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Determining rates of virus production in aquatic systems by the virus reduction approach

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Abstract

The reduction approach to assess virus production and the prokaryotic mortality by viral lysis stops new infection by reducing total virus abundance (and thus virus–host contacts). This allows for easy enumeration of viruses that originate from lysis of already infected cells due to the decreased abundance of free virus particles. This reoccurrence can be quantified and used to assess production and cell lysis rates. Several modifications of the method are presented and compared. The approaches have great potential for elucidating trends in virus production rates as well as for making generalized estimates of the quantitative effects of viruses on marine microbial communities.

Introduction

Since the rediscovery of the importance of viruses in marine environments (Bergh et al. 1989; Proctor and Fuhrman 1990; Suttle et al. 1990), researchers have worked to try and determine the quantitative nature of virus effects on marine microbial food webs. Originally documented in aquatic systems almost 100 years ago (Duckworth 1976) the implications of virus activity have remained elusive. Beginning in the early 1990s however, efforts on several fronts began to quantify the rate at which virus particles were produced and "turned-over" in primarily pelagic aquatic environments. Whereas many of these methods have not been set aside, an appreciation of the different options available to the aquatic viral ecologist is necessary.

Prior to understanding the methods that are available to estimate virus production rates in aquatic systems, it is perhaps best to understand how the information is important and will be used (as the intended fate of the information may, in part, dictate the manner of its collection). Virus production rates are most commonly used to infer the losses of primary or secondary production in aquatic systems due to the activity of viruses. In the case of direct estimates of particle production rate, knowledge concerning the number of viruses produced per lytic event (the burst size) allows for one to estimate the number of host cells destroyed by the activity of viruses. As such, estimates of virus activity need to be made over times scales that are on the same temporal order as the turnover rate of the host population.

TEM assessments of microbial mortality—One of the earliest attempts to estimate the mortality inferred on microbial communities was the percentage of visibly infected cells approach (Proctor et al. 1993). The approach is based on the assumption that intact virus particles are visible in infected cells for a certain percentage of the lytic cycle. By undertaking controlled infections within a lab setting, Proctor and colleagues (1993) were able to estimate the percentage of the lytic cycle that viruses were visible within infected cells. By extrapolating this relationship to microbial communities, estimates of the percentage of microbial cells that carried a visible virus infection could be made using a transmission electron microscope set to

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a high accelerating voltage. Scoring a sufficient number of cells, a researcher can then make estimates of the percentage of a population carrying a virus burden. This approach was applied using thin-sectioning (Proctor et al. 1993) as well as a whole cell approach with (Bratbak et al. 1992) or without "lysis-from-without" by streptomycin (Weinbauer and Peduzzi 1994).

Radioactive incorporation—Popular for assaying the production rate of bacteria in aquatic environments, the incorporation rate of radiotracers to estimate virus production was developed and proposed as a method in the early 1990s (Steward et al. 1992a, 1992b). In brief, the method mimics bacterial production assays by estimating the incorporate of a ^{3}H-, ^{32}P-, or ^{14}C-labeled radiotracer (thymidine or leucine) into virus particles. As such, the technique is highly dependent on the ability of the researcher to separate intact virus particles from both whole and lysed bacterial and algal materials. Mechanical separation (by filtration) is typically the method of choice for this approach because a high throughput rate of samples is needed for significant replicates to be processed. However, the size-range that viruses occupy (~50–750 nm) overlaps with the size-range of the operational exclusion range (>0.2 µm) leading to the loss of some portion of some samples. Moreover, filter "breakthrough" (the passage of particles greater than the operational cut off of the filters into samples) quickly contaminates this assay. This filtration step in the assay is critical, as even minor amounts of contamination from a couple of bacterial or algal cells can result in a significant error in the estimates of the amount of viral DNA or protein that is produced. As such, both the variance and opportunity for error associated with this approach reduce its attractiveness.

Indirect methods: virus decay rates—One approach to determine the rate of production of virus particles is to examine their loss rates from the water column. Given that virus particle abundance is static (a tenuous assumption in some cases), then the loss rate of virus particles should be balanced by the production rate. Several variations on this approach exist, including the use of tracer particles (Garza and Suttle 1998; Suttle and Chen 1992; Wilhelm et al. 1998a), the use of natural communities, and the arrest of virus production by adding poisons (Heldal and Bratbak 1991), and the addition of fluorescently labeled particles that can be tracked as a percentage of the population (Noble and Fuhrman 2000). In all cases, these approaches provide information on specific groups in natural samples, although the information comes at a cost of some tractability for the system in question.

What do we want from a virus production method?—Ultimately, the estimation of virus production rates should be as noninvasive as possible, and be able to provide information concerning the production rate of either total virus particles or specific groups within a sample. To this end, many labs now favor the dilution and reoccurrence approach that has been in use for the last 6 y (Weinbauer et al. 2002; Wilhelm et al. 2002). It has been suggested to use the name "virus reduction approach" (VRA) (McDaniel et al. 2002), because a dilution approach has been used for a long time for grazers (Landry and Hassett 1982) and has been recently applied to viruses (Baudoux et al. 2007; Evans et al. 2003).

In a comparison with the radiotracer incorporation and the fluorescently labeled viruses approach, Helton et al. (2005) conclude that the VRA should be the most widely applicable method because it is the least difficult and the most efficient method. In brief, the method involves the removal of free virus particles from a sample, and then documents their reoccurrence over time. The rate of this reoccurrence, when corrected for the relative abundance of potential host cells in a sample, allows for an estimate of the production rate of particles in the sample by direct counts using epifluorescence microscopy or flow cytometry. In addition, it is possible to estimate the percentage of infected cells (PIC) in the initial population. Adaptations, including the enumeration of specific particles (by qPCR quantification) or infectious particles (*via* plaque assays or MPN assays), allow for multiple components of the virus community to be assayed from individual experiments.

Materials and procedures

General remarks—The reduction and reoccurrence method for estimating virus production has become the new "gold standard" by which virus production rates have been measured. This approach has been tested in a number of environments and in different seasons. Whereas the approach itself is relatively simple, several different adaptations of the approach now exist. These adaptations are discussed below, each with their own variations. The areas of this process can be partitioned into the following areas: 1) methods to reduce the abundance of viruses, 2) incubation and sampling of samples, and 3) data processing and interpretation.

Methods to reduce the abundance of viruses—The major difference between all published approaches to measure virus production by the reduction and reoccurrence method is the process of reducing the abundance of free virus particles. Before collecting the host community, prefiltration can be used to avoid loss of newly produced viruses by attachment to large particles or grazing on infected cells. Like any filtration step, this also has the potential to lead to loss of hosts or viruses. As such, if prefiltration of samples is going to occur, it needs to be completed in a manner appropriate for the samples in question. As well, separation of the microbial (i.e., host) community from free viruses also requires filtration, which can lead to significant losses or changes in the efficacy of the approach. To this end, the choice of membrane material is important, and while some membranes (e.g., low protein binding-matrices) may be more expensive that others (e.g., glass fiber or cellulose nitrate) they offer advantages in reduced analytical variances that are well worth the extra expense.

In this paper, three different approaches to reduce the abundance of free virus particles are discussed. Whereas each method has its benefit and drawback, it is incumbent on the

users to understand these as well as to choose the method most appropriate for their question of interest.

Approach 1: Over filter virus reduction with continuous cell resuspension (Wilhelm et al. 2002)—In this approach, the microbial host community (~300 mL) is gently (vacuum pressures of <200 mmHg) collected over a 0.2-µm nominal pore-size low protein-binding filter (e.g., Durapore, Millipore Corporation) while virus-free (ultrafiltrate, UF) water is added to maintain the approximate sample volume. After three passages of sample volume through the filter, the retained microbial community is distributed ($n \geq 3$) for incubation (see below). During the filtration process, bacteria are gently and continually resuspended from the filter surface using a transfer pipette to resuspend cells that may become trapped on the membrane. Since the original approach for this assay, a number of adaptations have been made: these include the use of a tube and peristaltic pump to keep cells in suspension (Helton et al. 2005).

Approach 2: Tangential flow filtration (TFF) based concentration and resuspension of cells in virus-free water (Weinbauer et al. 2002)—Bacteria in a 200-300 mL water sample are concentrated using a 0.2-µm pore-size tangential flow filtration system (e.g., a Vivaflow 50 cartridge, 0.2-µm pore size, polysulfone; Vivascience operated by a peristaltic pump). The bacterial concentrate (*ca.* 10-15 mL; i.e., the retentate) is kept and the filtrate (permeate) containing the viruses is passed through a 30- or 100-kDa filter unit to generate virus free water. Note that some concentrate is in the cartridge and tubes but can be collected by removing the feed tube and pumping the concentrate into the retentate container. The bacterial concentrate is then mixed with the UF, and samples are distributed in triplicate into incubation tubes.

Approach 3: TFF virus reduction and continuous cell resuspension (Winget et al. 2005)—This approach is similar to approach 2, however, UF is made before and fed into the bacterial retentate to keep the volume constant. Filtered volumes are as in approach 1. One caveat is that passages of the sample volume through the filter have been found to marginally improve viral reduction over use of 3 passages of the sample volume (Winget et al. 2005).

Comments on microbial community collection and virus reduction—Ultrafiltered water can be made by a variety of cartridges that are available from several providers. Either 30 kDa or 100 kDa exclusion cartridges are typically used as they are in the generation of virus concentrates (Wilhelm and Poorvin 2001). In practice the 100 kDa should remove less dissolved organic matter and, as such, lead to fewer changes in dissolved solute concentrations. However, the 100 kDa cartridges might not retain very small viruses, such as some RNA viruses.

In all three approaches, the goal is to maintain the host population while reducing the abundance of free viruses. Typically viral abundance is reduced to ~10%–20% of the initial concentration, while bacterial abundance is reduced to ~50%. However, recovery efficiency can vary strongly. One would expect that the recovery efficiency differs among environments, but this has been not studied systematically. While not ideal, the reduction in host abundance reduces virus–host contact rates and the frequency with which new infections occur during the incubation stage. For approaches 1 and 3, the procedures require the separate generation of virus-free water prior to experimental set-up, and this can be time consuming as the virus-free water should be generated from the specific station where the incubation sample is collected. In practice, this time lag can be reduced by using a larger scale concentration system (e.g., the Amicon M12 system, Millipore), which can more rapidly generate virus-free water. One advantage of approach 2 is that the virus-free water can be generated in parallel with the collection of the microbial host community, allowing for more rapid pre-processing and experimental set-up (and as such allowing for multiple samples to be processed in parallel). However, this approach carries with it the caveat that cells are concentration up to 10-fold beyond their in situ abundances for a short period, and this increased cell density may have unknown effects on microbial metabolism (e.g., activation of quorum sensing pathways).

Experiment incubation and sample collection—To determine the rate of virus production, each of the above approaches requires that samples containing the reduced virus community be incubated under in situ conditions so that the microbial metabolism can proceed and viruses continue the lytic cycle. Several options are available here, including the use of environmental chambers that can control temperature. In the field, one of the most common approaches is to use flowing lake/seawater incubators. In this case, water is pumped from the sea surface (often exploiting existing equipment if on a research vessel, i.e., the ship's deck water or fire systems) into an on deck box incubator, and then allowed to return overboard by means of an overflow system. Care must be taken in these cases to ensure that the volumes and flushing rates of the incubators are sufficient to allow for complete incubation of sample bottles while cycling the flow-through fast enough to maintain surface temperatures (i.e., to avoid heating in the sun). One other question commonly raised concerns whether to carry out the incubations at in situ light levels or in darkness. To date most studies have focused on the heterotrophic bacterial community, and as such, have used darkened bottles or incubators for this step. Incubation under in situ light conditions can be completed and may favor virus production in photoheterotrophs or alga, but comes with the caveat of virus loss due to light effects. Please see the Assessment section for more details on the impacts of light versus dark incubations.

To determine the rate of virus production in the experimental sample, subsamples are collected from the incubation bottles at increments appropriate for the system being studied. In environments where the microbial community is rapidly turning over, this may be on the order of every 1.5 h, whereas in environments where microbial growth is slow this may be on the order of every 4-6 h. Typically, subsampling is best

Table 1. Formulae for inferring the production, turnover, and effects of viruses on marine microbial communities

Equation	Parameter	Units	Formula
1.	In situ virus production rates (VPR)	Particles per volume per time	Experimental virus production = (in situ $B_A/B_{Aex,\ T=0}$)
2.	Virus-inferred bacterial lysis	Bacteria per volume per time	Bacterial lysed = VPR/BS
3.	Number of lysed cells	Bacteria per volume	Number of lysed cells = Maximum minus minimum viral abundance/average burst size
4.	Percentage of infected cells	Percentage	PIC = Number of lysed cell divided by bacterial abundance
5.	Virus remobilized nutrients	Nutrients per time	Nutrients = Virus-inferred bacterial lysis × nutrient quota per cell

VPR, in situ virus production rate; B_A, in situ bacterial abundance; $B_{Aex,\ T=0}$, experimental bacterial abundance at T = 0; BS, burst size; VA, in situ virus abundance.

completed at 2.5-3 h intervals over a period of 10-12 h, although in environments of low trophic status/growth rate experiments can run 18-24 h. It is critical here that the precise time of sampling is noted, as this information is required to determine the rates of virus production within the samples.

Subsamples, once collected, need to be quickly processed or preserved for enumeration of the virus community. To date, the only published information using any of these approaches involves the enumeration of the total virus community within samples. Ongoing research, however, is focusing on the reduction and reoccurrence approach to enumerate the rates of production of individual virus groups (e.g., by plaque assay or quantitative PCR).

Data processing and interpretation—The processing and analysis of the data collected by the above experimental designs is as important as the choice of method to set up the experiment. In each case, the results of the enumerations result in 3 independent rates of virus production. These rates are determined from the slopes of plots of virus abundance versus time for the independent incubations. These in situ experimental production rates must then be corrected for the bacterial losses during sample set up: to do this one simply takes the ratio of in situ bacterial abundance to experimental (T = 0) bacterial abundance and multiplies this by the production rate (Table 1, Eq. 1). It is critical to determine these rates from the individual incubations and not from the mean of the virus abundance in the 3 separate samples, as the independent rates can be used to calculate a mean rate and an estimate of variance (the first standard deviation) for that measure.

Once the rate (and variance) of virus production is determined, a number of secondary calculations become available to the researcher beyond the variations in virus production rates under different environmental conditions or spatiotemporally. It is important at this juncture to note that each of these calculations comes with the caveats of not only this method, but also of the method used to determine the companion parameters discussed below.

The most basic calculation typically completed from the virus production data is to develop an estimate of the host cells lost. This estimate is calculated from the rate at which viruses are produced and an empirically (preferably) determined or estimated burst size (Table 1, Eq. 2). This calculation makes the assumption that the viruses produced within a sample are produced primarily from the lysis of heterotrophic bacteria. While this may not be completely correct, it is generally considered a safe assumption that aquatic viruses in most samples (>90%) are produced this way (Weinbauer 2004).

To estimate the percentage of the microbial community that was infected at the beginning of the experiment (% infected cells, PIC), the abundance of viruses produced during the observation is divided by the burst size to estimate the number of bacterial cells that were lysed (Table 1, Eq. 3). This represents a conservative estimate of the cells carrying a virus-burden at the onset of the experiment, as some cells in the early stages of the lytic cycle and with long lytic cycle times may not yet have lysed. The PIC is then calculated as 100 × the number of lysed cells divided by bacterial abundance at T = 0.

Furthermore, virus production can be related to viral-mediated mortality of bacterioplankton in several ways. For more detailed calculations, see also http://www.univie.ac.at/nuhag-php/vipcal/ (Luef et al. 2009). Virus production can be divided by the burst size and bacterial abundance at T = 0 to obtain a lysis rate of the standing stock: for example, as % of bacterial abundance per day. Using burst size estimates, viral lysis rate can also be compared with bacterial production and expressed as % mortality in the sense of % of production lysed. In the latter case, it is important to either correct for losses of bacterial abundance or measure bacterial production at T = 0 of the incubations.

The PIC can also be related to bacterial mortality using models. Two models have been used (Binder 1999; Proctor et al. 1993) to make these estimates from transmission electron microscopy measures. These models are predicated on the assumption that in steady state one of the two daughter cells originating from cell division is lost. Thus, in the model of Proctor et al. (1993) the percentage of infected cells is multiplied by two to obtain that ("factor-of-two rule"). Binder (1999) developed a more elaborate model that including grazing on infected cells to estimate the fraction of mortality from viral lysis. Note that in those studies the authors chose (we believe incorrectly) the term *frequency* instead of *percentage*, but the calculations are the same.

Table 2. A comparison of the pros and cons of the three virus reduction assay approaches

Approach	Advantages	Disadvantages
Over filter concentration approach (Wilhelm et al. 2002)	• Cells are not concentrated • High reduction efficiency (75 – 80% +) • Limited material requirements	• UF has to be made before the start of the incubations (adds 0.5–1 h to processing time) • Weak recovery of bacteria
TFF Concentration and resuspension (Weinbauer et al. 2002)	• Parallel sampling processing is easy (only one pump needed) • Most rapid approach • Volume needed: 200 mL • Good recovery of bacteria	• Bacteria are concentrated, which might increase infection and affect performance of cells and physiology • Low reduction efficiency • Requires 30 or 100 kDa filters to generate ultrafiltered water
TFF concentration with continual resuspension (Winget et al. 2005)	• Cells are not concentrated • High reduction efficiency (75 – 80% +) • Good recovery of bacteria	• UF has to be made before the start of the incubations (adds 0.5–1 h to processing time) • Multiple UF filters needed (0.2 and 30 or 100 kDA)

A final calculation that has become very relevant as of late is the production of estimates of nutrients "recycled" due to virus-mediated cell lysis. In both marine and freshwater environments, some knowledge of the biochemical impacts of viruses is desired to better develop models of geochemical budgets and cycles. In the current case, the abundance of cells lysed by viruses can be used to estimate carbon and nutrient regeneration rates by multiplying cells lysed by the cellular quota for the nutrient in question (Poorvin et al. 2004). One caveat to this calculation is that the fate of elements released by virus-mediated cell lysis remains unsure, as only a few studies (Gobler et al. 1997; Middelboe and Jörgensen 2006; Middelboe and Lyck 2002; Mioni et al. 2005; Poorvin et al. 2004) have carefully addressed this issue. That said, the role of viruses within these cycles is no doubt critical (Brussaard et al. 2008; Suttle 2007; Wilhelm and Suttle 1999), and potentially a fruitful area of future research.

Assessment

A series of factors to consider when choosing the approach that is most appropriate for a lab is given in Table 2. One of the problems with the virus reduction method is that the manipulation of the sample could influence virus production. For example, the loss of cells and release of organic compounds due to stress or cell breakage during filtration (Nagata and Kirchman 1990) could influence rates. This alteration could affect bacterial production and ultimately affect the burst size (Parada et al. 2006). While no changes in bacterial production were seen in early trials of the virus reduction approach (Wilhelm and Suttle, unpubl. data), this problem suggests that bacterial production rates should be measured at the start of the experiment and either during or at the end of incubations to determine if virus production is related to losses of heterotrophic production. In a previous virus-reduction type assay where this was tested, the burst size did not differ between in situ and mitomycin C–treated samples (Weinbauer and Suttle 1996). This is, so far, the only indication that the VRA does not influence burst size, however, it has to be noted that burst size was not checked in the untreated controls. Another potential problem is that many protistan grazers of prokaryotes are destroyed or inactivated by excessive handling. Because protists can ingest viruses (Gonzalez and Suttle 1993) and could preferentially graze on infected cells (Weinbauer and Peduzzi 1995), such losses could result in increased variance in the estimates of viral production although preliminary observations (unpublished) suggest that exclusion of grazers at the beginning of the experiment does not affect rates. Finally, viral decay rate is usually not measured during the incubations, although it can be important (Winter et al. 2004).

Only a few studies have compared different approaches to assess prokaryotic mortality by viruses (for example, summarized in Weinbauer 2004). Some studies have also compared various approaches of the VRA. Weinbauer et al. (2002) found no consistent differences between Approach 1 and 2 for five samples from coastal and offshore Mediterranean water when calculating FIC. Another comparison was done using approach 1 and 3 (Winget et al. 2005). In three experiments, one of the two methods yielded negative and the other method positive values. For the two where positive values were obtained, there was no significant difference between the two samples.

As part of the current assessment, a comparison of approaches 1 and 3 were completed during cruise transects in the Pacific and Atlantic Oceans (Fig. 1). Across 8 different stations, only one station (occupied in the North Atlantic) showed a significant difference in estimated virus production rates using these two techniques. That station, which was part of a larger survey of the North Atlantic described elsewhere (Rowe et al. 2008), was a general statistical outlier for a number of parameters.

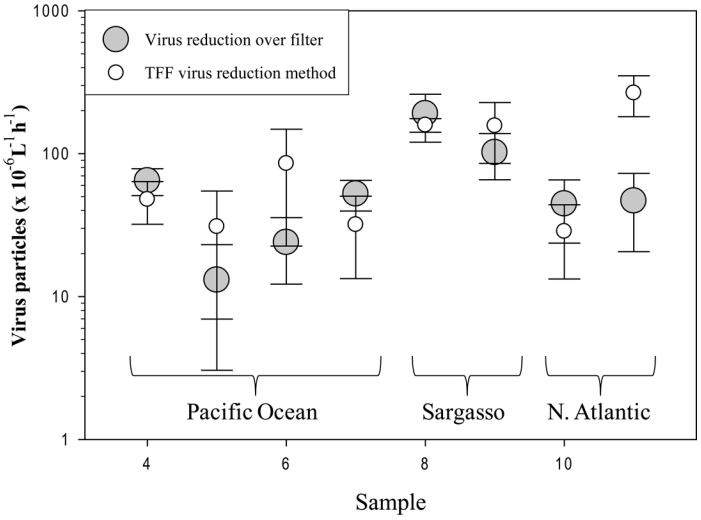

Fig. 1. Side-by-side comparisons of the over-filter method reduction approach (gray circles, Wilhelm et al. 2002) and the tangential flow filtration (TFF) method for the reduction of free virus particles (white circles, Winget et al. 2005). Samples were collected in the southeastern Pacific Ocean (January 2007) as well as the Sargasso Sea and the North Atlantic during May–June 2005 (Rowe et al. 2008). Experiments were completed at sea using the described protocols (n = 3, ±SD), and results are displayed as viruses produced (log scale). No significant differences were seen (Student t-test, 2-tailed, $P < 0.05$) except for station 11 (far right).

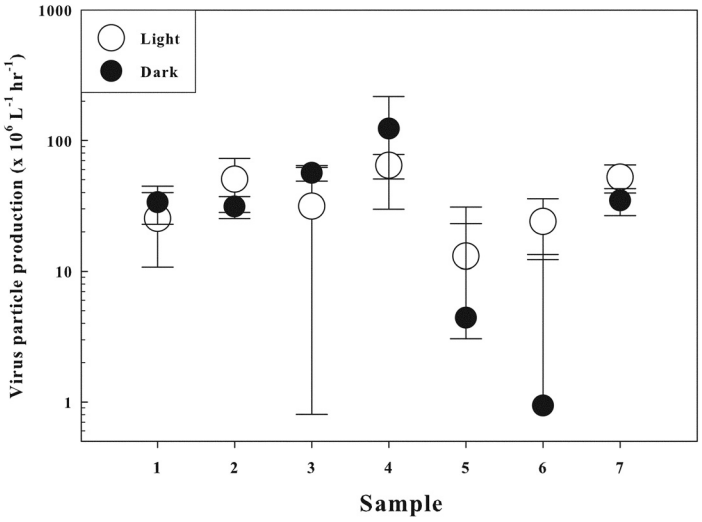

Fig. 2. Side-by-side comparisons of virus production rates determined using the over-filter method reduction approach (Wilhelm et al. 2002) with incubation stages completed in the dark (black circles) or reduced sunlight (white circles). For light exposed experiments, samples were incubated at ambient temperatures in a continuous flow incubator with solar intensity reduced to 30% using neutral density screening. Samples were collected in the southeastern Pacific Ocean (January 2007). Experiments were completed at sea using the described protocols (n = 3, ±SD) and results are displayed as viruses produced (log scale). No significant differences were seen (Student t-test, 2-tailed, $P < 0.05$).

Another area that has yet to be assessed in terms of estimates of virus production rates is the effect of light levels during the incubation process. While exposure to light may cause a loss of virus particles integrity or particle infectivity (Wilhelm et al. 2003; Wilhelm et al. 1998b), exposure to levels of photosynthetically active radiation may enhance host production rates or drive the photoreactivation of viruses that have experienced DNA damage (Weinbauer et al. 1997; Weinbauer et al. 1999). To date most studies have focused on estimating the production rates of infecting heterotrophic bacteria, so the incubation step has been completed with darkened bottles or incubators. To examine the effects of ambient light exposure, seven comparisons (using the over-filter approach) were completed during a transect from Hawaii to Australia. The assays were completed in a Plexiglas incubator (thereby reducing UV wavelengths) in bottles where the light field was reduced to 30% ambient (light) or completely darkened. As shown in Fig. 2, no significant difference was seen between the light and dark incubations in terms of estimated virus production rates. While not an exhaustive survey (and absent of information on the richness and evenness of viruses within the samples), the results demonstrate that virus production estimates appear to be independent of light field. One caveat to this is that changes in virus community structure were not examined in this study: it is possible that some virus populations increased in production whereas others were lost in the contrasting light and dark incubations.

Discussion

As new estimates of virus production rates appear, one overarching observation is that the production rates often seem to be too high to be sustainable by estimated bacterial production rates. The immediate effect of this is the generation of problems when extrapolating to food web or biogeochemical models. However, it has to be noted, that precisely quantifying both the production and mortality rates is difficult for microorganisms in general. This observation also illustrates the critical point that microbial communities are doubtless never in "steady-state" and as such (relatively) near-instantaneous observations of rates do not neatly describe community function (Hutchinson 1961). Moreover, this observation also suggests that the production of viruses from the lysis of phototrophs and/or protists may be important in some situations.

Given the above caveat, there remains an opportunity to employ the information generated by these measures to examine the effects of viruses on biogeochemical processes and food web interactions. For example, the virus reduction approach in its various forms has revealed ecologically relevant trends of viral infection such as diel cycles (Winter et al. 2004), seasonal variations, and changes along trophic gradients (Winget et al. 2005) or fronts (Wilhelm et al. 2002). Carbon and Fe release have also been estimated using this approach (Poorvin et al. 2004; Strzepek et al. 2005).

Comparisons to other mortality processes, such as grazing, can also be made to gauge how environmental parameters influence mortality mechanisms (Gobler et al. 2008; Weinbauer and Höfle 1998). In all, the availability of a method to estimate virus production rates provides researchers with an opportunity to begin to develop quantitative estimates of the effect of viruses on marine microbial communities.

Comments and recommendations

There are now many adaptations appearing for the above experimental approaches, and the reader is encouraged to review the literature prior to undertaking these experiments. New applications, including the use of infection assays and quantitative polymerase chain reaction (qPCR) estimates of virus abundance are now being used to allow researchers to focus on the production rate of specific viruses within whole community populations. As well, the virus reduction approach has also been employed to estimate virus turnover rates in marine sediments (Hewson et al. 2001; Mei and Danovaro 2004). This requires alterations to the protocols described above, and researchers are encouraged to seek out those references prior to attempting such a study.

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