Chapter 40
Measurement of Virus-Induced Phytoplankton Mortality

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Abstract Viruses are the most abundant biological entities in global ecosystems. Viral infection and lysis are one of the major causes of phytoplankton mortality. The methods used to measure the virus-induced phytoplankton mortality include the modified dilution assay and indirect estimations based on the measurements of viral production, viral infection rate, viral contact rate, and viral decay rate. The modified dilution method is the only approach that can determine and differentiate directly the virus- and grazer-induced phytoplankton mortality and does not require the use of conversion factors, thus avoiding introducing error. However, cautions should be made when applying the modified dilution method to natural assemblages of phytoplankton because the method requires accurate measurement of the phytoplankton abundance and significant difference between virus- and grazer-induced phytoplankton mortality.

Keywords Infection · Modified dilution assay · Mortality · Phytoplankton · Viruses

40.1 Introduction

Viruses are ultramicroscopic and infectious organisms that can multiply only in living cells of animals, plants, or bacteria/archaea and do not have a cellular structure, thus considered as "organisms at the edge of life." The sizes of viruses range mostly from 20 to 350 nm, averaging 100 nm. Viruses usually consist of the

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genetic materials and a protein coat, which surrounds the genetic materials. In some cases, an outside envelope of lipids or saccharides is also found in some viruses. A virus has either a DNA (single- or double-stranded DNA) or a RNA (single- or double-stranded RNA) genome but never both. While not inside the host cell or in the process of infecting a cell, viruses exist in the form of free-living independent particles. Once getting into the host cell, viruses release their genomes and direct host cells to ultimately produce viral proteins and genetic materials, packaging new viral particles. The new viruses can be released from the host cell by lysis and start new cycles of infection. Marine viruses mainly include bacteriophage, which infects bacteria, and phycovirus, which infects eukaryotic algae. The known phycoviruses are mostly double-stranded DNA viruses, with diameter ranging from 100 to 220 nm and genome sizes from 100 to 560 kb. Recently, single-stranded DNA viruses and single- or double-stranded RNA viruses have also been reported, which indicate the hidden diversity of unknown marine viruses (Tucker et al. 2011; Steward et al. 2013).

Viruses are ubiquitous and the most abundant biological agent in aquatic environments, with a typical surface water abundance of 10^6 virus-like particles per milliliter and a total of 4×10^{30} virus-like particles in the sea. It is widely accepted that viruses play key roles in regulating biogeochemical cycles in the ocean (Fuhrman 1999; Wommack and Colwell 2000; Weinbauer 2004; Suttle 2005, 2007; Brussaard et al. 2008a). Viral lysis has been proposed as one of the major causes of phytoplankton mortality. In the microbial loop, protists that graze on phytoplankton will transfer the energy and substances to higher trophic level. However, viral lysis of phytoplankton cells converts cells into viruses and cellular debris, the latter of which consists of dissolved molecules (monomers, oligomers, and polymers), colloids, and cell fragments, which is termed as viral shunt. It has been estimated that approximately 6–26% of primary production through photosynthesis has been channeled back to dissolved organic carbon pool in the ocean (Fuhrman 1999; Wilhelm and Suttle 1999). In the coastal as well as open ocean environments, when the spring/autumn algal blooms occur, phytoplankton populations rapidly increase, followed by the accumulations of viruses. It has been reported that some phytoplankton blooms can be terminated through large-scale lytic events (Bratbak et al. 1993).

40.2 Estimation of Viral Abundance

Three methods are generally used to estimate the abundance of viruses in aquatic samples, including transmission electron microscopy (TEM), epifluorescence microscopy (EFM), and flow cytometry (FCM). Viruses from the environmental samples or lab cultures are concentrated through filtering or ultracentrifuge and deposited onto TEM grids and stained with an electron-dense material (e.g., uranyl acetate) (Børsheim et al. 1990). In addition to the abundance of viruses, TEM observation can also provide information about the key morphological characters

of the viruses, such as the size of the capsid, the length of the tails, and fibers which are important to determine the type of the viruses. Direct observations of virally infected phytoplankton cells using TEM can be used to estimate virus induced mortality rates of phytoplankton. EFM is currently the most widely used approach for estimating the abundances of viruses, which was first used for viruses in 1990 (Suttle et al. 1990). A certain volume (typically 1 mL) of seawater is vacuum-filtered onto a filter with a 0.02 mm pore size, followed by staining with the nucleic acid dyes such as SYBR Gold/Green. The filter was then mounted on a slide and observed under an epifluorescence microscope which will give the abundance but not the size and classification of viruses owing to the intrinsic limitation of resolution given by the light microscopy. More recently, FCM has been used to estimate the abundance and biomass of viruses, which is also used to quantify or even sort subpopulations of viruses that differ in their characteristics of fluorescence after stained by the nucleic acid dyes such as SYBR Gold/Green (Marie et al. 1999; Mojica et al. 2014; Brussaard et al. 2010). For example, viruses (e.g., members in Phycodnaviridae family) infecting eukaryotic algae usually have more fluorescence and scatter and thus can be differentiated from virus-infecting prokaryotes that have relatively lower nucleic acid content.

40.3 Measurement of Virus-Induced Phytoplankton Mortality

Currently, studies on the virus-induced phytoplankton mortality and its impact on the local and global carbon cycling are rather scarce, with lack of method being one of the major reasons. To determine the impact of virus on its hosts, various approaches have been developed (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). However, most of these approaches have been designed for bacterioplankton and may not be applicable to phytoplankton. One of the conventional methods would be using TEM to quantify the frequency of virus-infected host cells to further estimate the number of cells lysed. In the other method, the rates of virus production are determined on the basis of the rate of ^{32}P-orthophosphate ($^{32}P_i$) or ^{3}H-thymidine (^{3}H-TdR) incorporation into the DNA of the viruses released from host cells, and the mortality rates of hosts are thus calculated (Steward et al. 1992; Weinbauer et al. 1993). To obtain the potential rates of viral infection, contact rate model of virus and host cells and decay rate of virus have been incorporated by Murray and Jackson (1992). However, the above methods all use viral abundance, production, or decay rates to estimate the virus-induced host mortality rate indirectly.

The modified dilution assay, which was adapted from the original dilution approach (Landry and Hassett 1982), is the only one at present that attempts to directly partition phytoplankton mortality into virus- versus grazing-induced

fractions (Gallegos 1989; Landry et al. 1995; Worden and Binder 2003). The original dilution method was designed for estimating the micro-zooplankton grazing impact on natural communities of marine phytoplankton. Three assumptions were made which serve as the basis of the method: (1) the growth of individual phytoplankton is not directly affected by the presence or absence of other phytoplankton within the same community; (2) the probability of a phytoplankton cell being consumed is a direct function of the rate of encounter of consumers with prey cells; and (3) change in the density of phytoplankton (P), over some time (t), can be represented appropriately by the exponential equation $P_t = P_0 e^{(k-g)t}$ (Landry and Hassett 1982). According to the protocol, half of sampled seawater was filtered to remove grazers and then combined with the remaining, unfiltered seawater in different ratios of unfiltered to filtered water to get a series of "diluted" grazer-containing incubations. After a certain period (usually 24 h) of incubation in in situ waters, instantaneous coefficients of phytoplankton growth (k) and micro-zooplankton grazing (g) could be obtained from least squares and linear regression analysis of the relationship between the rate of change of chlorophyll and the fraction of unfiltered seawater in the various bottles (Landry and Hassett 1982). In the grazer-free filtered seawater, most viruses are usually detained due to the size of filter used. In the modified dilution assay, further mixing step involving the virus-free and unfiltered seawater at certain proportions was generated. The phytoplankton mortality induced by grazer alone and grazer and virus combined was measured. The virus-induced mortality was thus obtained by deducting grazer-induced mortality from grazer and virus combined phytoplankton mortality rates.

Seawater with high primary production, undergoing a bloom event, or with agitated sediment substances needs to be prefiltered to prevent blockage during filtration. Slow filtration is expected to avoid formation of bubbles to impact the grazer, virus, and phytoplankton communities. Due to phytoplankton cell fission and potential diurnal change effects on viral infection, experiments should be set up at the same time during the same day. Filtration and setting up of experiments should be performed at the in situ temperature. The incubation is recommended to be performed in situ or under the same temperature and light conditions to simulate the in situ environment.

The success of the modified dilution method relies on the production of the dilution efficiencies of both grazing and viral lysis effects. One of the basic assumptions of the modified dilution method is that grazing and lysis impacts on phytoplankton cells vary in direct proportion to the dilution of grazers and lysis population abundances. However, phytoplankton cells may already be infected at the beginning of the experiment, and viral lysis of this group of cells cannot be serially diluted, and only new infection will be detected. Twenty-four hours of incubation period with no nutrition addition is preferred because the addition of extra nutrients can cause unnatural growth of phytoplankton cells.

Since its first application on the determination of viral lysis on a *Micromonas* spp. population (Evans et al. 2003), the modified dilution method has been successfully applied in various marine environments to directly determine virus-induced phytoplankton mortality including mesocosm (Evans et al. 2003), coastal area (Baudoux

et al. 2006; Kimmance et al. 2007), estuary (Tsai et al. 2015b), as well as oligotrophic open ocean (Baudoux et al. 2007; Brussaard et al. 2008b; Baudoux et al. 2008). It has been reported that during a mesocosm study, the virus-induced *Micromonas* spp. mortality rates ranged from 0.10 to 0.29 day^{-1}, and up to ~34% of *Micromonas* spp. production could be lysed daily by viruses. Baudoux et al. (2006) used this method to study virus-mediated mortality rates of *Phaeocystis globosa* during two consecutive spring blooms and found that during the bloom events, viral lysis was the major cause of *P. globosa* mortality (up to ~0.35 day^{-1}), which is comparable to that due to microzooplankton grazing (up to 0.4 day^{-1}). Later, Baudoux et al. (2007) studied the virus-induced mortality of picophytoplankton in the deep chlorophyll maximum layer and found that viral lysis was responsible for 50–100% of the cell losses of picoeukaryotic phytoplankton cells, with mortality rates ranging from 0.1 to 0.8 day^{-1}. In a recent study, Mojica et al. (2016) investigated virus-induced phytoplankton mortality rates along a latitudinal gradient within the North Atlantic Ocean. The study reported that virus-induced mortality was the major loss process at low and mid latitudes, whereas at higher latitudes microzooplankton grazing surpassed virus-induced mortality. Tsai et al. (2012) studied diel variations in the protozoan grazing and virus-mediated mortality of *Synechococcus* spp. in the coastal waters off Taiwan and subtropical western Pacific Ocean and found that grazing was the dominant cause of *Synechococcus* spp. mortality during daytime; however, virus-induced and nanoflagellate grazing-mediated mortalities were more balanced at night. A study performed in a subtropical estuary reported that viral lysis overran microzooplankton grazing to be the major cause of *Synechococcus* spp. losses upriver and in the estuary (Tsai et al. 2015a).

40.4 Evaluation of Techniques

The approach based on the frequency of infected cells using TEM does not need incubation and is a straightforward method to derive the virus-mediated phytoplankton mortality. However, relatively high number of viruses (at least 10^5 particles mL^{-1}) are required for TEM observation. Therefore, samples from oligotrophic environments with low microbial abundance should be concentrated before TEM observation, which will introduce bias (Weinbauer and Suttle 1997). In addition, this method relies on the knowledge of lytic cycle of host-virus system, such as the time periods when viruses are visible in cells and viral latent period. Unfortunately, these information for phytoplankton-virus system are very scarce (Proctor and Fuhrman 1990; Brussaard et al. 1996). Virus-induced mortality of the phytoplankton can also be obtained from the result of the viral production divided by the burst size. Viral decay rate can be used instead of viral production, and the assumption is that viral production and viral decay rates are balanced. This approach relies on the known burst size, as well as high-frequency sampling during incubation. It is worthy of pointing out that decay of viral particles will occur during the measurement of viral production, and this will introduce errors for the calculation of viral production

and then virus-induced mortality of the phytoplankton. The modified dilution approach is the only method that can simultaneously estimate virus- and grazing-induced phytoplankton mortality without the use of conversion factors. However, relative high abundances of phytoplankton and grazers/viruses are required to ensure a high encounter rate even at the lowest level of dilution. Accurate measurement of phytoplankton abundance is also needed. The detectable difference between the slopes of two regressions is fundamental for the calculation of virus-induced mortality rate, which is difficult in case of low virus-induced mortality, such as in oligotrophic environments (Baudoux et al. 2008; Kimmance et al. 2007; Brussaard et al. 2008b). In order to increase the utility of the modified dilution method to estimate the viral mortality of phytoplankton in natural communities, laboratory studies of virus-host systems at different density are urged to determine their encounter rates and infection dynamics.

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