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# Flow cytometric analysis of phytoplankton viability following viral infection

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ABSTRACT: Two flow cytometric assays using physiological probes were used on the phytoplankton species *Phaeocystis pouchetii* and *Micromonas pusilla* to examine the assays' utility in detecting viral infections. Dead cells were detected using the membrane impermeant nucleic-acid dye SYTOX-Green, which stains algal cells that have lost their membrane integrity. Live cells were detected using the membrane permeant dye Calcein-AM, which is hydrolyzed by intracellular esterases into a green fluorescent charged form. We found that both assays are easy to use, are reproducible and can indeed be used as markers of the viability of individual phytoplankton cells following infection by viruses. Cell death rates up to 0.8 $d^{-1}$ for *P. pouchetii* and 0.5 $d^{-1}$ for *M. pusilla* were calculated. The first day postinfection, death rates determined by the Calcein-AM assay were typically twice as high as those determined by the SYTOX-Green assay. Both viability tests were found to assess the physiological status of noninfected *P. pouchetii* cells, independent of viral infection. The optimal choice of viability assay depended on the phytoplankton species studied. Compared with existing assays, the protocols described permit examination of infected phytoplankton in more detail, yielding insight into the heterogeneity of the algal population.

KEY WORDS: Flow cytometry · Live/dead assays · Phytoplankton · Viability · Viral infection



## INTRODUCTION

With the increased awareness that viruses in aquatic environments can be important agents of cell death for phytoplankton and can play a significant role as regulators of ecological and biogeochemical processes (see reviews by Fuhrman 1999, Wilhelm & Suttle 1999, Wommack & Colwell 2000 and references therein), one of the main challenges in algal virus ecology is to detect virus-infected cells and quantify virus-induced mortality in pelagic ecosystems. Studies on virus-host interactions generally used the decline of algal cell abundance, *in vivo* fluorescence or photosynthetic rates as indicator of virus-mediated cell death for phytoplankton (Suttle et al. 1990, Suttle 1992, Bratbak et al. 1993, Milligan & Cosper 1994). However, these methods have the disadvantage that they are not single cell based and therefore do not provide information on the heterogeneity of viral infection within a phytoplankton population. Transmission electron microscopy to detect the fraction of visibly infected cells in thin sections (Bratbak et al. 1990, Brussaard et al. 1996, Proctor 1997) has the advantage of being a technique based on single cell analyses, but an important drawback is that it is very time-consuming. Flow cytometry combines high-speed data acquisition with multiparametric analyses of individual cells, unique technical properties that are potentially very suitable for research on viral ecology. Flow cytometry has not only been applied to the detection and enumeration of marine viruses (Marie et al. 1999, Brussaard et al. 2000), but has also been used to examine cellular changes in virus-infected marine phytoplankton species (Brussaard et al. 1999). In the latter study, the signals of forward and side scatter, the red autofluores-

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cence and the DNA fluorescence per phytoplankton cell allowed a clear differentiation between the virus-infected and the noninfected cultures. However, no information on the viability of the algal cells was provided.

Viability in the microbiological context refers to the ability of an organism to grow and reproduce under appropriate conditions. However, most viability assays are principally used to enumerate the proportion of live and dead cells in a population. The development of a number of sensitive viability fluorescent probes in the last decade actually resulted in ready-to-use live/dead viability assay kits for mammalian cells and bacteria (Molecular Probes Inc., Eugene, OR). The use of fluorescent dyes in the study of phytoplankton cells is difficult because of the red autofluorescence of chlorophyll. However, the combination of standard flow cytometry with a green fluorescent viability probe should permit discrimination between dead and live phytoplankton cells. To the best of our knowledge there are no reports on the viability of phytoplankton cells during viral infection using fluorescent dyes in combination with flow cytometry. The development of a live/dead assay for phytoplankton populations in combination with flow cytometry has the advantages of single cell analyses, high precision and good reproducibility, allowing a more detailed study of the effects of viral infection on phytoplankton biology.

Because cell death and viability are not easily defined in terms of a single physiological or morphological parameter, it is advantageous to develop different approaches. Cell death is accompanied by a series of processes, among which is the loss of membrane integrity. The most common stains for identifying dead cells are membrane impermeant nucleic acid stains. Nucleic acids are found in high concentrations in cells and, upon binding with a fluorophore, a large fluorescence enhancement can be detected. The nucleic acid stain SYTOX-Green (excitation 504 nm, emission 523 nm, Molecular Probes Inc.) has been recommended as an indicator of dead cells (Roth et al. 1997, Lebaron et al. 1998). It is a high affinity probe that easily penetrates cells with compromised plasma membranes but does not cross the membranes of live cells (Roth et al. 1997, Veldhuis et al. 1997, 2001).

Viability assessments of healthy cells frequently use electrically neutral or near neutral substrate molecules that can be passively loaded into cells. Once inside the cell, the nonfluorescent substrates are hydrolyzed by enzymes and become polar fluorescent products that are retained by cells with intact plasma membranes. The fluorogenic substrate Calcein-AM (Molecular Probes Inc.) has been recommended for staining live cells with metabolic esterase activity (Kaneshiro et al. 1993, Porter et al. 1995). Modification of carboxylic acids with acetoxymethyl (AM) ester groups results in uncharged molecules, which readily penetrate the cell plasma membranes. Once the colorless and nonfluorescent substrate is inside the viable cell, the lipophilic blocking groups are cleaved by nonspecific esterases to a charged green fluorescent (excitation 496 nm, emission 520 nm) membrane impermeant product. In contrast, dead cells cannot hydrolyze the Calcein-AM or retain the fluorescent product. Furthermore, it has a low extracellular fluorescence and has low pH sensitivity, together making it a favorable cytoplasmatic cell marker for use as a live cell function indicator.

Our objective of the present study was to develop easy-to-use flow cytometric assays allowing the detection and quantification of phytoplankton viability following a lytic viral infection. One assay identified dead cells with reduced cell membrane integrity using SYTOX-Green, while the other assessed living cells by detection of intracellular enzymatic activity using Calcein-AM. The applicability of both assays to virus-infected phytoplankton cultures was tested on the phytoplankton species *Phaeocystis pouchetii* and *Micromonas pusilla*.

## MATERIALS AND METHODS

**Culturing.** Axenic algal cultures of the prasinophyte *Micromonas pusilla* (Strain 38, culture collection at the Marine Research Center of Göteborg University, Sweden) and the prymnesiophyte *Phaeocystis pouchetii* (Strain AJ-01, culture collection at the University of Bergen, Norway) were used in this study. For details on isolation, culture conditions and axenity preparation we refered to Brussaard et al. (1999). Batch cultures of *M. pusilla* in modified ESAW medium (Cottrell & Suttle 1991) were incubated at 15 ± 0.5°C, at a light:dark period of 14:10 h with a photon flux density of 100 ± 20 µmol $m^{-2}$ $s^{-1}$ (Sylvania, T8/CW). Batch cultures of *P. pouchetii* in modified f/2 medium (Brussaard et al. 1999) were incubated at 8 ± 0.5°C at a 16 h light:8 h dark cycle with a photon flux density of 100 ± 20 µmol $m^{-2}$ $s^{-1}$ (Philips, TLD 33).

**Live/dead cell staining.** Calcein-AM and SYTOX-Green were purchased from Molecular Probes Inc. The commercial stock solution of Calcein-AM (1 mM in dimethyl sulfoxide) was diluted in 0.2 µm pore size (Supor mebrane Acrodisc syringe filters) filtered, autoclaved nutrient-poor seawater to a working stock concentration of 100 µM. SYTOX-Green (5 mM in dimethyl sulfoxide) was diluted in Milli-Q water to a working stock solution of 50 µM. For both stains working stocks were stored frozen at –20°C until use.

Before the experiments, the staining assays were optimized by testing dye concentration, incubation time and temperature. Both live algal cells and cells that had been killed by fixation with formaldehyde (1% final concentration for at least 1.5 h) were used for the optimization procedures. Formaldehyde was found to be superior to glutaraldehyde because the latter fixative generally shows higher specific background fluorescence (Roth et al. 1997, Veldhuis et al. 1997).

On the basis of the methodological results (see 'Results'), the samples for the SYTOX-Green assay were stained with 0.5 µM dye (final concentration) and incubated in the dark at room temperature for 10 min. As the fluorescent dye is light-sensitive, the samples were incubated in the dark. Samples for the Calcein-AM assay were incubated for 1 h after addition of the dye at a final concentration of 10 µM. Because the basic parameter of this assay is enzyme activity, samples were incubated under culturing conditions. Neither stain had a negative effect on the basic flow cytometric parameters (forward and side scatter, red autofluorescence) or abundance of the cells over a period of 24 h when the optimized final dye concentration for each stain was used.

**Experimental set-up.** At the start of the experiment comparing virus-infected with noninfected algal populations, batch cultures of *Micromonas pusilla* and *Phaeocystis pouchetii* were split into subcultures of 300 to 400 ml. One of the batch subcultures was infected with fresh lysate of the respective virus, whereas the other served as the noninfected control. The lytic virus (*M. pusilla* virus) isolate MpV-UF10-38 (MpV, E. Sahlsten pers. comm.) was added to *M. pusilla* cultures at a virus to algal cell ratio of 10. The lytic virus (*P. pouchetii* virus) isolate PpV-01 (PpV, Jacobsen et al. 1996) was added to *P. pouchetii* cultures at a virus to algal cell ratio of 40 to 80. Subcultures were incubated under conditions identical to those of the original algal cultures. The experiments were performed in duplicate for both algal species.

Samples were taken regularly at intervals of generally 2 to 6 h for 52 to 56 h. The total algal cell count and the viability assays were performed immediately after sampling on unfixed material using flow cytometry. Subsamples for virus count were fixed with glutaraldehyde at a final concentration of 0.5% for 30 min at 4°C, after which the samples were frozen in liquid nitrogen and stored at –70°C until analysis.

**Algal and virus counts.** Phytoplankton were enumerated on fresh, unfixed material directly after sampling using flow cytometry according to Brussaard et al. (1999). For virus enumeration, fixed frozen samples were thawed at room temperature and then diluted in TE buffer (Tris 10 mM, EDTA 1mM, pH 7.5) to obtain event rates between 100 and 1000 $s^{-1}$. For virus enumeration, after addition of the green fluorescent nucleic acid specific dye SYBR Green I (final concentration $0.5 \times 10^{-4}$ commercial stock) and incubation for 15 min in the dark at 80°C (Brussaard et al. 2000), samples were analyzed by flow cytometry according to Marie et al. (1999).

**Flow cytometry.** A FACSort and a FACSCalibur flow cytometer (Becton Dickinson), both equipped with an air-cooled argon laser (excitation 488 nm, 15 mW power) with the standard filter set-up, were used. Analyses were triggered on red autofluorescence and run for 1 to 4 min at a delivery rate of 50 µl $min^{-1}$. To avoid coincidence, algal cells were enumerated using an event rate between 100 and 400 cells $s^{-1}$. When needed, algal samples were diluted in 0.2 µm pore size filtered seawater. Sheath fluid for both assays consisted of 0.2 µm pore size filtered seawater.

Flow cytometric parameters of algal cells were collected as logarithmic signals. Data were computed with CYTOWIN 30 (see www.sb-roscoff.fr/Phyto/cyto.html). For analyses of the collected data, the discrimination between various subpopulations was set for a typical example and kept constant for all samples.

## RESULTS

### SYTOX-Green and Calcein-AM staining characteristics

To establish optimum experimental conditions, several factors such as the time of incubation, and the concentration of cells and dyes were tested on living and killed (formaldehyde-fixed) cells separately. The staining kinetics of SYTOX-Green (final concentration 0.5 µM) for *Micromonas pusilla* and *Phaeocystis pouchetii* showed that the killed cells stained more intensely than the live cells with intact cell membranes (Fig. 1A). The enhancement of fluorescence for the killed cells indicates a good discrimination between the dead and live cells. Staining equilibrium of the killed cells of both *M. pusilla* and *P. pouchetii* was reached within 3 min and remained constant for the remaining 15 min incubation time. After setting the optimum staining time at 10 min, we observed that the mean green fluorescence value increased with the SYTOX-Green concentrations used (0 to 5 µM, Fig. 1B). The fluorescent signal from live cells started to increase significantly above 1 µM SYTOX-Green. Below 1 µM SYTOX-Green final concentration, the maximum ratio of fluorescence of killed to live cells was obtained at 0.5 µM SYTOX-Green for *M. pusilla* and between 0.5 and 2 µM SYTOX-Green for *P. pouchetii* (data not shown). Incubation at the culture temperature (15°C for *M. pusilla* and 8°C for *P.*

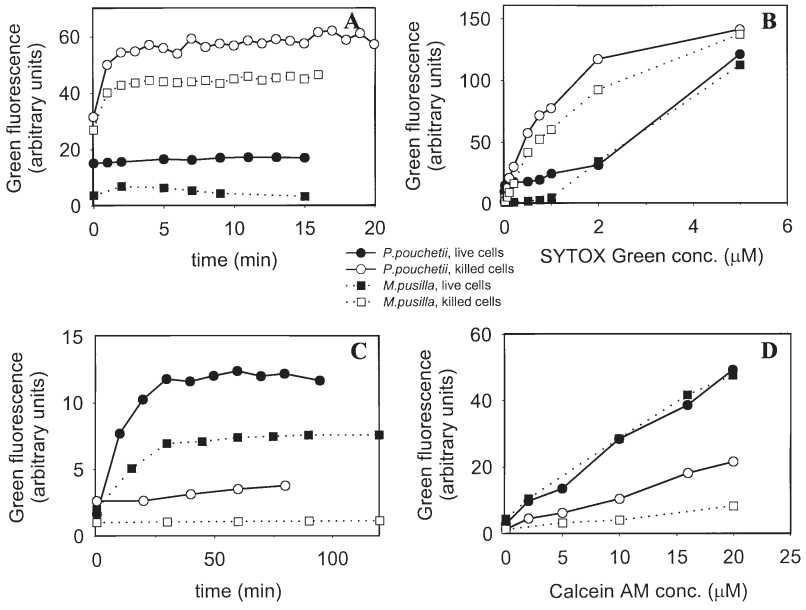


Fig. 1. Green fluorescence intensity (relative to 0.95 µm beads) of the marine phytoplankton species *Phaeocystis pouchetii* (circles, solid line) and *Micromonas pusilla* (squares, dotted line) after staining with the dyes (A,B) SYTOX-Green or (C,D) Calcein-AM as a function of (A,C) time and (B,D) the dye concentration. Filled symbols represent live and open symbols killed algal cells (by formaldehyde fixation). For the time kinetics, dye concentrations of 0.5 and 10 µM were used for SYTOX-Green and Calcein-AM, respectively. For the tests for optimal dye concentation, incubation time was 10 and 60 min for SYTOX-Green and Calcein-AM assay, respectively

*pouchetii*) had no effect on the results as compared with the incubation at room temperature (20°C). Quantitative flow cytometric analysis of the viability of both algal species using mixtures of live and killed algal cells in different proportions showed coherent data of relative abundance of live and dead cells (data not shown).

The staining kinetics of Calcein-AM (Fig. 1C) showed that both algal species reached staining saturation after 30 min at a final concentration of 10 µM Calcein-AM. The fact that the fluorescent signal from the killed cells was lower than that from the untreated cells indicated a good potential distinction between live and dead algal cells after staining with Calcein-AM. However, in mixtures of live cells and washed killed cells, discrimination between the 2 cell types was difficult (data not shown). Although the formaldehyde-killed cells were washed several times before being mixed with live cells, it might have been insufficient to prevent any negative effect of potentially remaining traces of formaldehyde on the live cells in the mixture. Killing the cells with alcohol or by heating, however, did not improve the results. Fluorescence of both live and killed cells was enhanced with increasing concentrations of Calcein-AM (0 to 20 µM final concentration; Fig. 1D). The highest staining ratio of live to dead algal cells for *Micromonas pusilla* was obtained with a 10 µM Calcein-AM final con centration, whereas for *Phaeocystis pouchetii* changes were minor over the entire range of concentrations.

## Viral infection experiments

### Cytograms

Figs 2 & 3 show typical biparametric plots of virus-infected and noninfected *Micromonas pusilla* and *Phaeocystis pouchetii* cultures with the red fluorescence signal from chlorophyll versus the green fluorescence signal resulting from staining with the dye SYTOX-Green or Calcein-AM, respectively. Both assays allowed apparent distinctions of stained and nonstained cells for the virus-infected cultures, although for the virus-infected cultures of *P. pouchetii* the population of SYTOX-Green stained cells (high green fluorescence) was generally less compact than that of SYTOX-Green stained cells of *M. pusilla*.

For both the Calcein-AM and the SYTOX-Green assay, *Phaeocystis pouchetii* cells showed higher green fluorescence signal than *Micromonas pusilla* cells (Fig. 1). This was most likely due to differences in cell size between *P. pouchetii* (diameter 4 to 6 µm) and *M. pusilla* (diameter 1.5 to 3 µm). Species-dependent fluorescence signals per phytoplankton cell have been reported while using the viability probe fluorescein diacetate (Dorsey et al. 1989).

### SYTOX-Green assay

The healthy physiological status of the well-growing noninfected control cultures of both *Micromonas pusilla* (Fig. 4A, average $\mu = 0.42$ d$^{-1}$) and *Phaeocystic pouchetii* (Fig. 4B, average $\mu = 0.23$ d$^{-1}$) was confirmed by the very low percentages of cells with compromised cell membranes (SYTOX-Green stained: for clarity defined as 'dead' cells; Figs 5 & 6). For both phytoplankton species, the percentage of dead cells in the virus infected cultures started approximately 12 h postinfection (Figs 5B & 6B). This coincided with the first increase in free viruses in the cultures (Fig. 4). In contrast to *M. pusilla*, with up to 55% of total cells dead after 56 h,

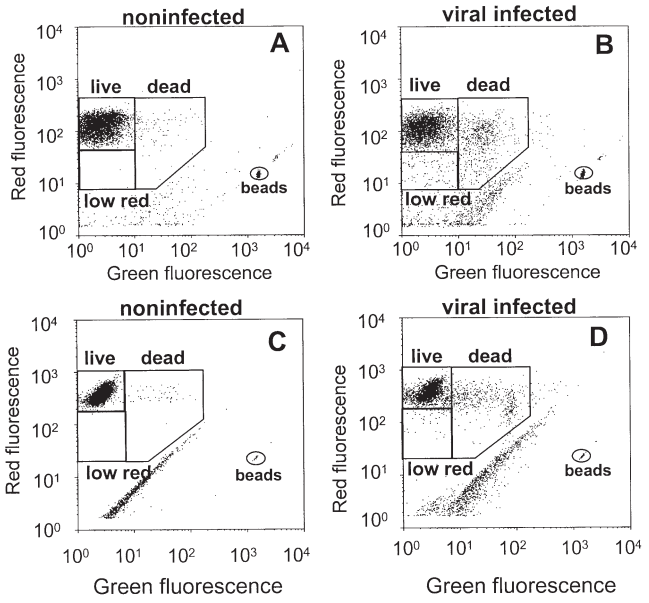


Fig. 2. Representative biparametric plot of green fluorescence versus chlorophyll red fluorescence representing axenic algal populations after staining with SYTOX-Green for 10 min in the dark at room temperature. The cytograms show noninfected and virus-infected cultures of (A,B) *Micromonas pusilla* and (C,D) *Phaeocystis pouchetii*. The regions represent populations of algal cells with normal red fluorescence and low green fluorescence ('live'), normal or reduced red fluorescence but enhanced green fluorescence (SYTOX-Green stained, 'dead'), and low red and low green fluorescence ('low red'). The discrimination between the 3 subpopulations was kept similar for all samples

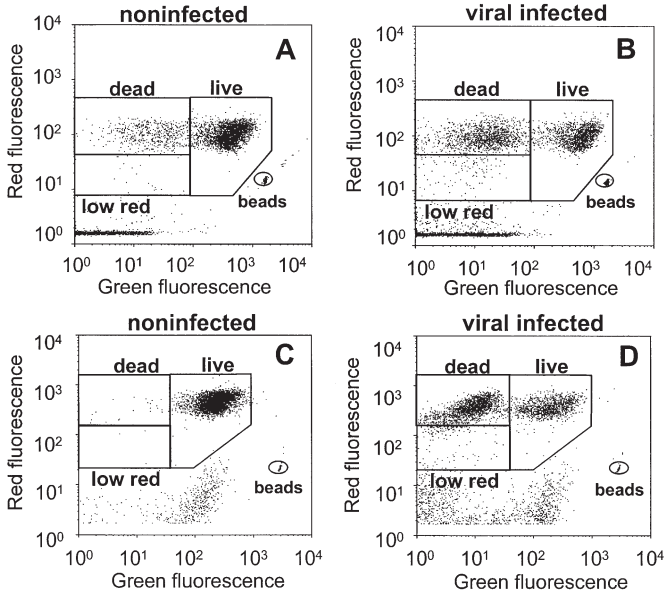


Fig. 3. Representative biparametric plot of green fluorescence versus chlorophyll red fluorescence representing axenic algal populations after staining with Calcein-AM for 1 h under culture conditions. The cytograms show noninfected and virus-infected cultures of (A,B) *Micromonas pusilla* and (C,D) *Phaeocystis pouchetii*. The regions represent populations of algal cells with normal red fluorescence and low green fluorescence (Calcein-AM stained, 'live'), normal or reduced red fluorescence but enhanced green fluorescence ('dead'), and low red and low green fluorescence ('low red'). The discrimination between the 3 subpopulations was kept similar for all samples

SYTOX-Green stained cells in the infected culture of *P. pouchetii* increased to only 25% at the end of the experiment (52 to 56 h). The increase in the percentage of dead SYTOX-Green stained cells in the virus-infected cultures did not match the steady loss of live cells (normal red autofluorescence and low green fluorescence, Figs 5A & 6A). The discrepancy was explained by the increasing percentages of cells with both low green and red fluorescence ('low red', Figs 5C & 6C) toward the end of the experiment (30 h postinfection).

In the same experiment with *Phaeocystis pouchetii* cultures in late exponential phase (Fig. 4B), enhanced percentages of SYTOX-Green stained dead cells were also found in the noninfected control cultures (Fig. 6B).

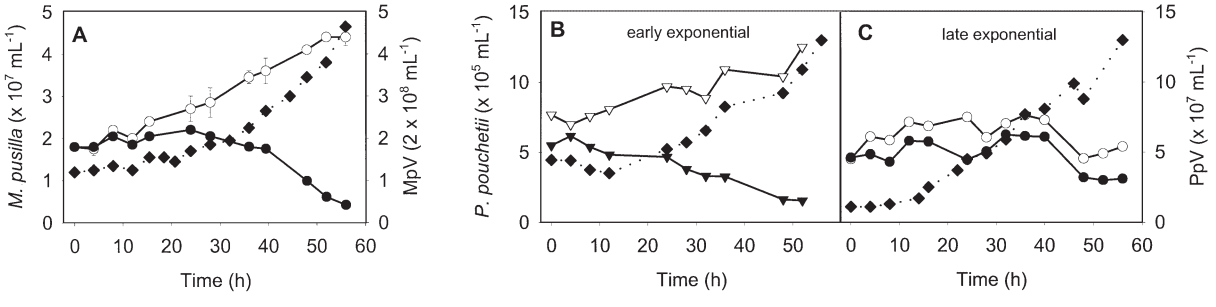


Fig. 4. Flow cytometric enumeration of the alga (A) *Micromonas pusilla* and *M. pusilla* virus (MpV), and (B,C) *Phaeocystis pouchetii* and *P. pouchetii* virus (PpV). Plots A and C are after Brussaard et al. (1999). The algal cultures were in early exponential (A,B) or late exponential (C) growth before infection. Open symbols on a solid line represent the noninfected algal cultures, closed symbols on a solid line the virus-infected algal cultures, and closed diamonds on a dotted line the viral population. Experiments were performed in duplicate; mean values are presented with standard error of the mean indicated by bars (if not visible, the error bar is smaller than the symbol)

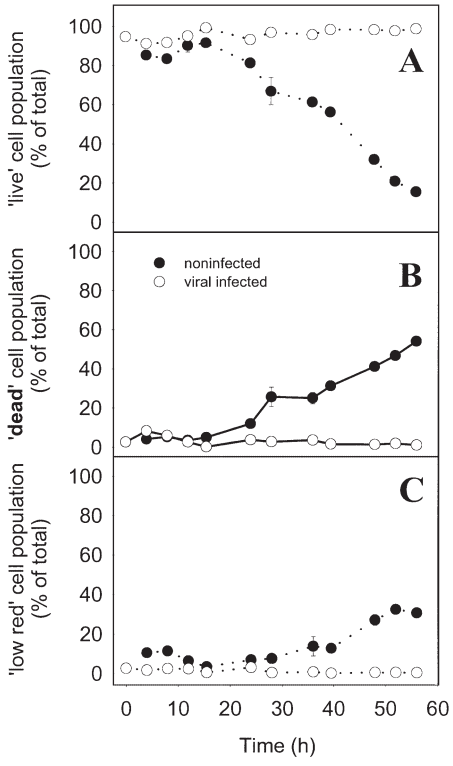


Fig. 5. Variations in cell viability of the virus-infected and noninfected cultures of *Micromonas pusilla* after staining with SYTOX-Green. Open circles represent the noninfected algal cultures and closed circles the virus-infected algal cultures. Experiments were performed in duplicate; mean values are presented with standard error of the mean indicated by bars (if not visible, the error bar is smaller than the symbol). Three populations were discriminated: algal cells with normal red fluorescence and low green fluorescence (A, 'live'), with normal or reduced red fluorescence but enhanced green fluorescence (B, SYTOX-Green stained 'dead'), and with low red and low green fluorescence (C, 'low red')

In contrast to the virus-infected cultures, however, no increase in the low red population was found for the dying control cultures (Fig. 6C). A late exponential growth before infection resulted in higher percentages of low red cells than of the cultures in early exponential phase (Fig. 6C).

Specific cell death rates calculated directly from changes in the total fraction of dead cells: those stained with SYTOX-Green, as well as the low red dead cells, ranged between 0.2 and 0.4 $d^{-1}$ during the first 36 h postinfection (12 to 36 h). For the remainder of the experiment death rates from 0.3 to 0.6 $d^{-1}$ were estimated.

## Calcein-AM assay

Using the Calcein-AM assay, we found for the virus infected cultures of both phytoplankton species a steady decline in the percentage of Calcein-AM stained live algal cells 4 to 12 h postinfection, depending on the algal species (Figs 7A & 8A). Concomitantly, there was an increase in the portion of dead cells, subsequently followed by enhanced portions of low red cells (Figs 7B,C & 8B,C). In the late exponential growth *Phaeocystis pouchetii* cultures, the proportion of low red cells was higher than in the early exponential growth cultures (Fig. 8C). In the noninfected control cultures of *P. pouchetii*, the percentage of live cells in the late exponential cultures was lower than in the early exponential cultures, indicating a poorer physiological status of the cells (Fig. 8A).

For the well-growing control cultures, the results of the 2 tested phytoplankton species were very different. The level of live *Phaeocystis pouchetii* cells was, as expected, more or less constant and very high during the entire experiment (Fig. 8A). *Micromonas pusilla*, on the other hand, showed clear diel oscillations in the

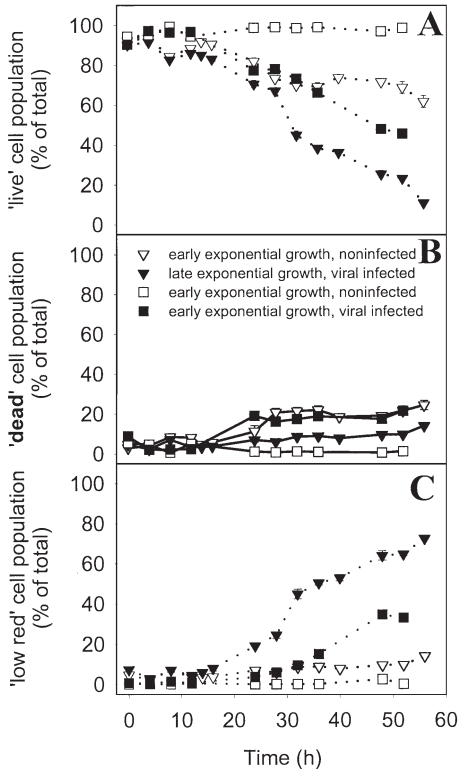


Fig. 6. Variations in cell viability of virus-infected and noninfected cultures of *Phaeocystis pouchetii* after staining with SYTOX-Green. The algal cultures were in early exponential (squares) or late exponential (triangles) growth before infection. Open symbols represent the noninfected algal cultures and closed symbols the virus-infected algal cultures. Experiments were performed in duplicate; mean values are presented with standard error of the mean indicated by bars (if not visible, the error bar is smaller than the symbol). See Fig. 5 for explanations

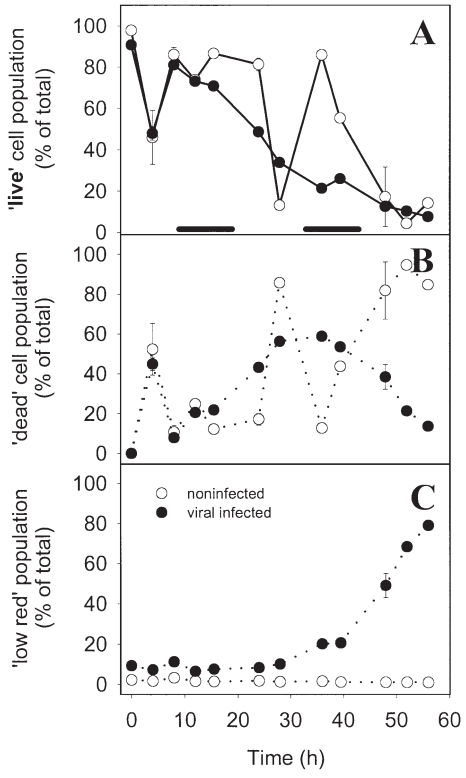


Fig. 7. Variations in cell viability of the virus-infected and noninfected cultures of *Micromonas pusilla* after staining with Calcein-AM. Open circles represent the noninfected algal cultures and closed circles the virus-infected algal cultures. Experiments were performed in duplicate; mean values are presented with standard error of the mean indicated by bars (if not visible, the error bar is smaller than the symbol). Bars on the *x*-axis in the top graph (A) indicate the dark period. Three populations were discriminated: algal cells with normal red fluorescence and low green fluorescence (A, Calcein-AM stained 'live'), with normal or reduced red fluorescence but enhanced green fluorescence (B, 'dead'), and with low red and low green fluorescence (C, 'low red')

percentages of Calcein-AM stained live cells with intracellular esterase activity (Fig. 7A). The lowest values were found during the light period, very likely indicating a reduced activity of intracellular esterases. These oscillations were not reflected in the percentages of low red cells (Fig. 7C).

Based on the Calcein-AM assay, specific cell death rates were calculated from the decline in the fraction of live instead of dead cells. Death rates of 0.4 to 0.8 $d^{-1}$ were recorded 12 to 24 h postinfection. During the following period, death rates declined strongly for the well-growing *Phaeocystis pouchetii* cultures (down to 0.2 $d^{-1}$) but to a lesser extent for *Micromonas pusilla* and the late exponential *P. pouchetii* cultures. There was, however, a shift in the populations, making up for these death rates, from the SYTOX-Green stained population to the low red population.

## DISCUSSION

### Methodological aspects

A shortcoming in the study of virus-infected phytoplankton is the lack of reliable assays to study the viability of the host cells. We examined assays using flow cytometric analysis of virus-infected phytoplankton populations. Because of the lack of an authoritative definition, cell viability or death has to be defined operationally. Two assays, based on membrane integrity and intracellular enzyme activity, were optimized for marine phytoplankton. The SYTOX-Green assay stains cells with compromised cell membranes, defined as dead cells (although it can't be ruled out that the process of reduced membrane integrity might still be reversible in cells with normal red chlorophyll autofluorescence). The Calcein-AM reveals intracellular esterase activity, defined as active or live cells. Both as-

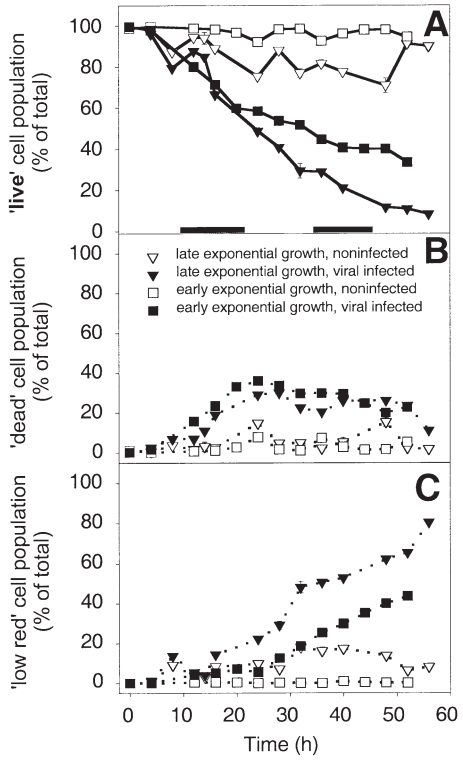


Fig. 8. Variations in cell viability of the virus-infected and noninfected cultures of *Phaeocystis pouchetii* after staining with Calcein-AM. The cultures were either in early (squares) or in late (triangles) exponential growth. Open symbols represent the noninfected algal cultures and closed symbols the virus-infected algal cultures. Experiments were performed in duplicate; mean values are presented with standard error of the mean indicated by bars (if not visible, the error bar is smaller than the symbol). Bars on the *x*-axis in the top graph (A) indicate the dark period. See Fig. 7 for explanations

says have inherent advantages and limitations. Theoretically the major advantage of using the Calcein-AM assay is that it allows earlier detection of nonhealthy cells than does the use of SYTOX-Green. The fluorescent cleavage product of Calcein-AM is membrane-impermeant and therefore cannot be retained by cells with compromised cell membranes. SYTOX-Green, on the other hand, can only penetrate cells that have lost the integrity of the plasma membrane, and therefore stains cells just before complete cell lysis. The 3-fold difference in response time between the 2 viability assays in the present study (the Calcein-AM assay first distinguished between control and infected cultures 4 h postinfection, whereas for the SYTOX-Green assay this happened only after 12 h) confirms this hypothesis.

Both the binding of SYTOX-Green to nucleic acids and the hydrolysis of Calcein-AM are affected by a number of factors including dye concentration, incubation time and temperature. The reproducibility was excellent for both viability assays, with standard error of the mean typically well below 5%. But the Calcein-AM assay required a relatively high amount of dye ($1 \times 10^{-2}$ commercial stock) and a longer incubation time, and was found to be more dependent on the incubation conditions than the SYTOX-Green assay. The fact that the intensity of staining of well-growing *Micromonas pusilla* cells upon incubation with Calcein-AM depended on the sampling time in the diel cycle indicates that nonstaining does not always reflect dying cells but can just reflect a reduction in intracellular esterase activity. Whether the Calcein-AM assay can be used routinely depends on the interspecific variability of possible fluctuations in intracellular esterase enzyme activity over the day, something that needs further testing. Although Calcein-AM has not yet been applied to several different genera of phytoplankton, another fluorogenic esterase substrate (fluorescein diacetate) has been successfully tested for many different phytoplankton species (Dorsey et al. 1989, Selvin et al. 1989, Jochem 1999). The small amount of SYTOX-Green dye needed ($1 \times 10^{-4}$ commercial stock) and the short incubation time (10 min) potentially make the SYTOX-Green assay a good candidate for routine assessment of phytoplankton populations. The use of SYTOX-Green as a viability indicator has not only been applied to a wide range of phytoplankton species in culture (Veldhuis et al. 1997) but recently has also been validated under natural conditions (Veldhuis et al. 2001).

### Experimental use

Time series of viability measurements allow the calculation of specific cell death rates. Typically, for the SYTOX-Green assay, the portion of stained dead cells should be used to estimate cell death rates, but the cells with reduced red chlorophyll autofluorescence that did not stain with SYTOX-Green (low red) may also be considered nonviable. Since reduced red autofluorescence is indicative of degradation of chlorophyll, these nonstaining cells (low red population) probably had already lost most of their nucleic acids due to partial cell lysis or DNA breakdown. From the present detailed time studies, it appears likely that there is a progression from cells with damaged membranes staining with SYTOX-Green to dead low red cells, and that the rate at which this occurs can be high (this holds true especially for virus-infected *Phaeocystis pouchetii*: maximum estimated transfer rate 0.3 $d^{-1}$). Specific cell death rates calculated directly from changes in the fraction of cells stained with SYTOX-Green might thus be underestimated as soon as the importance of the low red population increases. Compared with early exponentially growing cultures of *P. pouchetii*, cells in the late exponential phase before infection tend to transfer more rapidly and to a larger extent into the low red population (the last step before full cell lysis).

With the Calcein-AM assay, algal cell death rates are less likely to be underestimated because they are based on the decline in the fraction of live instead of dead cells. Algal cell death rates might even be slightly overestimated, indicated by our finding with well-growing noninfected *Micromonas pusilla* cultures that loss of intracellular enzyme activity does not necessarily mean that these cells were dead. As could be expected (see first paragraph of 'Discussion'), the Calcein-AM assay showed the highest death rates relatively early upon viral infection. The first day postinfection, the death rates based on the Calcein-AM assay were typically twice as high as those obtained using the SYTOX-Green assay.

Our estimates of death rates fall in the wide range of virus-mediated lysis rates for phytoplankton reported in the literature, obtained using a variety of approaches. During the decline of a bloom of *Emiliania huxleyi*, mortality rates of 0.1 to 2 $d^{-1}$ were reported based on visibly infected cells using transmission electron microscopy (Bratbak et al. 1993, Brussaard et al. 1996). For *Micromonas pusilla*, virus-induced mortality rates *in situ* were estimated from host and virus titers, virus kinetics and decay rates of infectivity (Cottrell & Suttle 1995). Depending on the adsorption coefficient of virus to *M. pusilla*, lysis rates between 0.1 and 0.5 $d^{-1}$ were recorded. We believe that the use of our viability assays during viral infection to infer the impact of viral infection on phytoplankton mortality is promising as it allows reproducible estimates relatively easily and quickly.

Noninfected cultures of *Phaeocystis pouchetii* in late exponential growth showed that the 2 viability assays allowed detection of algal cell death, independent of viral infection. Moreover, these assays can assess the physiological status of algal cells, showing distinct differences between late and early exponentially growing noninfected *P. pouchetii* cultures. These findings are important because there are other factors, biotic (e.g., allelopathy) and abiotic (e.g., nutrient depletion), that can also significantly affect phytoplankton cell mortality (Fritz & Nass 1992, Brussaard et al. 1997, 1998, Berges & Falkowski 1998, Wolfe 2000). Using SYTOX-Green, high percentages of dead cells have been recorded *in situ* for oceanic phytoplankton communities (Veldhuis et al. 2001).

One of the aspirations of the use of live/dead assays in the study of virus-host interactions was that they should allow us to obtain information on the viability of the phytoplankton cells before any decline in the number of living cells (or increase in dead cells) becomes evident. Algal cell death might still be balanced by population growth during the early stages of infection (Waters & Chan 1982, Brussaard et al. 1999). Thus, a decline in total cell count would not be detectable but changes in the viability of the infected algal cells would already be apparent. The present study showed that the earliest loss of viability of the algal cells in the virus-infected cultures was indeed not generally reflected in the total number of algal cells in the virus-infected cultures. The first differences in viability status of the algal cells in the virus-infected, compared with the control, cultures fell within the latent period for the 2 model systems (the time between infection and cell lysis was 14 h for MpV and 12 to 18 h for PpV; Waters & Chan 1982, Jacobsen et al. 1996).

The steady increase in proportion of SYTOX-Green stained cells of infected *Micromonas pusilla* indicates that the cultures were not infected synchronously (Brussaard et al. 1999). Since viral infections under natural conditions are generally also not examples of one-step infections (the entire population infected by viruses at the same time), the application of viability assays under such conditions offers the opportunity to address the physiological condition within an algal population before any signs of viral infection are evident.

The use of viability assays in combination with quantitative flow cytometry permits the study of individual cells and therefore provides insight into the heterogeneity of the algal population. This is a main advantage over existing methods indicative of the viability of algal cells (*in vivo* fluorescence, photosynthetic rates; Suttle et al 1990, Suttle 1992, Milligan & Cosper 1994), which are bulk measurements on a population scale instead of single cell based approaches. On the basis of chlorophyll autofluorescence and size, algal populations might seem uniform, but at the same time large differences in viability can be found (Veldhuis et al. 2001, present study). Especially when a substantial portion of the algal population is dying, as is found upon viral infection with a lytic virus, the subsequent large heterogeneity in viability within an algal population (present study) would affect population-based estimates of, e.g., photosynthetic activity, nutrient uptake and gross growth rates. It can be anticipated that this has implications for species dynamics and succession, as well as for food-web structure.

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