Video Article

Estimating Virus Production Rates in Aquatic Systems

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Abstract

Viruses are pervasive components of marine and freshwater systems, and are known to be significant agents of microbial mortality. Developing quantitative estimates of this process is critical as we can then develop better models of microbial community structure and function as well as advance our understanding of how viruses work to alter aquatic biogeochemical cycles. The virus reduction technique allows researchers to estimate the rate at which virus particles are released from the endemic microbial community. In brief, the abundance of free (extracellular) viruses is reduced in a sample while the microbial community is maintained at near ambient concentration. The microbial community is then incubated in the absence of free viruses and the rate at which viruses reoccur in the sample (through the lysis of already infected members of the community) can be quantified by epifluorescence microscopy or, in the case of specific viruses, quantitative PCR. These rates can then be used to estimate the rate of microbial mortality due to virus-mediated cell lysis.

Video Link

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Protocol

1. Ultrafiltration of Seawater to Generate "Virus-free" Water (Wilhelm & Poorvin 2001)

1. Approximately 20L of seawater / lakewater is collected as aseptically as possible.
2. Water is serially prefiltered through 142-mm diameter polycarbonate 0.8 µm filters that can be kept at -20 °C for community analysis. Larger pore size filters may be used before this step for very productive systems.
3. To obtain ultrafiltered water from an Amicon M12 system (Millipore) a 30 kDa-cutoff spiral cartridge is used to exclude all viruses, even small RNA viruses.
4. The samples are processed and concentrated at ~ 25% speed with ~15-16 kPa of backpressure.
5. The remaining sample of ~500 mL of water will contain a concentrated virus community (which can be saved for other experiments) while the remaining 19.5 L of virus-free water will be used for viral production assays.
6. After each day of use, the Amicon M12 system must be cleaned to prevent damage to the membrane of the filter cartridge.
7. If you are working with seawater, rinse the membrane out with at least 6L of Milli-Q water followed by a washing with 0.1N NaOH solution for 30-45 minutes.
8. Again rinse the cartridge with at least 6L of Milli-Q water.
9. When finished using the M12 system, the spiral cartridge should be stored in a 0.05M H_3PO_4 solution at 4°C.

2. Virus Reduction Method for Viral Production (Wilhelm *et al.* 2002)

1. Up to 500 mL of the seawater / lakewater sample with both host and viruses is obtained and placed in a sterifilter unit with a 0.2-µm nominal pore-size low protein-binding filter (e.g., Durapore) placed on it.
2. The sample is gently vacuum pressured at <200 mmHg while continually resuspending the sample using a sterile transfer pipette to inhibit bacterial cells from concentrating on the filter.
3. Slowly three volumes of ultrafiltrate are added to the bacterial suspension to significantly reduce the number of free viruses in the sample.
4. The bacterial fraction is diluted back to 500 mL with virus-free water and divided into three replicates of 150 mL each and are placed in clear 250ml polycarbonate bottles.

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3. Tangential Flow Filtration (TFF) Method for Viral Production (Weinbauer *et al.* 2002, Winget *et al.* 2005)

TFF represents an alternative approach to the viral reduction method.

1. Approximately 500 mL of natural sample is collected as described above.
2. This sample is concentrated using a 0.2-μm nominal pore-size tangential flow filtration system.
3. When the bacterial fraction is reduced to approximately 10-15 mL, ultrafiltered, virus-free water is added and distributed as above.
4. The replicate bottles are incubated at *in situ* conditions using environmental chambers.
    1. Light levels are altered to surface conditions by using blue-tinted acrylic or clear acrylic with screening net to decrease light intensity.
    2. Ambient surface temperatures are often obtained by using a flowing seawater deck incubator.
5. Samples for bacterial and viral abundance estimates are taken at time 0 with a final concentration of 2.0-2.5% sterile glutaraldehyde added into cryovials. These samples are immediately flash frozen with liquid nitrogen and stored frozen until processed.
    1. If liquid nitrogen is not available, microscopy slides may be prepared and processed immediately (see procedure below)
6. Subsamples are collected every 2.5 hours for at least 10 hours by the method described above.
    1. At this time water may be collected for quantitative PCR analysis. Up to 5 mL of the sample may be added to a cryovial with no fixative agent with immediate flash freezing in liquid nitrogen.

4. Viral Production Microscopy (Noble & Fuhrman 1998, Wen *et al.* 2004)

1. Frozen samples to be 0.02-μm filtered for microscopy should be thawed on ice.
2. Prepare a stock solution of SYBR Green by diluting the stock solution 1:10 with sterile water. Next, from the stock solution, prepare a working solution by adding 1mL of the stock solution to 39 mL of sterile water. A 50% glycerol, 50% phosphate buffered saline solutions (PBS, 0.05 M Na_2HPO_4, 0.85% NaCl, pH 7.5) and fresh 2.5% stock solution of p-phenylenediamine should also be prepared before beginning. Keep the 50% glycerol/50% PBS solution at 4°C and the p-phenylenediamine stock at -20°C in the dark until starting. Right before filtering add the p-phenylenediamine to the 50% glycerol/50% PBS to a final concentration of 0.1% to be used as the Antifade solution.
3. Place a 25 mm 0.02-μm Anodisc filter on top of a 0.45-μm MicronStep, cellulosic backing filter. Add 850 μL of the fixed sample to the top of the Anodisc and vacuum at 20 pKa until completely dried. Place 100 μL of SYBR Green working solution to a sterile Petri dish and, with the vacuum still on, carefully remove the Anodisc from the filter tower and place on the SYBR Green. Incubate the samples in the dark at room temperature for 20 minutes. Carefully remove the filter from the SYBR Green solution and wick the back of the filter with a Kimwipe to remove all residual dye. If desired, return the filter to the tower and pass up to 800 μL of 0.02-μm filtered water or sterile media through the filter to rinse off excess stain.
4. Add a small drop of antifade solution to a microscope slide and place a cover slip on top. Remove the cover slip and add the dried filter to the microscope slide wet with the antifade solution. Again, add a small amount of antifade solution to the cover slip and slowly place it on top of the filter, making sure to get rid of any bubbles that may form.
5. Immediately freeze the slides at -20° C until needed (these should be used within a few months to prevent fading and lowered virus counts)
6. Viruses are enumerated using fluorescence microscopy (in our case a Leica DMRXA microscope) with a wide blue filter set (λ_{Ex}= 450 to 490 nm, λ_{Em}= 510 nm with a suppression filter at λ= 510 nm). Each filter will have at least 20 fields of view counted, making sure to quantify total viruses from each field grid to ensure even distribution of viruses across the filter membrane.
7. Averaged rates of virus reoccurrence from the three independent replicates are then calculated and a standard deviation is determined from the production rates.

5. Representative Results

The raw data collected by the researcher requires minimal mathematical processing to generate reoccurrence rates of virus abundance. The primary data set resulting from this study is the reoccurrence rates of virus abundance in the subsamples from the incubations. These results form independent regressions of virus abundance vs time for each of the samples. For each sample the individual incubations act as one treatment, so by completing three replicate-incubations the researcher can calculate rates as well as an estimate of variation (e.g., standard deviation) (see Figure 1.)

One caveat of this process is that the reduction in virus abundance invariable leads to a reduction in the host cells in the sample that are carrying the virus burden. To offset this loss, enumeration of bacterial abundance from both the source (unfiltered seawater or lake water) and the T = 0 incubation sample are necessary. This information can be used to account for the percentage of cells lost: assuming that the process of reducing virus abundance is not selective for or against any members of the microbial community, this factor can then be used to estimate the *in situ* production rate of viruses.

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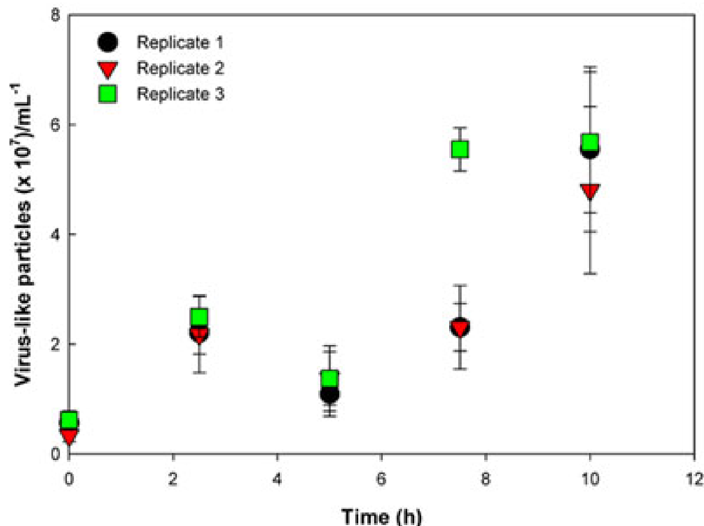

Figure 1. The production of virus-like particles over a 10 hour incubation at *in situ* conditions using epifluorescence microscopy. Samples were collected during a phytoplankton bloom off the coast of New Zealand in September of 2008.

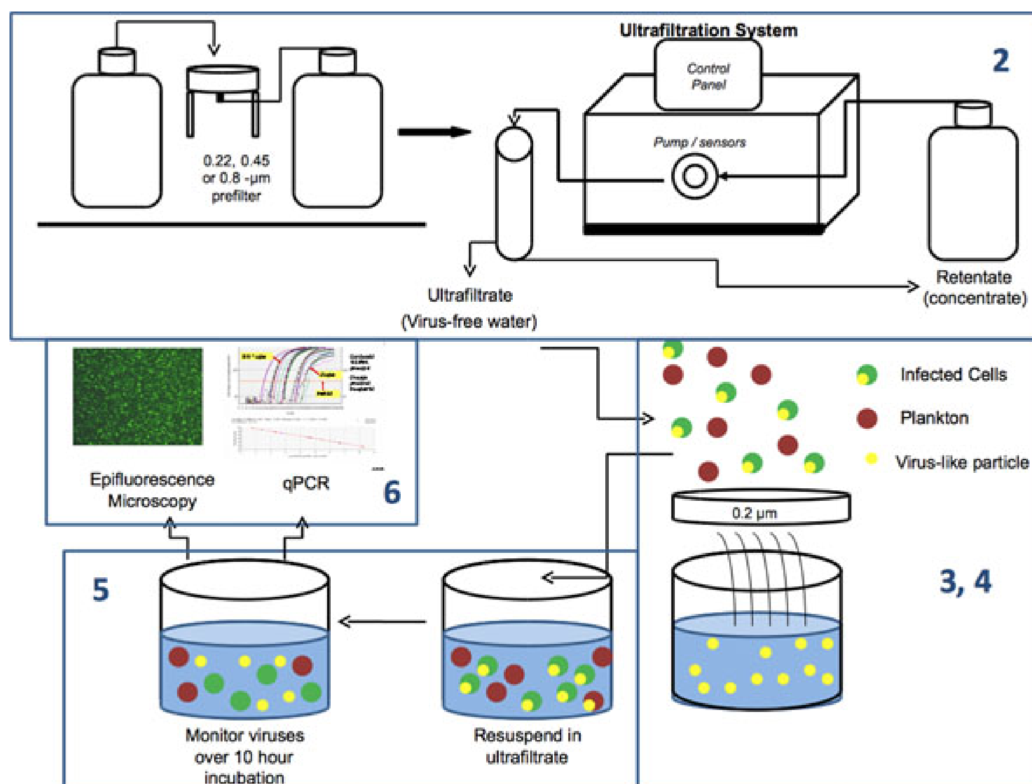

Figure 2. Schematic diagram of the work flow process for assaying virus production. The process starts with the ultrafiltration of sample water to generate virus-free water. This is completed using an ultrafiltration system. In parallel water samples are collected from the same site and the free viruses passed through a filter while the microbial community (containing a mixture of infected and non-infected cells) is retained. This community is then resuspended in the virus free water and incubated under *in situ* conditions. The reoccurrence rate of viruses is then monitored for the next 10 hours to determine rates of virus production.

Discussion

A critical component in the understanding how viruses influence marine microbial communities is to determine the rate at which virus particle are produced. Given that abundances are (more or less) static in most systems (Wilhelm & Suttle 1999, Weinbauer 2004), and that viruses are removed or rendered non-infectious quickly in aquatic systems (Wilhelm *et al.* 1998), then production rates must be relative rapid to replace lost particles.

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Estimating the mortality viruses cause to the microbial community requires a knowledge of how many viruses are produced every time a virus lyses a cell (the "burst size"). Virus burst sizes in natural samples can vary greatly. Burst sizes can be determined directly by transmission electron microscopy (e.g., Weinbauer & Peduzzi 1994), but this is often beyond the capabilities of a given laboratory or not always practical. In situations where they cannot be empirically determined, literature values of 24 viruses per lytic event may be used for marine systems and 34 for freshwater systems (Parada *et al.* 2006). If the rate of virus production is divided by this number, the result is the abundance of cells per volume destroyed by viruses on a daily basis. The microbes lysed value can then be divided by the standing stock of bacterial abundance resulting in the virus induced mortality for the system in question: existing estimates range from few percent to nearly the entire population and are often dependent on other factors in the system in question (Wilhelm & Matteson 2008). To determine the percentage of total mortality this number is often multiplied by two (working from the assumption that 50% of the cells go on to reproduce and 50% of the cells are lost, Weinbauer 2004).

Given that nutrient and trace element bioavailability (e.g., N, P, Fe) can limit the rate of primary productivity, and as such carbon flux, through aquatic systems, and understanding of the role of virus-driven microbial mortality in this process has become of interest to marine geochemists. Several estimates now exist that suggest viruses release a significant concentrations of nutrient elements back to the water column on a daily basis (Rowe *et al.* 2008, Higgins *et al.* 2009) and that these elements are rapidly assimilated by the microbial community (Poorvin *et al.* 2004, Mioni *et al.* 2005). The rate of nutrient flux to the environment can be determined by multiplying the number of cells destroyed by the amount of nutrient per cell (denoted "quotas"). This information can provide a critical component to our understanding of how microbial food webs function across aquatic systems.

Ongoing developments: Current efforts by a series of research groups involve adapting the above strategy to enumerate specific viruses within the community and, as such, to determine how specific organisms are influenced by virus activity. To do this researchers use the quantitative polymerase chain reaction (qPCR) to estimate the abundance of specific viruses groups or families in parallel to the estimates of the total virus community. The results are then directly applied to provide estimates of virus mortality, nutrient turnover, etc for specific plankton groups. This powerful new approach will allow researchers in the coming years to dig much more deeply into processes associated with the ecology of viruses and, for the first time, to quantify the interactions of specific virus-host communities beyond the constraints of laboratory systems.

Disclosures

No conflicts of interest declared.

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