MANUAL
of
AQUATIC VIRAL ECOLOGY

*MAVE* Chapter 7, 2010, 65–73


# Estimation of viral-induced phytoplankton mortality using the modified dilution method

*Susan A. Kimmance[1]* and Corina P. D. Brussaard[2]*

[1]Plymouth Marine Laboratory, Prospect Place, The Hoe, Plymouth, Devon, PL1 3DH, United Kingdom

[2]Department of Biological Oceanography, Royal Netherlands Institute for Sea Research (NIOZ), PO Box 59, 1790 AB Den Burg, Texel, the Netherlands

## Abstract

The modified dilution assay aims to partition phytoplankton mortality into virus- versus grazing-induced fractions and has previously been applied to several different environments to determine viral lysis rates of natural phytoplankton. The method involves creating a gradient of both grazing and viral lysis by dilution with different proportions of grazer- and virus-free filtrate, and assessing the subsequent impact on phytoplankton growth rates. We have conducted a critical evaluation of this method, and reviewed published data sets obtained using this approach to examine the utility of the modified dilution assay for estimating viral mortality rates. We provide modifications and improvements that have been incorporated into the method since it was first developed, and suggest recommendations for improving experimental success in less productive oligotrophic environments. Published data show that viral lysis rates vary between different algal groups and in response to environmental conditions. Results also suggest that this method has the potential to be a useful tool for estimating the impact of viruses on phytoplankton populations, but that the measurement of natural, low viral lysis rates (<0.1 $d^{-1}$) can challenge the application of this approach. Ultimately, however, the limitation of this method is associated with dilution of specific phytoplankton populations at low abundance.

## Introduction

Viruses are known to infect and lyse a wide range of autotrophs (*see* Brussaard 2004 for review). Because viral lysis can affect the carbon fixation rates of eukaryotic and prokaryotic primary producers, this suggests a significant effect of viruses on primary production-mediated carbon cycling (Suttle 2007). Modeling exercises have estimated that between 6% and 26% (Fuhrman 1999; Wilhelm and Suttle 1999; Ruardij et al. 2005) of primary production may bypass higher trophic levels because of viral-induced transformation of phytoplankton cells to dissolved organic matter (viral shunt). However, although viruses are predicted to have a significant impact on primary production, viral-induced mortality rates from natural waters are scarce.

Various methods have been developed to determine the impact of viruses on microbial populations; however, most were developed for bacterioplankton (Proctor and Fuhrman 1990; Heldal and Bratbak 1991; Steward et al. 1992; Weinbauer et al. 1993; Noble and Fuhrman 2000; Wilhelm et al. 2002; Parada et al. 2008) and may not be applicable for autotrophs. One of the most straightforward involves quantifying rates of cell lysis based on net increases in viral abundance over time (Bratbak et al. 1990). However, this method is limited because it relies on the virus of interest being distinguishable from other viruses in the sample, is only appropriate when viral numbers are increasing, and requires the use of assumed burst sizes to calculate lysis rates. Also, unless loss through viral decay is also accounted for, this approach provides only a minimum estimate of viral productivity (Noble and Steward 2001), and the resulting productivity estimates are highly influenced by the frequency and timing of sampling (Bratbak et al. 1990).

The approach based on the frequency of visibly infected cells (Proctor and Fuhrman 1990; Brussaard et al. 1996) is another relatively straightforward method with the advantage

*Corresponding author: E-mail: sukim@pml.ac.uk

***Acknowledgments***

Publication costs for the Manual of Aquatic Viral Ecology were provided by the Gordon and Betty Moore Foundation. This document is based on work partially supported by the U.S. National Science Foundation (NSF) to the Scientific Committee for Oceanographic Research under Grant OCE-0608600. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the NSF.

ISBN 978-0-9845591-0-7, DOI 10.4319/mave.2010.978-0-9845591-0-7.65

*Suggested citation format:* Kimmance, S. A., and C. P. D. Brussaard. 2010. Estimation of viral-induced phytoplankton mortality using the modified dilution method, p. 65–73. *In* S. W. Wilhelm, M. G. Weinbauer, and C. A. Suttle [eds.], Manual of Aquatic Viral Ecology. ASLO.

that incubations or culturing are not necessary. The method involves determining the frequency of virally infected cells using transmission electron microscopy to derive the fraction of the host population that is infected by viruses, and thus the potential number of cells that will be lost as a result of viral lysis. However, this method is reliant on correct identification of the host species from thin sections, which if not accurately determined may lead to over- or underestimation of the impact of viral infection. A second limitation of this method is the requirement for estimates of the proportion of the lytic cycle during which viral particles are visible and knowledge of the virus latent period; presently there are few data for virus-phytoplankton systems (Proctor and Fuhrman 1990; Brussaard et al. 1996). To obtain potential rates of infection, a third approach is to use the contact rate model of Murray and Jackson (1992); for example, see Suttle and Chan (1994). Assumptions must be made, however, that host abundance estimates are accurate and that all viruses have the potential to infect all hosts (Suttle 2000). In natural systems, specificity and range of viral-strain hosts may lead to deviations from the model, which may change the proportion of host cells that are infected (Suttle 2000).

Viral production rates have also been estimated from decay rates of virus communities (Heldal and Bratbak 1991). The principal of this approach is that host mortality rates are determined indirectly as the product of the decay rate of viruses divided by the burst size, and the assumption is that viral production and removal rates are balanced. By the use of fluorescently labeled viruses as tracers (Noble and Fuhrman 2000) rates of both virus production and removal can be simultaneously determined as the change in ratio of labeled versus unlabelled viruses over time. However, both of these methods rely on the caveat that burst size (the number of viruses produced upon host cell lysis) is known. Most studies apply burst size values reported in the literature to derive mortality rates; these reported values may not be appropriate because of the highly variable nature of the burst size parameter to environmental conditions (Wilson et al. 1996; Bratbak et al. 1998). In addition, methods using viral tracers and decay of infectivity (Suttle and Chen 1992) assume that viral tracers are representative of the natural virus communities and rarely take into account the effect of sunlight on viral DNA, which destroys infectivity, but not viral particles (Wilhelm et al. 1998). The latter technique also incurs an additional disadvantage because it is limited to dark incubations to protect the fluorescent stain from light degradation, which negates its use for autotrophic virus-host systems (Noble and Steward 2001). Extrapolation from viral DNA synthesis rates determined using radiolabelled inorganic phosphate can provide phytoplankton virus production rates (Steward et al. 1992). However, this method requires using isotopes with short half-lives, which are challenging to handle, and the use of poorly constrained conversion factors to convert radioactivity into viral production (Noble and Steward 2001).

All of the above methods attempt to derive viral-induced microbial mortality rates indirectly by assessing changes to the virus, i.e., changes in viral abundance, production, or decay. Measurements of viral replication rates must be inextricably linked to host mortality rates, so this seems to be a reasonable assumption; however, it does not provide us with the parameter that is most often lacking, i.e., a direct rate of host cell loss via viral lysis. Viral-induced mortality rates should ideally be measured as directly as possible without the use of inferred assumptions and conversion factors. The "modified dilution" approach was introduced to provide this direct measurement for specific phytoplankton. This method is an adaptation of the original dilution technique developed by Landry and Hassett (1982), which has been used extensively to provide estimates of phytoplankton growth and microzooplankton grazing (e.g., Gallegos 1989; Landry et al. 1995; Worden and Binder 2003). The original dilution approach is based on the theory that by combining whole seawater with grazer-free filtrate in different proportions, grazing impact will be progressively decreased with increasing dilution. Linear regression analysis (Fig. 1) of observed phytoplankton net growth rate over the 24-h incubation period versus experimental dilution ($D$) provides estimates of growth rate in the absence of grazing ($y$-intercept), and grazing mortality rate (slope). The grazer-free water still allows most free viruses to pass through the filter. The modified dilution approach includes an additional dilution step created by combining whole seawater with virus-free filtrate in different proportions, thus allowing the direct measurement of grazing versus grazer and viral-induced mortality. Viral mortality of the phytoplankton can be obtained from the difference in the two relationships (Fig. 2).

To date there are few published studies that have used the modified dilution approach to determine phytoplankton mortality (Evans et al. 2003; Baudoux et al. 2006; 2007; Kimmance et al. 2007; Baudoux et al. 2008; Brussaard et al. 2008), partly because these experiments can be difficult and time-consuming to set up and analyze (e.g., counting live phytoplankton). The labor-intensive nature of these experiments limits their use to perhaps one or two depths per day during field studies. However, at present this method is the only one that attempts to directly measure viral lysis rates of phytoplankton, therefore more studies need to be undertaken to fully validate and develop its utility. Note that this method is distinct from the "viral reduction" approach, which was developed to estimate viral-mediated bacterioplankton mortality (e.g., Parada et al. 2008). This method is often referred to as a dilution approach; however, although free virus abundance is diluted during experimental setup, the prokaryote hosts are concentrated. Viral-induced mortality rates are then obtained indirectly through changes in viral abundance over time. This procedure is distinctly different to the modified dilution method, in which both viruses and host are diluted to create a gradient of lysis pressure and viral lysis rates are derived directly from loss of phytoplankton cells.

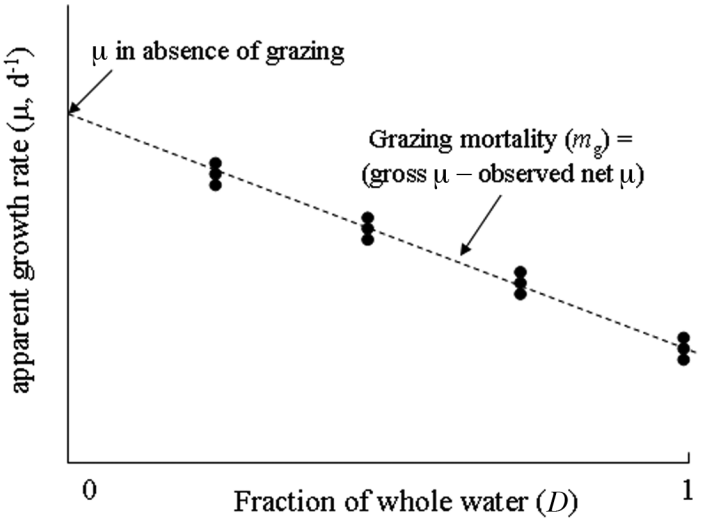


**Fig. 1.** Theoretical dilution plot of apparent phytoplankton growth rate versus fraction of whole water (*D*). Dilution of whole water with <0.45-μm filtrate creates a gradient of grazing, and thus $m_g$ = mortality rate due to grazing and *y*-intercept = gross μ; phytoplankton growth rate in the absence of grazing pressure (μ, d$^{-1}$). Net μ = actual observed phytoplankton growth rate (μ, d$^{-1}$).

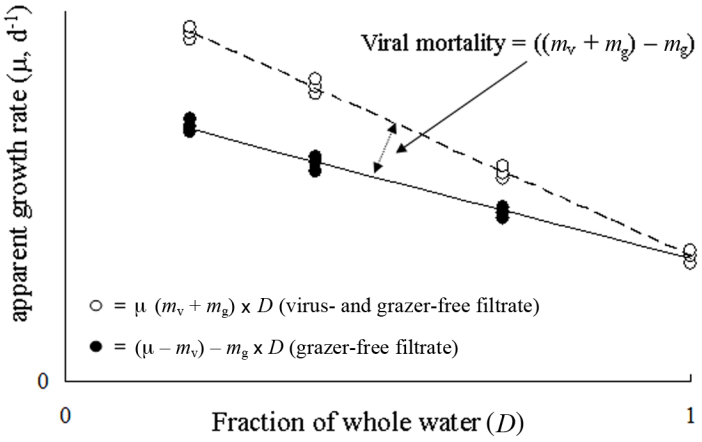


**Fig. 2.** Idealized dilution plots and analysis when whole water is diluted with either <0.45 μm filtered water (● = grazer-free) or kDa filtrate (○ = grazer- and virus-free) to make up the dilution series for incubation. Reducing viral mortality in the kDa dilution series (○) is expected to increase phytoplankton growth rates. Viral mortality rate of the phytoplankton is obtained from the difference in the relationship between μ and *D* in the parallel dilution series (μ = instantaneous growth rate of phytoplankton; $m_v$ = mortality due to viral lysis; $m_g$ = mortality due to microzooplankton grazing; *D* = fraction of whole water).

## *Materials and procedures*

Experiments are set up based on the original protocol of the modified dilution method of Evans et al. (2003), and the following method is appropriate for both natural and cultured phytoplankton–virus systems (Fig. 3). The approach is based around combining mesoplankton-free seawater (whole water) with either grazer-free or virus-free diluents. To prevent contamination of seawater samples by handling, vinyl gloves are worn throughout the water collection and experimental setup. To create mesoplankton-free whole water for experiments, seawater is gently siphoned through 200-μm mesh (or reverse sieved, Baudoux et al. 2006) using silicone tubing into clean, polypropylene carboys. Additional (reverse) prefiltration may be required, e.g., during sampling of colonial phytoplankton (Baudoux et al. 2006) or in productive, coastal, or sediment-filled waters, to prevent clogging during the later fine-scale filtration. Gentle filtration and avoidance of air bubbling are crucial to prevent destruction of the grazer, virus, and phytoplankton populations. To protect the phytoplankton communities within the seawater sample from excessive light (light-shock), experiments using natural phytoplankton populations are best set up predawn in dimmed-light conditions using light-proofed carboys. It is also recommended that experiments be performed at the same time of day because of the synchronicity of phytoplankton cell division and potential diel effects on viral infection processes. Filtration and handling should be conducted at in situ temperature. Handling time once the seawater has been collected should be kept to a minimum before the start of the experimental incubation. Ideally experimental setup should be within an hour of water collection if possible.

To create the grazer-free diluent whole seawater is gravity filtered through acid-washed (*see* below) 0.2–0.45 μm filters (e.g., PALL Acropak™ Supor® membrane capsules) into a clean carboy; a new filter should be used for each dilution experiment (Fig. 3). Prior to each experiment 0.2–0.45 μm filters, silicon tubing, carboys, and bottles should be acid-washed in 5% HCL and rinsed thoroughly with Milli-Q water and then twice with the sample. The 0.2–0.45-μm and kDa filters should be flushed prior to use with freshly prepared Milli-Q and the first few liters of both filtrates discarded. Ensure that all air bubbles are removed from the filter capsule and tubing during filtration by opening the filter valves. To speed up this process you may use two filters in parallel. After gravity filtration half of the grazer-free diluent is passed through a tangential flow filtration system with kilodalton (kDa) pore size to create the virus-free diluent. The designated pore size is variable but typically between 10–100 kDa (Evans et al. 2003; Baudoux et al. 2006; Kimmance et al. 2007). This pore size range is sufficient for removal of both bacteriophage and larger algal viruses such as *Emiliania huxleyi* viruses. The effectiveness of the modified dilution technique is dependent on the efficiency of the 0.2–0.45-μm filtration step to create a gradient of grazing pressure and the kDa filtration step to remove viruses from the 0.2–0.45-μm filtrate, thus creating an additional viral gradient. Assessing the differences in virus abundance between the diluents in comparison with natural samples determines how effective the additional filtration step was at removing viruses and thus producing a gradient of viral pressure.

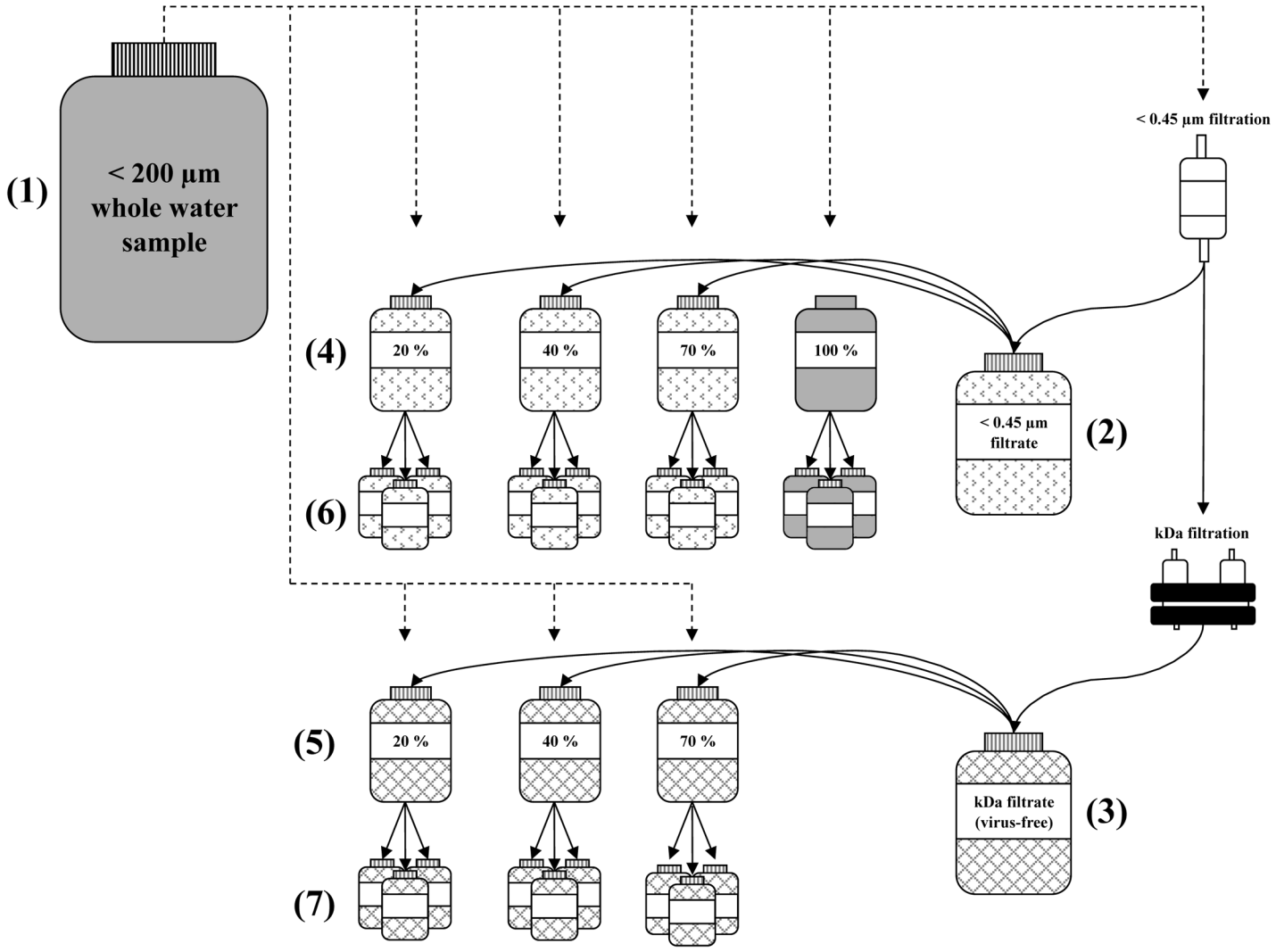


**Fig. 3.** Modified dilution assay experimental design. Mesoplankton-free whole water (1) is combined with either <0.45-µm filtrate (2) or kDa filtrate (3) in the correct proportions to create the parallel $t_0$ dilution series: <0.45µm series, with reduced grazing mortality (4) kDa series, with reduced grazing and viral mortality (5). Replicate sample bottles from the <0.45-µm and kDa dilution series are then created (6 and 7) and incubated under experimental conditions.

The grazer-free and virus-free diluents are added to 10-L polycarbonate bottles in the correct proportions to create the parallel $t_0$ dilution series, e.g., 20%, 40%, 70%, and 100% whole water. The mesoplankton-free whole water is then gently added by siphoning. From each of these $t_0$ bottles, triplicate 1-L polycarbonate bottles were rinsed twice and then gently filled by siphoning (to minimize physical damage to the grazers, viruses, and phytoplankton populations), ensuring that bottles are filled completely to avoid trapping air bubbles inside upon closure. After filling, triplicate experimental bottles are placed randomly into experimental conditions. For natural samples, if in situ incubation is not feasible, then the experimental environment should match the in situ temperature and light conditions (including light–dark period) as closely as possible. Therefore outdoor incubators should be temperature controlled and covered with neutral density screening to match natural light intensity at the sample depth. Laboratory assays should be set up as described above except that viral lysate capable of infecting the chosen host should be added (multiplicity of infection >1) to the cultured host before dilution (Baudoux et al. 2006) and experimental bottles incubated under the appropriate conditions for the chosen cultured virus–host system.

For determination of phytoplankton composition and abundance and virus abundance, triplicate subsamples (5 mL) are taken from the 10 L $t_0$ dilution bottles and the 0.2–0.45-µm and kDa diluents. However, an alternative method is to take samples out of each and every 1-L experimental bottle directly at $t_0$,

making sure that there are no air bubbles in the bottles upon closing. Final time-point samples are also taken from every experimental bottle at the end of the experimental period, 24 h. Initial ($t_0$) and final ($t_{24}$) phytoplankton composition and abundance estimates are typically determined by analysis of samples using flow cytometry (Evans et al. 2003; Baudoux et al. 2006; Kimmance et al. 2007). If flow cytometry is not available other cell-counting methods could be used (e.g., microscopy or COULTER COUNTER®). In the case of monoalgal blooms, chlorophyll measurements are a possible alternative (Evans et al. 2003). However, this is not recommended because of the highly specific nature of viral infection. It is more desirable to detect changes within specific phytoplankton groups, and with flow cytometry distinct groups can easily be discriminated by differences in fluorescence characteristics.

Apparent phytoplankton growth rates (μ, $d^{-1}$) are calculated from each experimental bottle as the changes in abundance during the incubation using the equation:

$$\mu = \ln (P_t/P_0)/t \quad (1)$$

where $P_t$ and $P_0$ are the final and initial measured phytoplankton abundance, respectively, and $t$ is the duration of the experiment. The actual dilution rate is calculated by dividing the $t_0$ phytoplankton abundance in each bottle by the averaged abundance of the replicate $t_0$ 100% counts (3–6 bottles). Model 1 linear regression analysis of apparent growth rates against fraction of whole water is applied to each of the dilution experimental series (0.2–0.45 μm and kDa) to estimate instantaneous growth and mortality due to grazing and/or viral lysis. As explained above, the regression coefficient of apparent growth rate versus fraction of whole water for the <0.45-μm dilution series represents the microzooplankton grazing rate [$m_g$ = (μ in the absence of grazing – observed net μ) = slope], whereas the regression coefficient from the kDa dilution series represents the combined mortality impact of both grazers and viruses ($m_g + m_v$), where $m_v$ = viral mortality (Fig. 2). The viral-induced phytoplankton mortality rate therefore is the difference between these two regression slopes, i.e., $m_v = [(m_v + m_g) - m_g]$. However, a significant rate can be derived only when there is a significant difference between the two mortality slopes. To determine this, experiments that produce significant regression slopes for both the 0.2–0.45-μm and kDa dilution series are further analyzed, e.g., using an F-test. Only then can the difference between the two slopes be considered a significant viral lysis rate.

## Assessment

The modified dilution approach is promising in that it is the only method that can potentially partition phytoplankton mortality into grazer- versus viral-induced fractions and does not require the use of conversion factors, which can introduce error. However, there are limitations associated with this method. As described above, one of the fundamental assumptions of the original Landry and Hassett dilution method (1982) is that phytoplankton mortality during the incubation period is a result of an encounter between grazer and prey, and the grazing impact will vary with dilution. The concept of prey (host) encounter is also fundamental to the modified dilution approach. However, phytoplankton cells may already be infected at the beginning of the experiment, and thus this component of cell lysis will not vary with dilution. Only new infection is detected during the experiment. The method is based on changes in phytoplankton cell abundance and thus the period to cell lysis of the algal host will dictate whether measurable cell lysis occurs during the incubation (Evans et al. 2003; Baudoux et al. 2006) and not the latent period of the virus. As long as the time to lysis of cells is longer than 12 h and shorter than 24 h we can determine the viral lysis rate. It does not matter whether reinfection is happening during this experimental time period because the cells that are infected by the newly released viruses will not undergo lysis before the end of the experiment. An incubation period of 24 h is optimum because a shorter time is not suitable for investigating phased algal growth, and a longer time period induces bottle effects due to containment. During long experimental incubations containment will influence the apparent growth rate estimates and affect the outcome of experiments.

Although nutrient adjustment has previously been suggested to ensure that phytoplankton growth rates are independent of the dilution effect (Landry et al. 1995), the addition of nutrients is not recommended here as it may produce unnatural growth rates. Care should be taken with the interpretation of results, especially under incubation conditions in which, e.g., nutrients may have become limiting. If nutrient depletion occurs to the same degree in all dilutions then growth rate may be underestimated but the grazing/viral mortality rate should not be affected. However, depletion of a recycled limiting nutrient would occur to a greater degree in the highest dilution with the fewest grazers/viruses; this would lower the slope of the regression and underestimate both grazing and growth rates (Dolan and McKeon 2004). Typically, dilution effects on phytoplankton growth rate are detectable when plotting net growth against dilution factor, particularly if combined with physiological assessment during the experiments. Therefore if nutrient limitation effects are visible then it may be acceptable to remove the data points at the highest level of dilution when determining the regression slopes.

The original dilution protocol was modified to include an independent measure of "relative grazing activity" to improve the accuracy of grazing rate measurements (Landry et al. 1995). This internal measure (uptake of fluorescently labeled prey) was introduced because of the potential violation of the assumption that grazing impact varies in direct proportion to the dilution of grazer population density. In theory the mechanism of viral infection is simpler than grazing in that a single virus infects only a single host. Therefore, the premise that grazer–prey interactions should follow a linear rather than

nonlinear response with dilution should also apply to virus–host encounter and infection. However, like grazers, viruses and their hosts are not just inert particles and so in reality may not follow simple encounter rate theory. Whether this hypothesis remains true throughout the experimental period has not yet been tested for natural virus–host communities to our knowledge; however, viral lysis has been shown to remain linear to dilution factor in a model *Phaeocystis globosa* system (Baudoux et al. 2006). This is an important assumption of the modified dilution technique that needs further clarification using natural populations. Other factors such as threshold host concentrations and host defense mechanisms may also cause a deviation from the linear viral lysis model. Gallegos (1989) showed that dilution regressions can become nonlinear as a result of prey density thresholds for microzooplankton grazing. The possibility of threshold concentrations also needs to be considered during modified dilution experiments, and the level of abundance of both phytoplankton and grazers/viruses must be adjusted to allow a high enough encounter rate even at the lowest level of dilution. The effect of reduced viral mortality because of a host threshold in the dilute treatments could produce a nonlinear response and thus an underestimation of viral lysis rate. Another factor to consider is the possibility that grazers may preferentially graze viral-infected cells. As yet this theory remains to be tested for other phytoplankton species and in natural populations; however, Evans and Wilson (2008) suggest that under culture conditions grazers may actively select for infected versus noninfected phytoplankton prey. If this does occur typically during the dilution experiments, it would cause an underestimation of mortality rates and have serious implications for the use of the modified dilution approach to measure viral lysis rates.

Ultimately, the success of this approach is reliant on the production of two significant mortality rates ($m_v = [(m_v + m_g) - m_g]$) and thus it is vital to have an appropriate sample number in both dilution series to be able to detect a significant difference between the two regression slopes. The accuracy of the mortality data are dependent on the precision of the apparent growth-rate measurements, which ultimately depends on the phytoplankton cell counts. The ability to quantify initial $t_0$ and $t_{24}$ phytoplankton abundance within acceptable levels of precision (i.e., <5 % standard error) is fundamental for the success of this method. Thus it is critical that analyses of $t_0$ and $t_{24}$ phytoplankton cell counts conducted using fresh samples are analyzed as quickly as possible. Relatively precise estimates of initial and final abundances are needed to minimize the error generated through calculations of population rates of change. Sources of error can arise from imprecise subsampling and variability between replicates at the same dilution. Furthermore, attempting to enumerate distinct subpopulations can be particularly challenging in areas of low productivity, because detecting mortality rates based on small changes in cell concentrations is not easy. Phytoplankton abundance is relatively low in oligotrophic waters, which creates a problem because of the high levels of dilution required for this assay. As mentioned above, cell counts are typically analyzed using flow cytometry and live samples. The use of flow cytometry instead of chlorophyll *a* is recommended because more detailed information can be obtained for viral-induced mortality of specific algal groups. Because viruses are specific to phytoplankton species and at times even strains, it is important not to use bulk measurements such as chlorophyll *a*. However, in some environments sample analysis can take too long because of the difficulty of gaining a statistically viable cell count at the most dilute level of dilution. This difficulty has serious implications because it extends the analysis time for the whole dilution series (21 samples if experiment has triplicate bottles at four dilution levels). During this long analysis time there may be changes in phytoplankton host physiology or loss of cells, which may affect the outcome of the experiments. Therefore fixation is occasionally necessary. However, because it is well established that fixation in itself causes some degree of cell loss (e.g., Vaulot et al. 1989), this effect of fixation should be tested to ensure that the percentage of cell loss is the same regardless of dilution factor.

As described above, the ability to measure a significant viral lysis rate during modified dilution experiments is dependent on the presence of a detectable difference between the two regression slopes, and when viral mortality rates are low the detection of this difference becomes difficult. Therefore to increase the chance of detecting low rates it is critical that the experimental design is appropriate for the phytoplankton communities studied. To detect significant differences between the two slopes, experiments need to have a large enough *n* number (number of samples in each parallel dilution series). Slight slopes or low grazing rates are difficult to detect with regression analysis using the small *n* values commonly employed during dilution experiments (Dolan and McKeon 2004). This can be tested, however, and to alleviate this problem Kimmance et al. (2007) suggested adding a sensitivity test to the modified dilution assay. This additional analysis may improve the efficiency of dilution experiments by demonstrating the level of modification that needs to be made to the experimental design, i.e., determining the appropriate *n* value for production of significant results (Kimmance et al. 2007). However, even if the analysis is not applied before experiments are conducted it can be useful for determining retrospectively the level at which the regression analyses and subsequent F-tests would have had the power to detect differences between the mortality slopes. From the literature studies conducted so far, testing the sensitivity of the experimental approach indicates that viral lysis rates <0.1 $d^{-1}$ were not effectively estimated using the modified dilution method (Kimmance et al. 2007, Baudoux et al. 2008).

## Discussion

Since its development in 2003 the modified dilution assay has been applied to several different environments, including

seminatural mesocosm (Evans et al. 2003), coastal (Baudoux et al. 2006; Kimmance et al. 2007), open ocean (Brussaard et al. 2008), and oligotrophic (Baudoux et al. 2007; 2008), to test its ability to determine viral lysis rates of natural phytoplankton communities. Results from these published studies clearly show that rates vary in response to environmental conditions and between different algal groups. The modified dilution method was first tested during such conditions in a nutrient-enriched mesocosm experiment. Using this approach Evans et al. (2003) successfully determined viral-induced mortality rates of *Micromonas* spp. of 0.10–0.29 $d^{-1}$ and showed that up to 34 % of *Micromonas* spp. production could be lysed daily by viruses. More recently Baudoux et al. (2006) demonstrated that this approach could also be applied successfully during a bloom of *P. globosa*. In this more productive, coastal system the viral-induced mortality was higher, accounting for up to 66% of the total *P. globosa* mortality, with maximum lysis rates of 0.35 $d^{-1}$ measured. However, in both of these studies there was a dominant phytoplankton species and viruses were the major mortality agents.

Other published data sets suggest that in more diverse communities significant viral lysis rates are not always as readily attainable with this method (Kimmance et al. 2007; Baudoux et al. 2008). In microbial systems in which low biomass and high diversity predominate, the chances of successfully measuring lysis events using the modified dilution approach may be lower. Recently Baudoux et al. (2007) and Brussaard et al. (2008) tested this method in less productive, oligotrophic waters. In the subtropical northeastern Atlantic, Baudoux et al. (2007) found that significant viral lysis rates could be obtained at five of six stations, and lysis of 1 group of picoeukaryotes was responsible for 50–100% of the total cell mortality with rates of 0.1–0.8 $d^{-1}$ (Baudoux et al. 2007). However, these experiments were restricted to phytoplankton populations from the deep chlorophyll maximum where cell numbers were sufficiently high to avoid the problems associated with a three- to four-fold dilution, which is essential for this methodology (Baudoux et al. 2007). Substantial viral lysis rates (0.16–0.23 $d^{-1}$) were also obtained for picoeukaryotes during true oligotrophic conditions in the North Sea, but lower viral lysis rates were not significantly different from zero (Baudoux et al. 2008). Kimmance et al. (2007) were unable to measure any significant viral-induced mortality rates at a coastal site in the western English Channel where picophytoplankton were the dominant phytoplankton group. Low, nonsignificant lysis rates (0.09–0.18 $d^{-1}$) were also obtained for phytoplankton populations in the Southern Ocean (Brussaard et al. 2008). Such low rates of viral lysis may be common in natural nonbloom conditions (which may present a challenge for the use of this method in some environments). Therefore more studies need to be conducted to determine the ability of the modified dilution assay to accurately detect such low viral lysis rates. However, we should also investigate virus dynamics at an array of host densities using laboratory cultured systems. By modeling virus–host encounter rates and infection dynamics from a variety of cultured phytoplankton groups we can determine the sensitivity range, i.e., the highest and lowest viral lysis rates that can be measured using this method. Thus, we can assess the suitability of this approach for estimating viral lysis rates in both low and high productivity environments.

Unfortunately there are limited data available to compare the rates obtained using the modified dilution assay to those obtained with an alternative method. Of the viral lysis studies published so far most do not include a comparative assay for estimating the viral impact on phytoplankton. However, Baudoux et al. (2006) demonstrated the utility and validity of the viral dilution method using both a cultured system and natural populations. Comparison of total cell lysis rates (obtained using the dissolved esterase activity assay) and growth-cycle lysis experiments to viral-induced mortality rates (derived from the viral dilution assay) showed good agreement. Thus, the consistency of the viral lysis rates obtained by this method with other means for assessing cell lysis provides confidence in the suitability of this method to infer the impact of viruses on phytoplankton mortality (Baudoux et al. 2006).

## Comments and recommendations

Although there are assumptions associated with the modified dilution approach that have not yet been tested and further application of this technique needs to be conducted with natural populations of phytoplankton, at present it is the only method that can potentially directly derive viral lysis rates from phytoplankton mortality. It is also the only single method that can in theory provide concurrent estimates of grazing and viral-induced mortality. However, low (natural) levels of viral mortality will challenge this method unless it can be modified to include more replicates and better precision. By improving experimental design through increased number of replicates, diluting biomass to an appropriate level to allow virus–host encounters even at the lowest level of dilution, prolonging counting time for the more diluted samples (provided the in situ temperature can be maintained), assessing taxon-specific mortality rates, and combining this approach with methods that assess host/viral diversity and host specificity, this method may be able to provide realistic estimates of viral mortality in natural waters. Other practical issues to consider include: ensuring precision in measurements of variables used to determine phytoplankton apparent growth rates, considering the use of nonstandard levels of dilution appropriate to abundance levels of natural host/virus populations, assessing the physiological state of host populations during incubation, and determining the sensitivity of the regression analyses by assessing the power of the test to provide an indication of the accuracy and error associated with the analyses, and thus provide a more powerful indication of "real" versus experimentally induced variation.

This report highlights the importance of monitoring various parameters during experimental incubations, particularly when trying to estimate phytoplankton growth and mortality rates from natural waters. Certain aquatic environments present more challenges than others and it may be that the modified dilution assay is best suited to productive waters. Experiments conducted in oligotrophic conditions may require modifications from the typical methodological approach, such as fixed versus live cell counts (*see* "Assessment" above). Although the modified dilution approach requires adaptations to suit the environmental conditions being investigated and needs further testing in natural waters, it has the potential to be a useful tool for estimating the impact of viruses on phytoplankton populations. Furthermore, because of the removal of grazers and viruses, these studies have the potential to provide estimates of other variables of interest to marine microbiologists; factors such as nutrient recycling and dissolved organic matter production can be assessed in the presence and absence of key food web components. What must be maintained, however, is the statistical rigor that is fundamental to this method. Only then will the rates obtained from this approach improve our understanding of the role of viruses on primary production-mediated carbon cycling.

## References


Baudoux, A.-C., A. A. M. Noordeloos, M. J. W. Veldhuis, and C. P. D. Brussaard. 2006. Virally induced mortality of *Phaeocystis globosa* during two spring blooms in temperate coastal waters. Aquat. Microb. Ecol. 44:207-217.

———, M. J. W. Veldhuis, H. J. Witte, and C. P. D. Brussaard. 2007. Viruses as mortality agents of picophytoplankton in the deep chlorophyll maximum layer during IRONAGES III. Limnol. Oceanogr. 52:2519-2529.

———, ———, A. A. M. Noordeloos, G. van Noort, and C. P. D. Brussaard. 2008. Estimates of virus- vs. grazing induced mortality of picophytoplankton in the North Sea during summer. Aquat. Microb. Ecol. 52:69-82.

Bratbak, G., M. Heldal, S. Norland, and T. F. Thingstad. 1990. Viruses as partners in spring bloom microbial trophodynamics. Appl. Environ. Microbiol. 56:1400-1405.

———, A. Jacobsen, M. Heldal, K. Nagasaki, and F. Thingstad. 1998. Virus production in *Phaeocystis pouchetii* and its relation to host cell growth and nutrition. Aquat. Microb. Ecol. 16:1-9.

Brussaard, C. P. D. 2004. Viral control of phytoplankton populations: A review. J. Euk. Microbiol. 51:125-138.

———, R. S. Kempers, A. J. Kop, R. Riegman, and M. Heldal. 1996. Virus-like particles in a summer bloom of *Emiliania huxleyi* in the North Sea. Aquat. Microb. Ecol. 10:105-113.

———, K. R. Timmermans, J. Uitz, and M. J. W. Veldhuis. 2008. Virioplankton dynamics and virally induced phytoplankton lysis versus microzooplankton grazing southeast of the Kerguelen (Southern Ocean). Deep-Sea Res. II 55:752-765.

Dolan, J. R., and K. McKeon. 2004. The reliability of grazing rate estimates from dilution experiments: Have we overestimated rates of organic carbon consumption? Ocean Sci. Disc. 1:21–36.

Evans, C., S. D. Archer, S. Jacquet, and W. H. Wilson. 2003. Direct estimates of the contribution of viral lysis and microzooplankton grazing to the decline of a *Micromonas* spp. population. Aquat. Microb. Ecol. 30:207-219.

———, and W.H. Wilson. 2008. Preferential grazing of *Oxyrrhis marina* on virus infected *Emiliania huxleyi*. Limnol. Oceanogr. 53:2035-2040.

Fuhrman, J. A. 1999. Marine viruses and their biogeochemical and ecological effects. Nature 399:541-548.

Gallegos, C. L. 1989. Microzooplankton grazing on phytoplankton in the Rhode River, Maryland: Nonlinear feeding kinetics. Mar. Ecol. Prog. Ser. 57:23-33.

Heldal, M., and G. Bratbak. 1991. Production and decay of viruses in aquatic environments Mar. Ecol. Prog. Ser. 72:205-212.

Kimmance, S. A., W. H. Wilson, and S. D. Archer. 2007. Use of the modified dilution technique to estimate viral versus grazing mortality of phytoplankton: limitations associated with method sensitivity in natural waters. Aquat. Microb. Ecol. 49:207-222.

Landry, M. R., and R. P. Hassett. 1982. Estimating the grazing impact of marine microzooplankton. Mar. Biol. 67:283-288.

———, J. Kirshtein, and J. Constantinou. 1995. A refined dilution technique for measuring the community grazing impact of microzooplankton, with experimental tests in the central equatorial Pacific. Mar. Ecol. Prog. Ser. 120:53-63.

Murray, A. G., and G. A. Jackson. 1992. Viral dynamics: A model of the effects of size, shape, motion and abundance of single-celled planktonic organisms and other particles. Mar. Ecol. Prog. Ser. 89:103-116.

Noble, R. T., and J. A. Fuhrman. 2000. Rapid virus production and removal as measured with fluorescently labeled viruses as tracers. Appl. Environ. Microbiol. 66:3790-3797.

———, and G. F. Steward. 2001. Estimating viral proliferation in aquatic samples, p. 67-84. *In* J. H. Paul [ed.], Marine microbiology. Academic Press.

Parada, V., A.-C. Baudoux, and E. Sintes. 2008. Dynamics and diversity of newly produced virioplankton in the North Sea. ISME J. 2:924–936.

Proctor, L. M., and J. A. Fuhrman. 1990. Viral mortality of marine bacteria and cyanobacteria. Nature 343:60-62.

Ruardij, P., M. J. W. Veldhuis, and C. P. D. Brussaard. 2005. Modeling the bloom dynamics of the polymorphic phytoplankter *Phaeocystis globosa*: Impact of grazers and viruses. Harmful Algae 4:941-963.

Steward, G. F., J. Wikner, D. C. Smith, W. P. Cochlan, and F. Azam. 1992. Estimation of virus production in the sea, I: Method development. Mar. Microb. Food Webs 6:57-78.

Suttle, C. A. 2000. Ecological, evolutionary, and geochemical consequences of viral infection of cyanobacteria and

eukaryotic algae. p 247-296. *In*: C. J. Hurst. 2000. Viral ecology. Academic Press, London.

———. 2007. Marine viruses-major players in the global ecosystem. Nature Rev. Microbiol. 5:801-812.

———, and A. M. Chan. 1994. Dynamics and distribution of cyanophages and their effect on marine *Synechococcus* spp. Appl. Environ. Microbiol. 60:3167-3174.

———, and F. Chen. 1992. Mechanisms and rates of decay of marine viruses in seawater. Appl. Environ. Microbiol. 58:3721-3729.

Vaulot, D., C. Courties, and F. Partensky. 1989. A simple method to preserve oceanic phytoplankton for flow cytometric analyses. Cytometry 5:629-635.

Weinbauer, M. G., Fuks, D. and Peduzzi, P. 1993. Distribution of viruses and dissolved DNA along a coastal trophic gradient in the Northern Adriatic Sea. Appl. Environ. Microbiol. 59:4074-4082.

Wilhelm, S. W., M. G. Weinbauer, C. A. Suttle, and W. H. Jeffrey. 1998. The role of sunlight in the removal and repair of viruses in the sea. Limnol. Oceanogr. 43:586-592.

———., and C. A. Suttle. 1999. Viruses and nutrient cycles in the sea: Viruses play critical roles in the structure and function of aquatic food webs. Bioscience 49:781-788.

———, S. M. Brigden, C. A. Suttle. 2002. A dilution technique for the direct measurement of viral production: a comparison in stratified and tidally mixed coastal waters. Microb. Ecol. 43:168-173

Wilson, W. H., Carr, N. G., and Mann, N. H. 1996. The effect of phosphate status on the kinetics of cyanophage infection in the oceanic cyanobacterium *Synechococcus* sp. WH7803. J. Phycol. 32:506-516.

Worden, A. Z., and B. J. Binder. 2003. Application of dilution experiments for measuring growth and mortality rates among *Prochlorococcus* and *Synechococcus* populations in oligotrophic environments. Aquat. Microb. Ecol. 30:159-174.