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

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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.

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Guidelines

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. 1). 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.

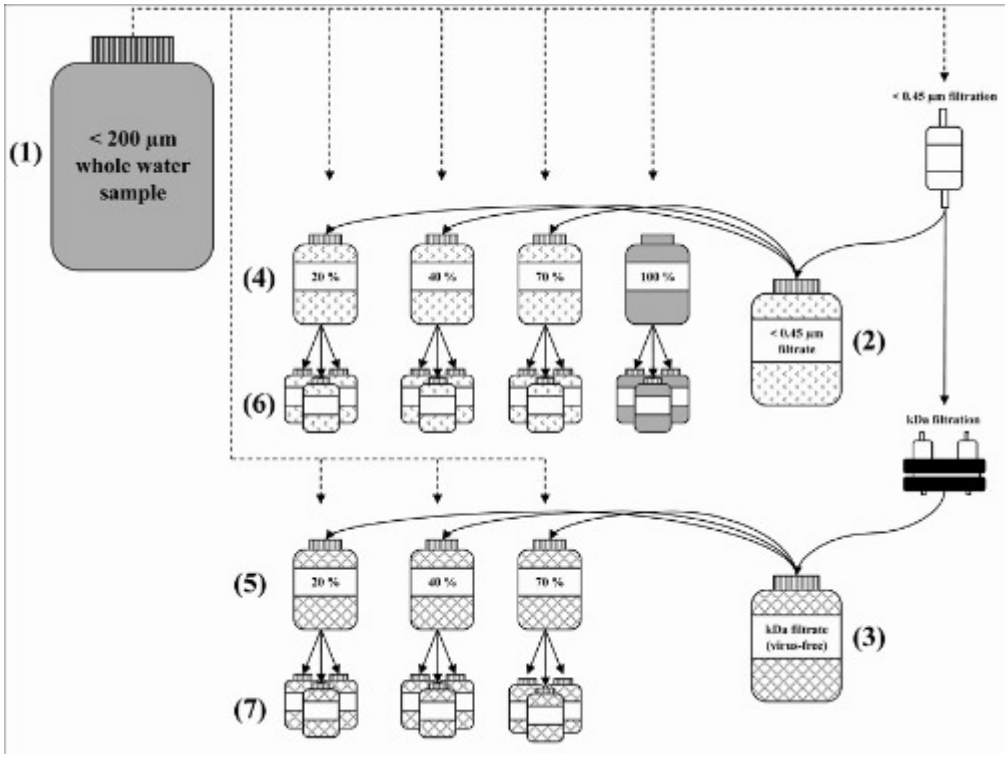

Figure 1: Modified dilution assay experimental design. Mesoplankton-free whole water (1) is combined with either <0.45-µm filtrate (2) or kDa filtrate (3) in the correct proportions to create the parallel t0 dilution series: <0.45µm series, with reduced grazing mortality (4) kDa series, with reduced 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.

References

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Before start

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.

Protocol

Step 1.
To create the grazer-free diluent whole seawater is gravity filtered through acid-washed 0.2–0.45 µm filters (e.g., PALL Acropak™ Supor® membrane capsules) into a clean carboy.

NOTES

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A new filter should be used for each dilution experiment.

Step 2.
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.

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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.

Step 3.
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.

Step 4.
The mesoplankton-free whole water is then gently added by siphoning.

Step 5.
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.

Step 6.
After filling, triplicate experimental bottles are placed randomly into experimental conditions.

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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.

Step 7.
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.

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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.

Step 8.
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).

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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.

Step 9.
Apparent phytoplankton growth rates (µ, d^{-1}) are calculated from each experimental bottle as the changes in abundance during the incubation using the equation:

$$\mu = \ln(P_t/P_o)/t$$

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where P_t and P_0 are the final and initial measured phytoplankton abundance, respectively, and t is the duration of the experiment.

Step 10.
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).

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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.