sealing the lid to the bottom of the plate helps to slow down evaporation of well contents, particularly the ones located at the plate perimeter.

Isolation of cyanophages by liquid enrichment assay: If low titers are expected, the viruses in the sample can be concentrated via TFF to make a virus concentrate (VC) (Suttle et al. 1991; Wommack et al. 2010, this volume). To increase the detection limit, several different VCs can be combined and added to the same culture. Another convenient way to screen larger sample volumes is to perform liquid enrichment cultures (Suttle 1993). By this approach, larger volumes of water samples can be screened for cyanophages, thus enabling detection of "rare" viruses. Similar to the use of enrichment cultures for bacteriophages, the disadvantages include the fact that lysis of the host cells are not always obvious, especially if the initial titer is low or if the host culture is not clonal or unialgal. Also, more steps are required to dilute out nonreplicating viruses to obtain pure clonal isolates. Because it is an end-point dilution assay, only the most abundant phages will be isolated. The principle is the same as for the isolation of bacteriophages mentioned earlier except that the sample volumes screened are in the order of liters instead of milliliters. Here is a typical procedure for cyanophage liquid enrichment cultures.

1. Prefilter at least 3 L water sample through a glass fiber filter (e.g., Advantec type GC50, Whatman type GF/C, or Gelman type A/E), followed by a 0.2 µm or 0.45 µm low protein binding PVDF filter (e.g., Millipore Durapore filter).
2. Dispense the filtered water samples (e.g., 0.5 L or more) into culture vessels, e.g., 1 L or larger Erlenmeyer flasks.
3. Add nutrients to the filtered water to support growth of the target cells. For example, F/2 nutrients for marine cyanobacteria or BG-11 nutrients (Rippka et al. 1979) for freshwater cyanobacteria.
4. Seed the filtered water with 1% to 10% v/v of host culture. Target cells must be in exponential growth to avoid loss of potential viral infection (e.g., viruses adsorbed to dead or dying cells will not cause infection and subsequent production of progeny virus).
5. As a control, replace the filtered environmental sample with virus-free (0.02 µm filtered or heat-killed) water sample. The volume of this culture need not be as large as the experimental flasks. This control is to make sure that there is not anything in the water sample that would inhibit growth of the target cells (e.g., chemical inhibition).
6. Incubate the flasks at the temperature and light conditions appropriate for the cyanobacteria and look for signs of lysis. This could take 2 to 3 weeks, depending on the growth rate of the host as well as the initial titer of cyanophages. It is recommended that the in vivo chlorophyll fluorescence be monitored regularly. A small

decrease in relative fluorescence could indicate the presence of a lytic virus.
7. Remove an aliquot of the enrichment culture, and pellet remaining cells by centrifugation (e.g., 20 minutes at 6000*g*).
8. Filter the supernatant through a 0.22 µm or 0.45 µm PVDF filter, and store the lysate at 4°C until further analysis.
9. Verify the presence of lytic phages by liquid assay (or plaque assay).

To propagate/amplify the lytic agent, the liquid bioassay is repeated using the putative lytic agent as the test sample:
1. Set up bioassay using 5 mL or larger culture tubes in triplicates. Add between 5 to 50 µL of each sample below to target cells in log phase.
   a. Whole lysate (unfiltered)
   b. Filtered lysate (0.22 or 0.45 µm)
   c. Negative control (no addition, or use filtered media)
2. Monitor in vivo chlorophyll fluorescence for about 1 week, look for decrease in relative fluorescence compared with control cultures.
3. If the cultures lyse, then the lytic agent is most probably a virus.
4. Propagate the lytic agent several times to dilute out non-replicating viruses.
5. Filter the lysate and use it to obtain pure clonal stocks (*see* below).

Isolation of cyanophages by plaque assays: More than 40 years ago, Safferman and Morris (1963) used the plaque assay method to isolate the first cyanophage that infects a freshwater filamentous cyanobacterium, *Plectonema boryanum*. Since then, this approach has been used successfully to detect and isolate a number of different phages infecting marine *Synechoccocus* and *Prochlorococcus* (e.g., Suttle and Chan 1993; Waterbury and Valois 1993; Wilson et al. 1993; Sullivan et al. 2003).

The advantages of this method are that results are easily interpreted as plaques formed on pigmented lawns can be easily identified. Since a plaque is the result of a single infection event, the virus can be easily purified and cloned. The disadvantages include the following: The target cells must be able to grow on solid media; small bacteria that pass through the filter can interfere with lawn formation of slow growing cyanobacteria while some bacteria can also cause plaques on cyanobacteria lawns. The higher temperature of the molten soft agar can inhibit or destroy temperature sensitive viruses or inhibit growth of the host, and the sample volume that can be tested is limited (<1 mL). The first part of the procedures described as follows: (1) preparation of base plates and (2) preparation of top agar/agarose are applicable also for bacteriophage plaque assays.
1. Prepare base plates: For example, purified agar or agarose (1% w/v) is added to your media of choice and autoclaved. This will provide a support base for the top agar/agarose overlay as well as nutrients for the host cells. For best results, use plates within 1 week of pouring.
   a. Add 5 g purified agar or agarose to 500 mL culture media in a 1-L Erlenmeyer or media bottle.
   b. Gently stir to disperse the agar/agarose.
   c. Autoclave for 20 to 25 min to sterilize.
   d. When cooled to about 60°C, dispense 15 to 20 mL per plate.
   e. To reduce condensation forming on the insides of the lids, leave lids slightly ajar to allow escape of steam or stack the plates immediately after pouring
   f. Invert plates once the agar has solidified to prevent condensation from dripping onto the surface of the agar.
   g. Plates can be used about 12 h after pouring if the agar surface is not wet; a longer time is needed if conditions are humid.
   h. *Warning*: If the surface of the bottom agar is too moist, the top agar/agarose will not stick to the bottom plate and will slide off when the plate is inverted.
   i. Tip: plates can be fast-tracked: dry plates at 37°C; leave lids slightly ajar; monitor closely to prevent over drying.

Considerations: Depending on the composition of the media used, the addition of solidification agents (in particular the combination of high salinity seawater-based media and common agar such as Bacto Agar) can often result in the formation of precipitates when autoclaved together. These "flocks" can sometimes interfere with interpretation of the plaque assay. Moreover, impurities in common agar can negatively affect the growth of the host cells. Here are some suggestions on how to reduce the formation of these precipitates. Some testing may be required to determine the best combination to use for your particular situation.
   a. Do not use common agar; rule of thumb—the whiter the agar, the "cleaner" it is.
   b. Use commercially available purified agar or agarose; or clean common agar using a washing procedure such as the one outlined in Waterbury and Willey (1989).
   c. Reduce the salinity of seawater media with purified water; e.g., to 20–25 psu.
   d. Add purified agar or agarose to autoclaved media aseptically and then melt the agar/agarose in the microwave (bring to a short boil 2–3 times to completely dissolve the agar/agarose).
   e. For cells that will grow in artificial media, prepare media and gelling agent at 2× concentration and autoclave separately. When cooled to ca. 60°C, gently mix the gelling agent into the media and dispense immediately.
   f. In the case of artificial media, add agar/agarose to filter-sterilized media and melt the gelling agent in the microwave
2. Prepare top agar/agarose: Prepare 100 mL portions of 0.4 to 0.5% (w/v) of purified agar, agarose or low-melting point (LMP) agarose (i.e., Invitrogen #15517-022) in your media of choice. Although LMP agarose can be quite expensive, it is recommended for temperature sensitive samples and

cells, since it solidifies at ca. 25°C. Purified agars, as well as low-melting point agars and agaroses are available for a range of lower temperatures (consult the following websites for more details: www.sigmaaldrich.com and www.invitrogen.com)

a. Autoclave or microwave sterilize on the day of the assay.
b. Dispense 2.5 to 3 mL into 13-×-100–mm glass disposable culture tubes (Fisher Scientific #1496127).
c. Transfer tubes to a water bath or dry heat block set at the appropriate temperature, (e.g., ca. 30 to 32°C for LMP agarose, ca. 40–42°C for purified agar or agarose).
d. Cover tubes with foil or cap, allow for temperature to equilibrate.
e. For each water sample, prepare triplicate tubes; control tubes containing cells are only used to monitor lawn growth.
f. For best results (smooth lump-free top agar), use freshly prepared top agar/agarose since repeated re-melting of solidified agar/agarose can give inferior results.

3. Prepare target (indicator) cells: Grow the cyanobacteria in liquid media, harvest in exponential growth and adjust cell density to about 10^7 to 10^8 cells/mL. If necessary, cells can be concentrated by gentle centrifugation and resuspended in media. Preliminary testing may be required to determine the best cell density to use for your particular host organism. The objective is to start with a lawn of cells that will have the capacity for additional growth during the length of the assay. Depending on the growth rate of the target cells, one can expect plaques to appear on the lawn as early as 3 to 4 d to weeks after infection. The initial lawn of cells will be very faint in color. However, the lawn will develop into an evenly distributed dense layer of cells within 7 to 10 d. If the lawn is too thin, plaques will go undetected. If the lawn is too thick, the cells could run out of nutrients prematurely which may result in poorly developed plaques.
4. Prepare the sample: Environmental samples should be prefiltered as described earlier. If high titers are expected, serial dilutions of the sample may need to be performed.
5. The assay:
   a. Adsorb 50 to 100 µL sample (as is, and 10-fold serial dilutions, up to ca. 3 levels) to 0.5 mL target cells under the usual culturing conditions (e.g., for *Synechococcus* sp. strain DC2, constant 5–25 µmol quanta $m^{-2}s^{-1}$, at 25°C), agitate occasionally to encourage adsorption of phage to host.
   b. After 1 h, transfer virus: host mixture to 2.5 mL soft agar. Quickly and gently vortex the mixture and pour the entire tube contents onto the surface of the agar plate. Working rapidly, gently rock and swirl the plate to spread the mixture evenly onto the plate surface before the agar starts to gel. Set aside on a flat surface to harden (about 1 h). For best results, the total volume of cells + virus + soft agar is between 3 to 4 mL. Larger volumes would make it easier to pour, but is not recommended as the top layer would be too thick, and plaques could form on top of one another.
   c. Prepare a control plate containing only cells; this plate will allow you to monitor cell growth.
   d. Seal plate with parafilm, flip plates upside down. Incubation of plates under constant low light conditions (5 to 25 µmol quanta $m^{-2}s^{-1}$) will produce darker lawns thus allowing for easier detection of plaques. Plaques will appear within 1 to 2 weeks, depending on the growth rate of the host cells.
   e. Note the number of plaque forming units (PFUs), plaque size, and morphology.
   f. Choose a well-isolated plaque on a plate that contains less than 100 PFUs.
   g. Harvest the plaque using a Pasteur pipette: gently press the tip of the pipette into the plaque to the bottom agar; using gentle suction, remove the plug.
   h. Transfer the plug to 1 mL media and vortex briefly to break it up.
   i. Place the tube at 4°C and allow the phages particle to elute from the plug overnight to form a plaque lysate.
   j. Vortex and centrifuge the sample (ca. 12,000*g* for 10 min) to pellet cyanobacteria and agar.
   k. Transfer the supernatant to a new tube; typical titer of the plaque lysate can be 10^4 to 10^5 PFU mL^{-1}.
   l. Repeat steps f to k for a minimum of 3 plaques. Choosing plaques with different morphologies may result in the isolation of different phages.

Obtaining pure cyanophage stocks (liquid assay):

1. Determine the titer of the lysate using the 96-well assay as described earlier.
   a. Prepare end-point dilution series (10-fold serial dilutions, 5 to 6 levels).
   b. Monitor plates for lysis every few days, recording the number and position of clear wells on the plate.
   c. When clear or nearly clear wells no longer appear for 1 week, record the final "score" for each dilution level.
   d. Use the MPN Assay Analyser program (Passmore et al. 2000) to determine the most-probable-number (Taylor 1962) of infective phages in the lysate.
2. Once the cyanophage titer is determined for the stock tube, proceed to purify a clonal virus:
   a. Use 13-×-100–mm culture tubes (or 24-well plates).
   b. Prepare exponentially growing target cells (e.g., >100 mL).
   c. Dilute some of the titered lysate to 1 infective virus/mL.
   d. Add 0.2 mL (0.2 infectious units) to each of 20 tubes of susceptible host cells.
   e. Monitor tubes for 2 to 3 weeks.
   f. Cultures in which lysis occurs are assumed to be the result of a single-virus infection; the probability that more than 1 infective unit occurred in a given culture is 0.0176.
   g. If lysis occurs in 4 tubes or less of 20, it is assumed that

lysis in each tube was caused by one infectious unit, therefore each tube would contain a separate phage clone.
   h. Propagate an aliquot from all the tubes to confirm the results.
   i. If lysis occurs in more than 4 tubes, repeat the clone out procedure by reducing the volume of diluted lysate added to the 20 tubes (e.g., add 0.1 mL instead of 0.2 mL)
   j. Scale up each phage clone to make primary phage stocks; e.g., add 5µL of the lysate to 40 mL of cells
   k. Centrifuge, filter, and titer the stock, store at 4°C in the dark.

Obtaining pure cyanophage stocks (plaque purification):

1. Make a dilution series of the lysate (assume 10^4 to 10^5 PFU per mL in the plaque lysate), and use this to perform a second round of plaque assays (steps 1 to 11) to purify the phage.
2. Repeat the plaque purification procedure 2 more times to ensure that the cyanophage isolated is clonal.
3. Finally, prepare a primary cyanophage stock using lysate from the final purification via one of the following methods:
   i. Liquid amplification: add some of the lysate to target host in liquid culture. After the culture has lysed, remove cell debris via centrifugation, filter sterilize the stock, and store at 4°C until further analysis.
   ii. Plate amplification: Prepare plaque assays (*see* above) with a dilution series of lysate from the final purification. Plates with confluent lysis of the host lawn (typically ca. 10^4 PFUs) can then be used to obtain cyanophage stocks by elution of phages from the plates. Add 5 mL sterile seawater to the plate, scrape off the top agar layer into the seawater, and leave at 4°C overnight. Remove agar and cell debris by centrifugation, filter sterilize the stock and store at 4°C until further analysis.
4. Titer the final stock via plaque assay.
5. Cyanophage stocks stored at 4°C in the dark are stable for at least a year.

Life cycle characterization of cyanophages—

Adsorption of phage to cyanobacteria: The first step in the life cycle of a virus is adsorption to host cells. If the virus does not adsorb to a viable host, infection will not take place. Where bacteriophage adsorption rates are often in the order of minutes, cyanophages usually adsorb to their host cells at a much slower rate; and not all contacts result in an infection. Suttle and Chan (1993) found that it took 45 min for 80% of cyanophage BBC1-P1 to be adsorbed to its host. Some factors that can affect adsorption rates are (1) host abundance (a minimum of 10^4 cells per mL are needed to have a sufficiently high rate of adsorption); (2) physiological state of the host (which could affect the availability of receptor sites); (3) physical environment (temperature, viscosity); (4) chemical environment (ions, salts, co-factors); (5) light (adsorption rate is light dependent in some species (Clokie et al. 2006); and (6) host strain. If every contact results in an infection, this would have tremendous ecological impact. Understanding how these factors affect the rate of virus adsorption would help one design better experiments, interpret data, and construct better models for virus–host cell relationships.

Several methods have been presented by Adams (1959) to determine adsorption kinetics of bacteriophages. One way to measure the adsorption efficiency of cyanophages is to assay for free unadsorbed phages (Suttle and Chan 1993). The principle of this assay is to add a known quantity of viruses to host cells (e.g., at an MOI = 0.01 to 0.1). Over a period of 1 to 2 h, small subsamples of the virus:host solution are removed ca. every 15 min and diluted 100-fold to stop further adsorption. The host is then separated from free viruses by centrifugation, and the number of free viruses remaining in the supernatant determined by plaque or end-point dilution assays. A plot of the abundance of free viruses remaining in solution as a function of time should produce a straight line. The slope of this line is then used to calculate the adsorption rate.

Under constant environmental and cultural conditions of the host cell, the rate of adsorption can be described using the following equation:

$$K = 2.3/(B)t \times \log (p_0/p)$$

where B = concentration of cyanobacteria (cells mL^{-1}), p_0 = phage assayed at time zero, p = phage not adsorbed at time t (min), K is the velocity constant (ml min^{-1}).

Because of the short sampling intervals, it is very important to have everything ready before you begin. Having media equilibrated to the appropriate temperature, pre-labeled agar plates and tubes, tables to record the time etc will facilitate acquisition of better data.

This procedure can be adapted for other host–virus systems. The example given is for host BBC1 and cyanophage BBC1-P1 (Suttle and Chan 1993).

1. Have everything ready to perform plaque assay (allow for 8 time points, in triplicate).
   a. Plating cells, aliquoted, and set aside.
   b. Bottom plates labeled (24 + plates).
   c. Top agar aliquoted and temperature equilibrated.
   d. Dilution tubes—these contain 1.5 mL media, labeled and kept on ice.
   e. Cyanophage stock diluted into 1–5 mL media.
2. Set up a table to record times such as Table 2.
3. Set up adsorption cultures (e.g., 250 mL polycarbonate Erlenmeyer flasks with screw cap). Cyanobacteria should be in exponential growth, e.g., for BBC1, about 10^6 to 10^7 cells/mL (The actual numbers should be predetermined by microscopy).
4. Fill flask with 100 mL host cells.
5. Add cyanophage stock of known titer to host at an MOI of ca. 0.01 and quickly mix to disperse the virus. For example, for a host concentration of 1×10^7 cells mL^{-1} (i.e., total

Table 2. Suggested tabler for recording sampling times and details for adsorption kinetics experiments.

Time point (min)	Replicate	Time: subsampled from tube	Time: centrifuged	Time: tube placed on ice	Time: added to plating cells	Volume titered	Results: # of PFUs	Average # of PFUs
T=0	A							
	B							
	C							
T=15	A							
	B							
	C							
Etc.								

number in 100 mL = 10^9 cells), a total of 10^7 infectious viruses is needed to achieve an MOI = 0.01. Thus, if the virus stock is 1×10^9 infectious units mL^{-1}, add 0.01 mL to the host culture. If the virus stock is highly concentrated, we recommend diluting the virus into a larger volume before adding to the host cells. This will enhance rapid dispersal of the viruses.

6. Immediately remove a subsample and dilute 100× for time zero: Transfer 15 µL to a tube containing 1.5 mL of ice cold media, vortex to mix, pellet host for 5 min at ca. 16,000*g* and 4°C; note the time.
7. Carefully remove a small aliquot (50 µL) of the supernatant to a new tube and keep cold for plaque assay; note time.
8. Place adsorption cultures under usual conditions (e.g., light and temp)
9. Repeat sampling at 15 min intervals for 1 to 1.5 h.
10. Determine the concentration of viruses remaining in the supernatant for each time point by plaque assay.

One-step growth experiments (cyanophages): The procedure is similar to that for bacteriophages with one major difference. Where bacteriophage growth is measurable in the order of minutes, cyanophage growth curves are measured in terms of hours. The burst sizes are similar, being in the tens to hundreds.

As for bacteriophages, infection should be done at a MOI between 0.1 and 0.01. In this instance, the total infective center = the phage input because the proportion of multiply-infected host cells is small. At higher MOI, the probability of cells infected by more than one virus would increase and the total infective centers (TIC = total number of infected cells + free viruses) become less than the phage input.

Described below is the procedure used to perform a one-step growth curve for cyanophage BBC1-P1 via plaque assay on *Synechococcus* sp. BBC1 (Suttle and Chan 1993).

Procedure—

1. Set up triplicate adsorption tubes (AT) in 1.5 mL microtubes: Add phages of a known titer to the cyanobacteria host in exponential growth (cell concentration determined by microscopy; $Cyano_{input}$) at an MOI of approximately 0.02 (e.g., 0.9 mL hosts [1×10^7 cells mL^{-1}] + 0.1mL phages [2×10^6 PFUs mL^{-1}] = MOI of ~0.02).
2. Allow the phages to adsorb to the hosts for 60 min. at room temperature and an illumination of 25µmol quanta $m^{-2}s^{-1}$. Flick tubes a couple of times at 30 min.
3. Set up control tubes (CT) as above, except
   a. positive controls: replace host cells with media (this gives input phage numbers).
   b. negative controls: omit virus.
4. After 60 min, remove unadsorbed phages (T = 0).
   a. centrifuge briefly to pellet host cells (e.g., 5 min, 16,000*g*, 4°C).
   b. remove supernatant, resuspend cells in fresh media.
   c. repeat washing step.
   d. assay washes to determine the number of unadsorbed phages (Free $Phage_{T=0}$) for calculation of efficiency of adsorption.
5. Prepare nine 15-mL centrifuge tubes containing 10 mL algal growth media (6 labeled "FGT" [first growth tube] and 3 labeled "SGT" [secondary growth tube]).
6. Add 100 µL from AT to FGT (10^{-2} dilution). Mix and remove 0.1 mL sample for T = 0. Perform plaque assay immediately to determine total infective centers ($TIC_{T=0}$).
7. Add 100 µL from FGT to SGT (10^{-4} dilution).
8. Incubate FGT and SGT tubes at room temperature and ca. 25 µmol quanta $m^{-2}s^{-1}$.
9. Remove 100 µL samples every 3 h from FGT (at 3, 6, 9, 12, 16 h) and SGT (from 12 h onwards) for ca. 30 h and determine the TIC via plaque assay.
10. Add 100 µL from positive control tube (CT) to FGT-control (10^{-2} dilution) and determine the total phage input ($Phage_{input}$).

The dilution factor in going from the adsorption tube to FGT is chosen so that a reasonable number of plaques (ca. 50-200) will form on the plates during the latent period. The dilution factor in going from the FGT (first growth tube) to SGT (second growth tube) is chosen on the basis of the expected increase in plaques at the end of the rise period so that later platings of SGT samples will also yield countable numbers. As mentioned in the bacteriophage section, it is recommended to

perform a preliminary one-step experiment to estimate the possible length of the latent period and burst size. Then repeat the experiment with more frequent sampling with adjustments to the dilution factors to gain more precision. Sampling times may need to be adjusted for each phage–host system.

In summary:

1. Determine total phage input ($Phage_{input}$) and total cyanobacteria input ($Cyano_{input}$).
2. At T = 0 determine the titer of unadsorbed phages (Free $Phage_{T=0}$) and total infective centers ($TIC_{T=0}$ = initial total infected cells).
3. During the latent period and rise, determine the titer of total infective centers (TIC = total infected cells + any phages released).
4. At the end determine the titer of total progeny (TIC_{final}).

Calculations.

Adsorbed phages = $Phage_{input}$ – Free $Phage_{T=0}$

Percent adsorption = Adsorbed phages/ $Phage_{input}$ × 100

Average MOI = Adsorbed phages/$Cyano_{input}$

Burst size = ($TIC_{final}/TIC_{T=0}$)

Assessment

Detection limit of the methods—In principle, the presence of a single infective virus in an enrichment culture or in a sample that is spotted on a bacterial or cyanobacterial lawn should be detected by the proposed methods, as a single infection theoretically is sufficient to initiate propagation of a given virus, which could then be isolated. However, not all infections are successful, and infectivity depends on a suite of conditions, such as the encounter rate between viruses and their hosts, the adsorption rate, host susceptibility, host growth conditions, virus decay rates etc; all factors which may determine whether a given virus will propagate in a given host community. Moreover, there exists, to our knowledge, no systematic study on the efficiency and detection limit of the current methods for isolating environmental phages.

We have done a series of experiments to evaluate the efficiency of the spot assay and the enrichment culture approach in detecting low densities of specific bacteriophages in a sample. Obviously, the enrichment approach has the advantage of the ability to screen a large volume of sample for viruses, but here we also wanted to test whether there are systematic differences in the two methods' ability to detect the presence of a given amount of viruses spotted to a lawn or added to an enrichment culture, respectively.

A dilution series of a stock of the specific phage (Vir#12) infecting the marine *Cellulophaga* sp group (Holmfeldt et al. 2007) was performed and the number of infectious units was determined in triplicate in each dilution by both plaque assay and spot assay on lawns of *Cellulophaga* sp. #12. The number of PFU in the Vir#12 dilutions ranged from $2.9 \times 10^4 \pm 1.4 \times 10^3$ PFU mL^{-1} (10^{-2} dilution) to $30 \pm 60 \times 10^2$ PFU mL^{-1} (10^{-5} dilution) obtained in the plaque assay and from $9.3 \times 10^2 \pm 3.5 \times 10^2$ (10^{-3} dilution) to no clearing observed (10^{-5} dilution) in the spot assay (Table 3). From these dilutions, a series of dilution culture experiments were established in duplicate 50 mL batch cultures with MLB medium inoculated with 10 µL of each of the dilutions (Table 3), as well as a positive control with undiluted virus stock and a negative control without addition of viruses. This corresponded to the addition of a range of infectious units from 0 PFU (negative control) to ~300 PFU (10^{-2} dilution) and ~30,000 PFU (positive control), corresponding to initial phage concentrations in the enrichment cultures of 0 to 6 PFU mL^{-1} (Table 3).

Two sets of experiments were set up in duplicate: In Experiment 1, the phage dilutions were added together with 1.5 mL of an overnight culture of the host bacterium *Cellulophaga* #12 (originally used to isolate Vir#12), corresponding to a start cell density of approximately 1.5×10^7 cells mL^{-1}. In Experiment 2, the phage dilutions were added to a mixture of 3 *Cellulophaga* sp strains in equal densities (*Cellulophaga* #12, *Cellulophaga* #3, and *Cellulophaga* NN16038). As for Experiment 1, the initial total bacterial density was 1.5×10^7 cells mL^{-1}, however the two new strains had reduced susceptibility to Vir#12 relative to *Cellulophaga* #12 (not shown). In Experiment 1, 10 µL undiluted Vir#12 stock (approximately 30,000 PFU) was added to another culture of the host bacterium as a positive control to be certain to see the effect of viral addition on OD measurements.

Samples were collected every 3-8 h during the 47-h incubation for OD measurements and for detection of phages by spot assay. For the spot assay, 10 µL sample was spotted on a lawn of *Cellulophaga* #12, and incubated for at least 24 hours for detection of a clearing zone.

The results from the spot assay showed that in Experiment 1, which contained only the most susceptible host, phages were propagating relatively fast. Here phages reached detectable numbers already after 3 h in cultures with an initial phage concentration of 6 PFU mL^{-1} and after 11 h with initial concentrations of 0.6 and 0.06 PFU mL^{-1} (Fig. 3). This means that the phage concentration had increased from ~6×10^{-2} PFU mL^{-1} to > 100 PFU mL^{-1} (i.e., corresponding to >1 phage in the 10 µL spotted) in 11 h in the culture where 3 phages had been added initially (Fig. 3). Addition of 30,000 PFU (the positive control) had a significant controlling effect on the bacteria, and after 9 h, no net increase in OD was observed in the culture (Fig. 4). In Experiment 2 with a combination of hosts with variable susceptibility to the phage, spot detection was first observed in cultures with initial phage concentrations of 0.6 and 0.06 PFU mL^{-1} after 17 h incubation (Fig. 3). In neither experiment did an initial phage density of 0.006 PFU mL^{-1} result in any systematic phage production, and consequently, the phage did not build up significant populations in these cultures during the incubation.

Overall, the data showed that a concentration of approximately 0.06 phages mL^{-1}, corresponding to the presence of 3 phages in enrichment cultures with potential host cells was sufficient to detect the phage by the enrichment assay, and

Table 3. The number of infectious units obtained in a dilution series of a specific phage (Vir#12) by plaque assay and spot test, respectively. From these dilutions, 10 L were added to a series of dilution culture experiments in duplicate 50 mL batch cultures with the host strain (Cellulophaga #12) corresponding to the addition of a range of infectious units from 0 PFU (negative control) to ~300 PFU. Thus, the initial phage concentrations in the enrichment cultures ranged from 0 to 6 PFU mL^{-1} and a positive control containing 600 PFU mL^{-1}.

	Number of infectious phages (PFU mL^{-1})					
Dilutions	**10^0 (Positive control)**	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**Negative control**
Plaque assay		2.9×10^4	2.7×10^3	280	30	0
Spot assay			9.3×10^2	70	0	0
Initial phage concentration in enrichment cultures	600	6	0.6	0.06	0.006	0

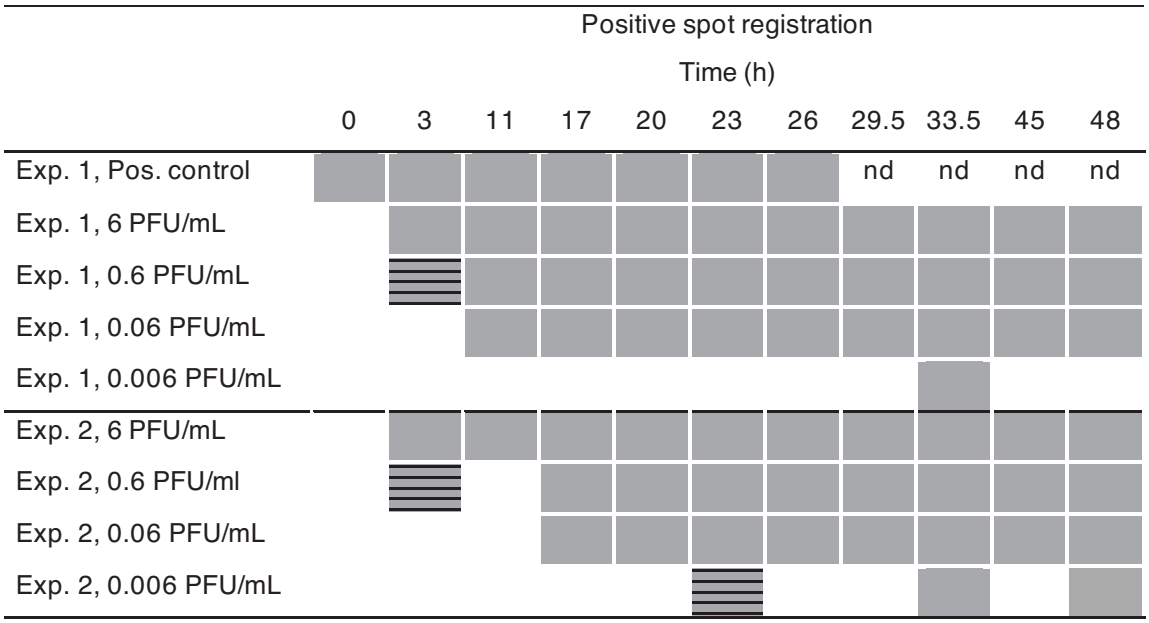

Fig. 3. Results from the 10 µL spot assays performed over time in Experiment 1 and 2 to detect the presence of viruses in the dilution cultures. White squares indicate that plaques were not detected, gray squares indicate positive plaque formation (horizontal lines indicate that plaques were only obtained in one of the replicate cultures). nd, not done.

subsequently, isolate it by spot assay. The detection limit was, in this case, independent on host strain composition; however, the faster propagation of phages in Experiment 1 indicated that the composition and susceptibility of potential host strains in the culture may influence the detection limit of viruses for other virus–host systems. Similar, for the spot assay approach, it was shown that the 10^{-4} dilution (3 phages in 10 µL) resulted in positive reactions in the spot assay, whereas 10^{-5} dilution did not produce a clearing zone (Table. 3). Consequently, the two methods had very similar detection limits and were in principal in both cases capable of detecting a single infectious unit.

Interestingly, viral proliferation in the enrichment cultures as verified by the spot assay did not result in a reduction in OD in all cases (Fig. 4). In fact, in Experiment 2, only the highest initial virus concentration (6 PFU mL^{-1}) resulted in a significant OD reduction, whereas in Experiment 1, initial concentrations as low as 0.6 PFU mL^{-1} affected OD in cultures relative to the control (Fig. 4). These results clearly demonstrate the point made above, that OD measurements are not necessarily sufficiently sensitive to detect the presence of viruses in enrichment cultures and that viruses therefore may be isolated from cultures even without observations of a reduction in OD. This is particularly the case if the host cells only have a low susceptibility to the phages in the sample or if a mixture of host cells is used for viral isolation. However, we recommend OD measurements as a rapid way of getting a first indication about whether cell lysis is occurring in the enrichment cultures.

Discussion

An essential property of a virus isolation procedure is which fraction of the total viral assemblage that is targeted by the procedure and what is the detection limit of the procedure (i.e., how many of a specific virus are needed in the original water sample to detect it by the various methods). Various types of studies may require different knowledge about the

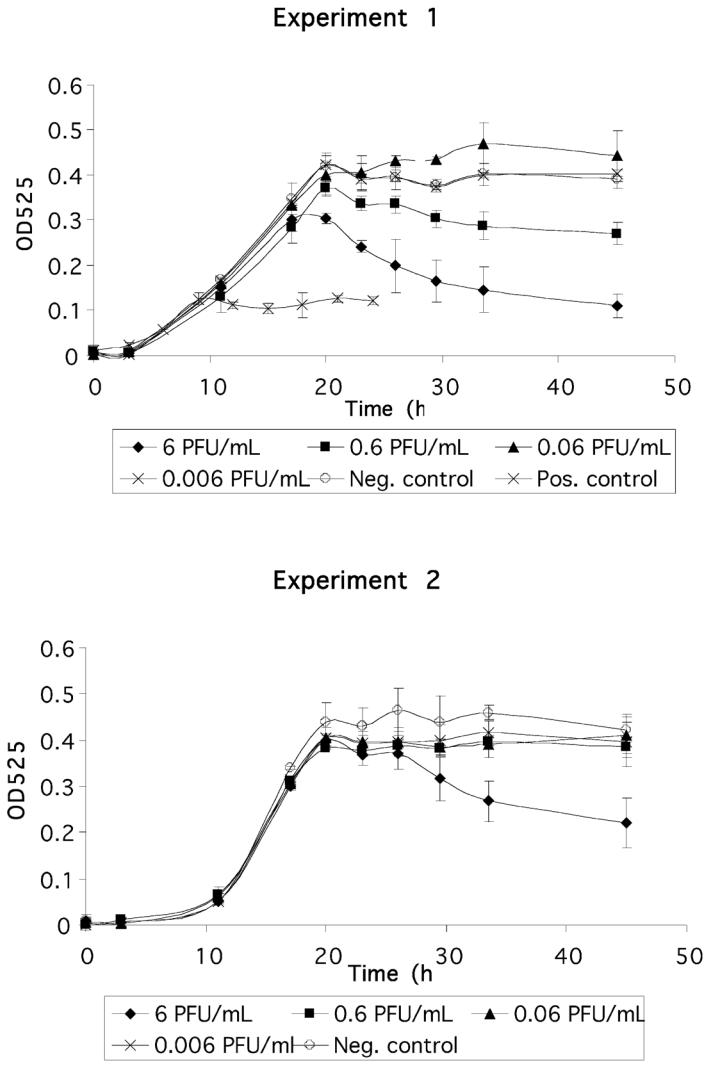

Fig. 4. Measurements of optical density (OD_{525}) in dilution cultures of host strains after addition of different numbers of the lytic phage #12 (range 0-6 PFU mL^{-1}. Experiment 1: Dilution cultures contained only the highly susceptible host strain *Cellulophaga* #12. Experiment 2: Dilution cultures contained a mixture of 3 *Cellulophaga* sp. strains in equal densities (*Cellulophaga* #12, *Cellulophaga* #3, and *Cellulophaga* NN16038) of which two strains (*Cellulophaga* #3 and *Cellulophaga* NN16038) had reduced susceptibility to the phage relative to *Cellulophaga* #12.

actual targets of the isolation procedures. If the purpose is simply random isolation of heterotrophic virus-bacteria systems, the methods described here are usually quite efficient with a success rate of 10-50% (i.e., it is usually possible to isolate lytic viruses against 10-50% of culturable bacteria isolated from the same water sample). However, if one is looking for, or wants to quantify, naturally occurring viruses against specific bacterial hosts, it is important to know the detection limit of the procedure. In that context, it is relevant to know the recovery efficiency of the method, i.e., the extent of loss of phage particles and/or phage infectivity during the various steps in the concentration procedures, etc.

The first and most obvious limitation of the presented isolation procedures is that the method only works with bacteria and cyanobacteria that are culturable. By far, most of the work on viral isolation has been carried out with aerobic heterotrophic bacteria and cyanobacteria, culturable both on agar plates and in enriched liquid cultures. In principle, however, it is possible to isolate viruses under anaerobic conditions or for bacteria that cannot grow in enriched cultures or on plates (e.g., the SAR 11 cluster), however, this requires modifications and adaptations of the standard protocols described here. Consequently, the methods presented here may need adjustments to the type of hosts or phages that are targeted. A second limitation is that the procedure selects for lytic viruses whereas isolation of temperate viruses is less straightforward and requires quite different techniques to obtain in culture (e.g., Dillon and Parry 2007).

It is, therefore, important to be aware that the method does not provide a general screening for lytic phages but rather a screening for phages infecting specific target hosts, or in the broader approach, hosts that are culturable under the given set of growth conditions and present in significant numbers and activities to allow propagation of a viral population. Also, the isolation procedure introduces a competition between infective phages for the applied host, and will therefore favor specific viruses (e.g., broad host range phages, phages with high affinity for the host, high burst size, etc.), at the expense of low-efficiency viruses, and will therefore not necessarily provide a representative selection of the viruses that are able to infect a given target bacterium.

The methods for characterization of adsorption kinetics and life cycles of phages are tedious and often require some adaptation for individual phage–host systems. However, we have presented some basic procedures that are known to work on certain types of phages and may function as a starting point in the development of more specific procedures for characterization of phage–host systems of interest.

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