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PHYTOPLANKTON, PHYTOBENTHOS, AND PHYTOPERIPHYTON

Effect of Viral Infection on the Functioning and Lysis of Black Sea Microalgae *Tetraselmis viridis* (Chlorophyta) and *Phaeodactylum tricornutum* (Bacillariophyta)

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Abstract—Experimental studies have been performed on *Tetraselmis viridis* (Rouchijajnen) R.E. Norris, Hori & Chihara (Chlorophyta) and *Phaeodactylum tricornutum* Bohlin (Bacillariophyta) cultures of Black Sea microalgae using TsV-S1 and PtV-S18 algal virus strains isolated from the Black Sea ecosystem. The study assesses the effects of light intensity and the initial abundance of microalgae cells on the onset of their infection by viruses and the decline in their abundance. As early as on the second or third day, the cells changed their shape and increased in volume in the cultures infected by viruses. At this time, a decrease was observed in chlorophyll *a* red autofluorescence, variable fluorescence per cell, and photochemical efficiency of photosystem 2. In the studied cultures, a reduction in cell abundance due to viral lysis was noted 1 to 2 days later. The threshold cell abundance at which this process was observed was independent of light conditions and amounted to 3×10^5 cells/mL for *T. viridis* and 18×10^5 cells/mL for *Ph. tricornutum*. The complete lysis of algae cells was detected by the end of the fourth to sixth day. In the dark, the effect of viruses on microalgae was insignificant.

Keywords: marine microalgae, algal viruses, phytoplankton, Black Sea
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INTRODUCTION

Viruses are among the most important components of aquatic ecosystems. There are from 10^5 to 10^9 viral particles in every 1 mL of seawater (Stepanova, 2017; Suttle, 2007). Most of them attack bacteria, as well as macro- and microalgae. The main causes of the functionally active cell death in marine phytoplankton include consumption by zooplankton and lysis due to exogenous viral infection (Beckett and Weitz, 2018; Bidle et al., 2007; Pasulka et al., 2015). Viral lysis is considered the primary cause of death of ~3–5% cells in phytoplankton daily (Wommack and Colwell, 2000); this number can peak at 25–30% during particular periods of time (Evans et al., 2003; Mojica et al., 2016). The rather rapid termination of the algal bloom, which is caused by particular microalgae species, may result from large abundance of viruses in the bloom zones and, therefore, the viral lysis of plant cells (Suttle, 2007; Cottrell and Suttle, 1995; Bratbak et al., 1993, 1996; Baudoux et al., 2006, 2008; Vardi et al., 2009; Lehahn et al., 2014). Phytoplankton organic matter, entering the external environment as a result of the viral lysis of its representatives, is primarily consumed by bacteria. This process has become known as the viral shunt (Wommack and Colwell, 2000; Pagarete et al., 2015).

A general assessment of the proportion of phytoplankton dying due to viral lysis currently involves the modification of the dilution approach (Evans et al., 2003). The latter, however, is unwieldy and has not yet gained widespread use in hydrobiology on the practical side. No clear or generally accepted research methodology has been developed with respect to the effects of viruses on phytoplankton. One reason for this appears to be associated with the high species specificity of viruses targeting a narrow range of hosts. One virus strain commonly has a single host, that is, one algal species (Kim et al., 2015), though a few of the Black Sea algal viruses have been found to have two each and more (Stepanova, 2016). The primary reasons are not well-understood, notwithstanding numerous publications and literature reviews about the contribution of the environmental conditions effects in promoting the viral lysis of phytoplankton cells (Wommack and Colwell, 2000; Horas et al., 2018). To identify the role of the individual factors, such as light, temperature, and biogenic substances in

Abbreviation: F_0/cell – the initial level of variable fluorescence; F_v/F_m – photochemical efficiency of photosystem 2.

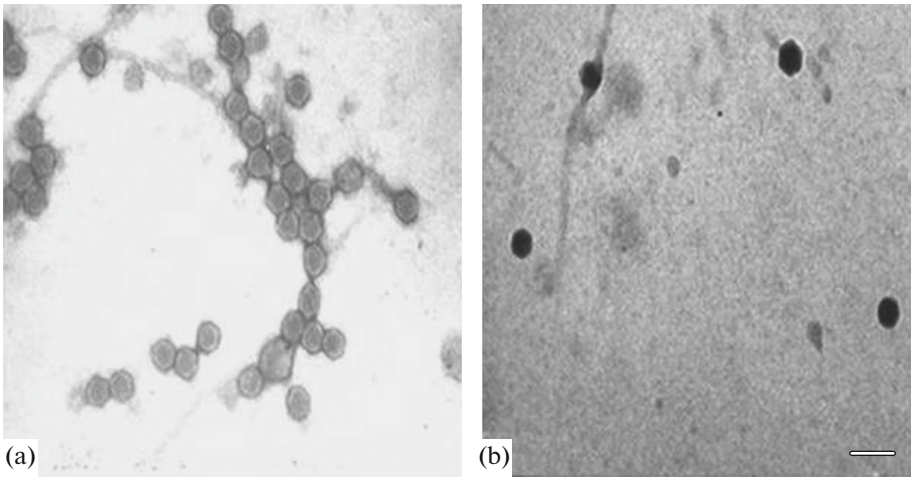

Fig. 1. Electron microscopy photo images of algal viruses in microalgae (a) *Tetraselmis viridis* (strainTvV-S1) and (b) *Phaeodactylum tricornutum* (strain PtV-S18). Dimension (Fig. 1b) 103 nm.

this process regulation, it is logical to employ findings of the laboratory experiments on interactions between viruses and their hosts, i.e., microalgae. In addition, the results can further be extrapolated on a situation with the freshwater and seawater phytoplankton, as well as employed in the development of the research methodology to determine a role of viruses in the functioning of phytoplankton and its individual representatives.

The goal of the present study is to evaluate the impact that viral infection has on some structural and functional characteristics of two Black Sea microalgae species, namely, *Tetraselmis viridis* and *Phaeodactylum tricornutum*, in the conditions of their growth in the enrichment culture under different light intensities and in the dark.

MATERIALS AND METHODS

The study examined algologically pure cultures of microalgae *Tetraselmis viridis* (Rouchijajnen) R.E. Norris, Hori & Chihara (Chlorophyta) and *Phaeodactylum tricornutum* Bohlin (Bacillariophyta) isolated from the Black Sea plankton and stored in the algal repository at Ecological Physiology Department of the Federal Research Center Kovalevskii Institute for Biology of the Southern Seas, Russian Academy of Sciences. The average volume of cells is 260 ± 40 μm^3 for the first species and 80 ± 20 μm^3 for the second one. These algal species are commonly used as models, possessing a high viability in the cell culture process. Before starting the experiment, each culture was adapted for 2 or 3 days to two values of continuous light intensity. One of them (I) limited the algal growth, while another (II) provided for the maximum growth rate at 20 μE/(m^2 · s) (I) and 60 μE/(m^2 · s) (II) in *Tetraselmis viridis* and at 20 μE /(m^2 · s) and 100 μE/(m^2 · s) in *Phaeodactylum tricornutum*, respectively. The culture of *Tetraselmis viridis* was adapted to the dark for 3 days in the third set of the experiment.

Each adapted culture was divided into three portions (50 mL each) and placed into 150-mL Erlenmeyer flask. The first one (control) was filled with 50 mL of sterile seawater with growth-supporting medium f/2 (Guillard and Ryther, 1962). The second and third (experimental flasks) were each admixed with 50 mL of virus suspension with a titer 10^7 virions/mL for the virus of the alga *Tetraselmis viridis* (strain TsV-S1) and 10^6 virions/mL for the virus of the alga *Phaeodactylum tricornutum* (strain PtV-S18). The titer (concentration, abundance) of algal viruses in viral suspension was determined according to the patented proprietary methodology (Stepanova, 2016).

Strain TsV-S1 has been earlier found to have icosahedron shape 56–60 nm in diameter (Fig. 1a) using the electron microscopy technique. Strain PtV-S18 is similarly an icosahedron with a diameter of 45–48 nm (Fig. 1b). They were identified to belong to family Phycodnaviridae based on the outcomes of research into the Black Sea algal viruses, including those employed in viral-strain work, and genetic analysis (Stepanova, 2016).

Before the experiment, the viral suspension was stored at −20°C. For virus activation, the host culture in the log phase was admixed (1 : 1 in terms of volume) to the thawed suspension and exposed to scattered light at 18°C for 10–14 days. Following the viral lysis of the culture and after the viral titer had been established, the suspension was preparatorily purified by centrifugation at 3000 rpm for 10 min and passed through track-etched membrane (pore diameter 0.2 μm). This resulted in pure viral suspensions of strains TsV-S1 and PtV-S18, which were used in the experiment.

The control and experiment flasks positioned on a lattice light sheet were illuminated from the bottom

using light-emitting diodes. Illuminance was measured with a U116 lux meter; the conversion factor from illuminance expressed as lux to light intensity is 10^4 lx = 170 μE /(m^2 s) (Parsons et al., 1982). The experiments were conducted using water temperature at 18°C, which is optimal for the development of the algae species under consideration.

The experiment lasted 4 to 6 days, depending on the light intensity. The 5-mL samples for control and experiment were collected from 9:00 a.m. to 5:00 p.m. at 4- to 8-h intervals. For the experiment with the *Tetraselmis viridis* culture kept in the dark, samples were collected over 10 days at 48–96 h intervals due to a delay in the lysis of algal cells in the dark as a result of a decline in physiological activity, affecting the ability to produce viruses.

A Micromed 3LUM light microscope was used to keep track of cell numbers and for photo imaging in bright and dark fields. The microscope was additionally equipped with a fluorescent unit with a 100-watt mercury lamp and a ToupCam UCMOS 14000 KPA camera. A blue filter was used for exciting red chlorophyll *a* autofluorescence (fluorescence excitation spectrum 410–490 nm). Region >515—700 nm was used to observe the red luminescence of the object (in reflected light) after passing through a stop-band light filter (515 nm).

The cell count was performed in a bright field and replicated three times in a Goryaev counting chamber. The total magnification of the microscopic system was ×100 during the cell count and ×1000 at their registration (photo imaging). The relative error of the cell count in parallel samples did not exceed 10%. The specific daily rate of growth in microalgae and the rate of their die-away were calculated based on growth or loss of cell population in samples from the equation

$$\mu = \ln(N_t - N_0),$$

where μ is specific rate of growth or die-away of microalgae, day^{-1}; N_0 is initial cell abundance; and N_t is their abundance after one day.

The F_v/F_m in microalgae was estimated with dual-flash fluorimeter, the operating principle and design of which were developed at the Chair of Biophysics, Department of Biology, Moscow State University (Pogosyan et al., 2009). Measurements were performed after the 30-min adaptation of microalgae to the dark for reactive centers of photosystem 2 to transfer into an open state. The fluorimeter employs a technique for measuring the initial level of variable fluorescence (F_0) by means of short probe flashes and its maximum level (F_m) with a saturating flash. The measured parameters served to calculate variable fluorescence $F_v = F_m - F_0$ and F_v/F_m. The relative error of the F_v/F_m value determinations did not exceed 5%. Normally, the F_0 value is well-correlated with the chlorophyll *a* concentration in microalgae cells (Tsylinskii et al., 2018). Therefore, we used this parameter as an indicator of chlorophyll variability in cells of the studied algae in the process of their growth.

The statistical processing of data involved finding the mean values of the indicators and their standard deviation.

RESULTS

Figures 2a and 2c present a view of cells in the initial cultures of microalgae *Tetraselmis viridis* and *Phaeodactylum tricornutum*. In the cultures infected by the corresponding viruses, the cells changed their shape and increased in volume 3–4 times when compared with the initial culture as early as on the second day under growth-saturation light intensities and on the third day under low-light intensities (Figs. 2b, 2d). Their numbers were gradually growing. The increase in microalgae cell sizes led to their subsequent destruction, accompanied by the release of cellular content into the environment.

Experiment with *Tetraselmis viridis* culture. Under the algal growth-limiting light conditions, cell abundance in *T. viridis* was observed to increase gradually over the first 4 days in the control, as well as the experiment setting (Fig. 3a) with a specific growth rate of 0.24–0.48 day^{-1} (Table 1). By the end of day 4, cell abundance in the infected culture reached its maximum (3×10^5 cells/mL), which is ~4 times higher than its initial values. A subsequent rapid decline in cell abundance terminated fully by the end of the 6th day. With that, the specific rate of the algae die-away was -1.20 day^{-1} on the fifth day of the experiment; it increased ~5.5 times that on day 6 (Table 1). During this time, in the control, the cell abundance of *T. viridis* continued rising and reached the stationary phase only by the end of day 5.

The initial level of the F_0/cell on a per cell basis and F_v/F_m in the process of *T. viridis* culture growth declined both in the control and the experiment. Notwithstanding, the reduction was mild in the control; in the experiment, the values of both parameters dropped to zero as early as in the end of the fifth day (Figs. 3a, 3b).

In the course of the experiment, red chlorophyll *a* autofluorescence in *T. viridis* changed little in the control; that is, the glow entailed a larger portion of the cell. The culture infected by a virus exhibited a similar view only in the first day of the experiment. Red chlorophyll *a* autofluorescence weakened in the predominant (in terms of numbers) altered cells at the end of the second or third day and was inappreciable on the fifth day.

In the control and experiment settings, the abundance of *T. viridis* cells increased approximately at the same rate under the light intensity optimal for their growth (Fig. 3c). On the second day, the specific rate of the algae growth in the experiment was slower than in the control by a factor of 2.8 (Table 1). As a result, the total abundance of cells in the infected culture was

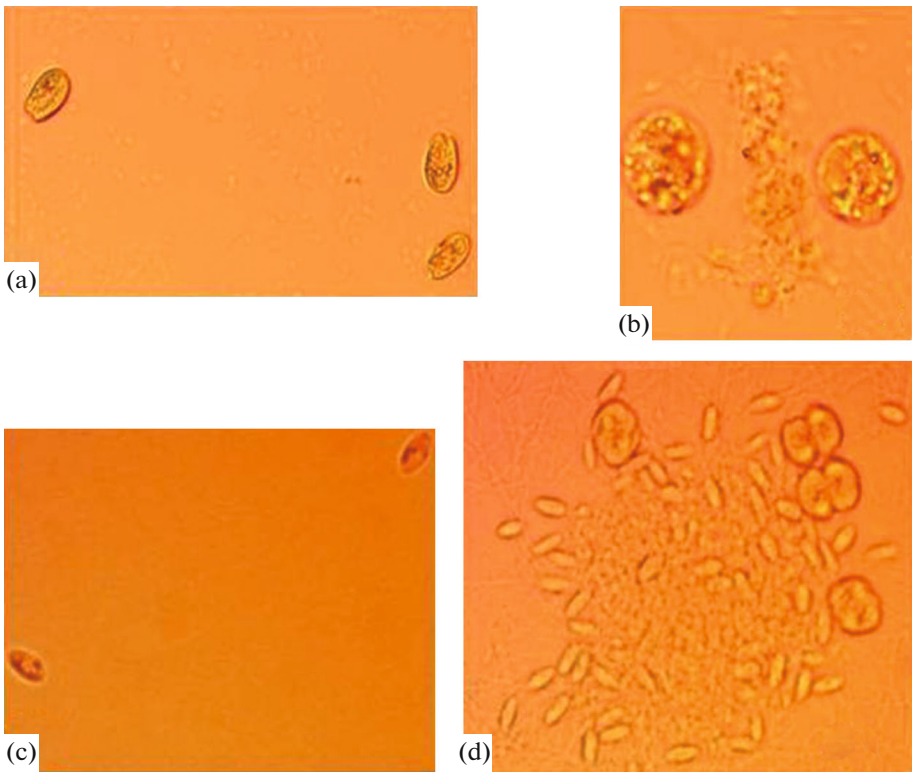

Fig. 2. Microalgae cells (a, c) before and (b, d) after introduction of viral infection: (a, b) *Tetraselmis viridis* and (c, d) *Phaeodactylum tricornutum*.

~30% smaller than in the control and amounted to 3×10^5 cells/mL by the end of the second day (Fig. 3c). Subsequently, the infected culture was dying away rapidly, resulting in complete lysis by the end of the fourth day. The control culture was in the exponential phase of growth during the same period. In the latter, values of F_0/cell and F_v/F_m slightly declined in the process of enrichment culture growth; in the experiment they went down to zero as early as by the end of the fourth day (Figs. 3c, 3d).

During 10 days in the dark, the abundance of *T. viridis* cells in the control varied within the limits of a few percent. In the infected culture, it reduced by just 20% of the initial one on the 10th day. The F_0/cell and F_v/F_m indicators did not change in the control and decreased insignificantly in the experiment (Fig. 4).

Experiment with *Phaeodactylum tricornutum* culture. Cell abundance was observed to increase in the control under conditions of light limited culture growth throughout the experiment (Fig. 5a). The specific growth rate peaked at 0.49–0.91 day^{-1} (Table 1). In the infected culture, intensive growth was noted only in the beginning of the experiment (0.69 day^{-1}). On day two, the growth rate was insignificant (0.13 day^{-1}), while cell numbers reached their maximum at 18×10^5 cells/mL, which is 2.3 times higher than the initial values. The algae began to die away intensively as early as on the third day; their complete lysis occurred on day 4. The parameters F_0/cell anf F_v/F_m changed little in the control (Figs. 5a, 5b) and gradually decreased to zero in the infected culture.

In the first day cell abundance was growing under the light intensity optimal for *Ph. tricornutum* culture growth in the both settings (control and experiment) (Fig. 5c). Specific growth rate of the culture, interacting with viruses, was lower in relation to the control by a factor of 2.8 at the beginning of the second day (Table 1). By the end of the second day, cell abundance in the infected culture was lower than in the control by a factor of ~3.8 (18×10^5 cells/mL (Fig. 5c). This was followed by the rapid die-away (Table 1), resulting in the complete cell lysis by the end of the fourth day. In the process of the culture growth, decrease in values of F_0/cell and F_v/F_m in the control was significantly slower than in the experiment; the values reached zero in the virus-infected culture by the end of the experiment (Figs. 5c, 5d).

DISCUSSION

In recent years there have been studies conducted in the various regions of the World Ocean on the effect of viral lysis on the phytoplankton mortality using the modified dilution method (Baudoux et al., 2008; Pasulka et al., 2015; Mojica et al., 2016;). It has been

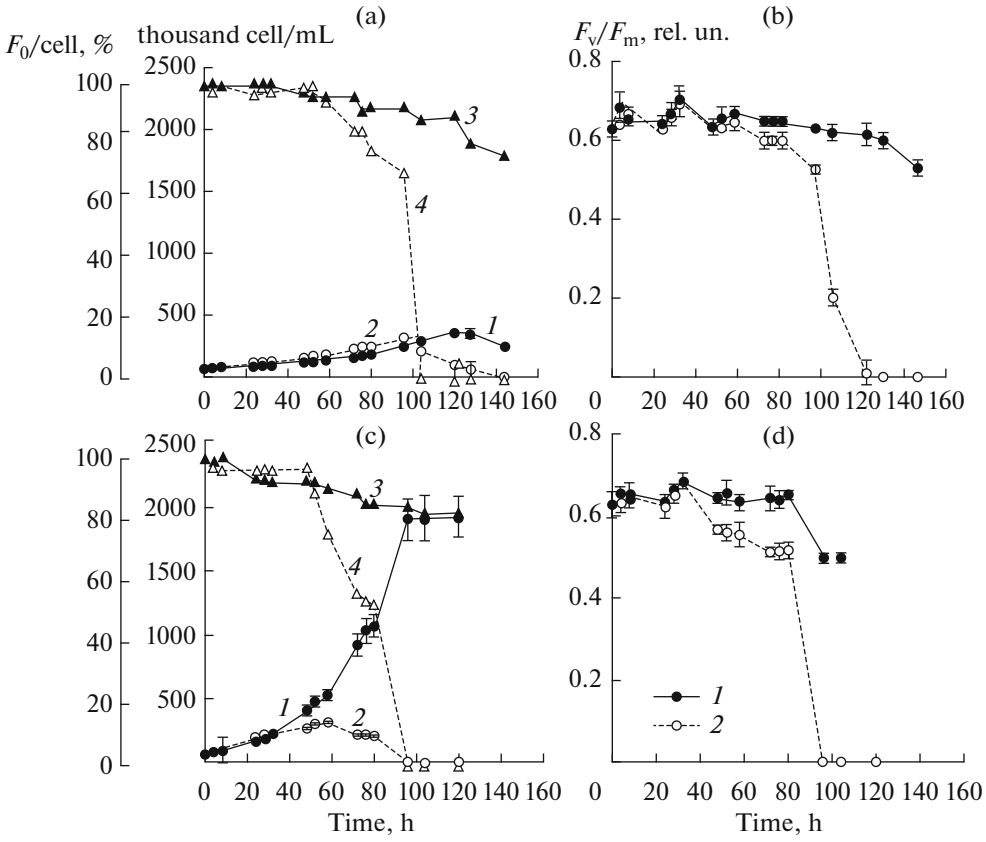

Fig. 3. Cell-abundance dynamics, F_0/cell, and F_v/F_m in culture *Tetraselmis viridis* at (a, b) low and (c, d) high light intensities: (a, c) cell abundance in the (*1*) control and (*2*) experiment; F_0/cell in the (*3*) control and (*4*) experiment; (b, d) F_v/F_m in the (*1*) control and (*2*) experiment. Mean values and their standard deviation are shown.

shown that an average of 8% of organic carbon synthesized by phytoplankton was released to the environment in the North Sea over the summer 2003 as a result of viral lysis in picophytoplankton; its content peaked at 32% in some instances (Baudoux et al., 2008). Phytoplankton mortality was on average 38% higher under the combined effects of microzooplankton and viruses than microzooplankton alone in the California Current in October 2008 (Pasulka et al., 2015). In the Northern Atlantic in July–August 2009, the death rate of picocyanobacteria and eukaryotic picophytoplankton caused by viral lysis approached 0.14–0.23 day^{-1}, which corresponds to 20–30% of their maximum specific growth rate (Mojica et al., 2016). The findings suggest an important role of algal viruses in marine ecosystems. Until present, however, there has been no clear understanding of the effects of environmental abiotic factors and light, in particular, on the rate of lysis of phytoplankton cells. It is very difficult to determine this in the sea due to the technical and methodological challenges. The situation can be elucidated with the laboratory experiments with particular algal species in controlled conditions.

The present research conducted on marine microalgae *Tetraselmis viridis* and *Phaeodactylum tricornutum* involved the maximum concentrations of algal viruses, whose numbers exceeded the algae by one or two orders of magnitude. This approach is commonly applied in experiments studying the interaction between algal viruses and their hosts, that is, microalgae (Pagarete et al., 2015). Generally, a high concentration of viruses in a medium provides for the most immediate onset of infection in algae. Based on our data, the onset of infection in *Tetraselmis viridis* and *Phaeodactylum tricornutum* was dependent on light conditions, which, if optimal, promoted a rapid increase in abundance and activity of the algal virus TsV-S1 and PtV-S18 host cells. Under the algae growth-saturating light intensity, the altered cells presumably infected by the virus were noticeable as early as in a day after the start of the experiment. A decrease in the light intensity by a factor of 3–5 delayed the process for 1 day. Determining the fluorescent parameters served to reveal a reduction in intracellular chlorophyll *a* content in the algae as their infecting by the viruses intensified. A change in shape and sizes of the infected cells, as well as degradation of intracellular pigments, was demonstrated earlier by the case of cultures of coccolithophore *Emiliania huxleyi* (Lohmann) W.W. Hay & H.P. Mohler (Bidle et al., 2007), green

Table 1. Specific rate of growth and loss (μ) of cells in algae cultures *Tetraselmis viridis* and *Phaeodactylum tricornutum* under the growth-limiting (I) and saturation (II) light intensities

Duration, day	μ, day^{-1}	
	I	II
	Tetraselmis viridis	
1	$\frac{0.24 \pm 0.04}{0.48 \pm 0.08}$	$\frac{0.88 \pm 0.15}{1.02 \pm 0.20}$
2	$\frac{0.24 \pm 0.05}{0.24 \pm 0.06}$	$\frac{0.87 \pm 0.17}{0.31 \pm 0.08}$
3	$\frac{0.48 \pm 0.09}{0.48 \pm 0.070}$	$\frac{0.81 \pm 0.15}{(-0.23) \pm (0.06)}$
4	$\frac{0.24 \pm 0.04}{0.24 \pm 0.03}$	$\frac{0.72 \pm 0.13}{(-6.28) \pm (-0.28)}$
5	$\frac{0.10 \pm 0.03}{(-1.20) \pm (-0.32)}$	$\frac{0}{-}$
6	$\frac{(-0.36) \pm (-0.07)}{(-6.68) \pm (-0.22)}$	–
	Phaeodactylum tricornutum	
1	$\frac{0.91 \pm 0.02}{0.69 \pm 0.07}$	$\frac{1.39 \pm 0.09}{0.83 \pm 0.07}$
2	$\frac{0.87 \pm 0.07}{0.13 \pm 0.04}$	$\frac{0.78 \pm 0.06}{(-0.05) \pm (-0.02)}$
3	$\frac{0.63 \pm 0.04}{(-0.11) \pm (-0.03)}$	$\frac{0.67 \pm 0.03}{(-0.16) \pm (-0.03)}$
4	$\frac{0.49 \pm 0.03}{(-3.84) \pm (0.22)}$	$\frac{0}{(-8.00) \pm (-0.75)}$

Control is above the line and experiment is below the line. The mean values and their standard deviation are shown; a dash indicates that data is not available.

microalgae *Tetraselmis striata* Butcher (Pagarete et al., 2015), and diatom algae *Chaetoceros tenuissimus* Meunier (Kimura and Tomaru, 2015).

Structural changes in the studied virus-infected algae cultures were accompanied by a decrease in F_v/F_m. According to Bidle et al. (2007), they transform metabolism in algal cells and activate the expression of metacaspase protein, which is essential for the reproduction of viral particles. The rupture of virus-infected algal cell membrane generally occurs at the moment when the number of viruses in the host cell cytoplasm reaches ~100 virions per cell (Kim et al., 2015). Notwithstanding a slowdown in functional activity of the infected algae *Tetraselmis viridis* and *Phaeodactylum tricornutum*, their cell number was noted to increase further up to a particular abundance level, which can be conditionally defined as the threshold. Their rapid die-away due to viral lysis was noted not earlier than cell abundance in the studied culture reached this threshold. The interval between the moment when the viruses were introduced into the medium and their complete lysis amounted to 4 to 6 days. Similar findings were received earlier from the cultures of coccolithophore *Emiliania huxleyi* (Bidle et al., 2007), green microalgae *Tetraselmis striata* (Pagarete et al., 2015), and diatom alga *Chaetoceros tenuissimus* (Kimura, Tomaru, 2015). In the present study, no growth in cell abundance was observed in the dark; therefore, the effect of viruses on microalgae was insignificant. Apparently, under unfavorable conditions, e.g., insufficient lighting, the active virus infection, causing the host cells lysis, goes into a state of lysogeny (virus survival inside its host cell) (Wommack and Colwell, 2000).

When extrapolating our own data and data published earlier by other authors (Kimura and Tomaru, 2015; Bidle et al., 2007; Pagarete et al., 2015) on the natural phytoplankton population, it can be presumed that the degradation of algal bloom by phytoplankton can occur in days. It appears though that the viral lysis of microalgae cells would occur intensively only in the upper portion of the photic zone where the light does not limit the growth of phytoplankton representatives. This will result in a drastic reduction of the flow of matter and energy from phytoplankton to the highest trophic levels. Additionally, a large amount of organic matter released into the environment due to the viral lysis of algae can serve as the basis for the enhanced growth of bacteria and other unicellular organisms (Stepanova, 2017; Wommack and Colwell, 2000). Viruses appear to play an insignificant role in phytoplankton death at the base of the photic zone.

CONCLUSIONS

Providing even the high concentration of viruses in the experiments, the onset of *Tetraselmis viridis* and *Phaeodactylum tricornutum* cell infection recorded based on an increase in their volume, as well as the reduction of red chlorophyll *a* autofluorescence, variable fluorescence, and photochemical efficiency of photosystem 2, has been shown to depend on the light conditions and the initial abundance of microalgae cells. An increase in the latter two enhanced the aforementioned processes, which was detected on the second or third day after the beginning of the experiment. A decline in the algae numbers due to viral lysis was recorded 1 or 2 days later. The study found the

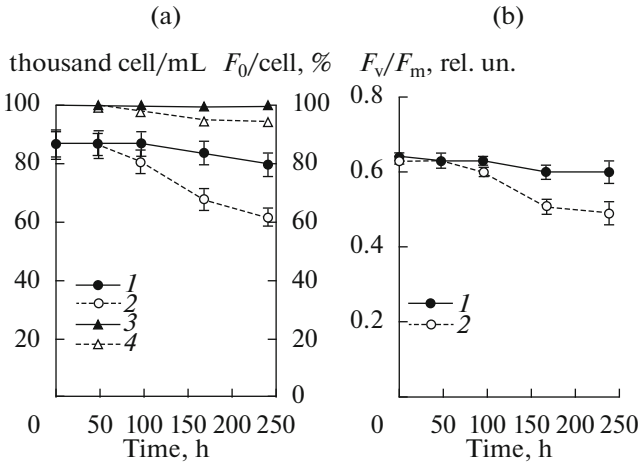

Fig. 4. Cell-abundance dynamics, F_0/cell and F_v/F_m in culture *T. viridis* in the dark: (a) cell abundance in (*1*) the control and (*2*) experiment, F_0/cell in the (*3*) control and (*4*) experiment, and (b) F_v/F_m in the (*1*) control and (*2*) experiment. Mean values and their standard deviation are shown.

microalgae threshold cell abundance at which the onset of their destruction was observed under exposure to viruses. Regardless of light conditions, it was 3×10^5 cells/mL for *T. viridis* and six times that for *Ph. tricornutum*. The complete lysis of cells was detected in the studied cultures by the end of the fourth to sixth day. Virus infection has little if any effect on the die-away of the algal cell in the dark.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare that they have no conflict of interest.

Statement on the welfare of animals. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

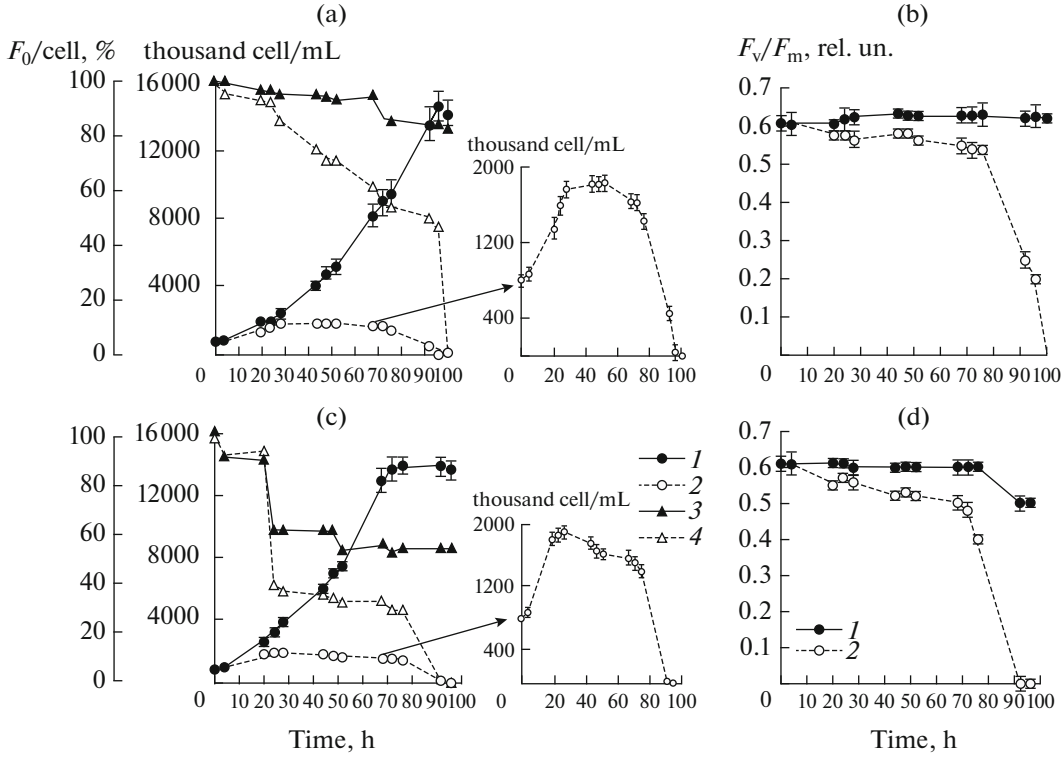

Fig. 5. Cell-abundance dynamics, F_0/cell and F_v/F_m in culture *Phaeodactylum tricornutum* at (a, b) low and (c, d) high light intensities: (a, c) cell abundance in the (*1*) control and (*2*) experiment, F_0/cell in the (*3*) control and (*4*) experiment, and (b, d) F_v/F_m in the (*1*) control and (*2*) experiment. Mean values and their standard deviation are shown.

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