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MICROBIOLOGY

Black Sea Algal Viruses

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Abstract—Monitoring of the Black Sea algal viruses in Sevastopol bays and Crimean water areas has been carried out since 2002. Based on the methods that were developed and patented by the author, more than 200 strains of algal viruses of five species of microalgae that are new to science were isolated: TvV (*Tetraselmis viridis* virus), DvV (*Dunaliella viridis* virus), PtV (*Phaeodactylum tricornutum* virus), PpV (*Prorocentrum pusillum* virus) and IgV (*Isochrysis galbana* virus). For the first time in the Black Sea, the *Emiliania huxleyi* virus (EhV) of microalgae was isolated. Using the method of electron microscopy, the Black Sea algal viruses were identified as icosahedral virions with respective sizes of 56–60, 45–48, 50–53, 88–92, and 128–132 nm, for the TvV, PtV, DvV, PpV and IgV viruses. The EhV size, as determined by the method of filtration, was within the range of 50–200 nm. In the IgV and EhV viruses we revealed a viral envelope. Based on their characters the isolated algal viruses were attributed to the Phycodnaeviridae. The maximum number of algal viruses was observed in the spring and autumn seasons, which is typical for their host phytoplankton species. The Black Sea algal viruses, TvV, PpV, IgV, and EhV, displayed no strict species specificity and have a wide range of available hosts.

Keywords: algal viruses, microalgae, Black Sea

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INTRODUCTION

Aquatic, including marine, viruses are the least studied but most numerous among the hydrobionts. Understanding the role of viruses in the circulation of organic carbon in the hydrosphere in the functioning of food chains and biodiversity is the basis for assessing the stability of marine ecosystems and the predictability of the impact of global changes on biogeochemical processes in the oceans [11, 12]. The algal viruses, which take a leading part in the control of phytoplankton abundance, are of particular interest to researchers. Viruses or virus-like particles have been revealed in 44 taxa of eukaryotic algae belonging to 10 of the 14 known classes of seaweed [13].

The monitoring of Black Sea algal viruses was carried at the base of the Kovalevsky Institute of Biology of the Southern Seas (IBSS) at the National Academy of Sciences of Ukraine (NASU) since 2002; however, from 2015, the research was continued at the Institute for Natural and Technical Systems of the Russian Academy of Sciences (RAS). The purpose of the present work was to search for, isolate and study algal viruses of the Black Sea and analyze the results of their monitoring.

MATERIALS AND METHODS

Searching for and isolating algal viruses, the determination of their concentrations in samples and titration in viral suspensions, as well as several experimental studies were carried out using the easily replicable techniques that were developed and patented by the author [2, 4, 5]. These methods are based on the cytopathogenic effect of the virus on a cell culture of indicator microalgae. The experiment creates the conditions for contact between an indicator microalgae and a sample in which a virus is probably available. The virus in the test sample, which is specific regarding the indicator host (a culture of microalgae) leads to visually observed lysis of the culture, which becomes transparent and colorless compared to that in the control. In subsequent passages, the incubation period is shortened and then stabilized.

The number of viruses (concentration or titer in 1 mL) in the samples of seawater and virus suspensions was determined by their tenfold dilution and further counting of the virus in infectious units per 1 mL. To facilitate the determination of the concentration of the virus we may assume that a single infectious unit corresponds to the infectious action of at least one virus. Thus, the number of infectious units per 1 mL can be equated to the number of virus particles in 1 mL.

Microalgal cultures of *Tetraselmis viridis* (Rouchijajnen) R.E. Norris, Hori & Chihara, 1980, *Dunaliella viridis* (Teodoresco, 1905), *Phaeodactylum tricornutum* (Bohlin, 1897), *Prorocentrum pusilla* (Schiller) Dodge and Bibby, 1973, *Isochrysis galbana* (Parke, 1949), *Emiliania huxleyi* (Lohmann) Hay and Mohler,

Table 1. The frequency of isolation of the TvV, PtV, DvV, PpV, IgV and EhV algal viruses at different stations in the Sevastopol bays and Crimean water areas (May 2002–February 2015), depending on the type of sample

Material	Number of samples/samples with isolated algal viruses, % of samples positive for algal viruses available						
	(TvV + PtV)			DvV, 2008–2012	PpV, 2010–2013	IgV, 2012–2013	EhV, from 2014 to February 2015
	2002–2006	from 2007 to September 2011	from October 2011 to September 2012				
Gills of fish	107/8 (8 + 0), 7.5% (7.5% + 0%)	8/0, 0%	Not studied	Not studied	Not studied	Not studied	Not studied
Mussels	11/8 (6 + 2), 72.7% (54.5% + 18.2%)	41/18 (2 + 16), 43.9% (4.9% + 9.0%)	4/3 (2 + 1), 75.0% (50% + 25%)	36/18, 50.0%	23/5, 21.7%	10/7, 70.0%	1/1, 100%
Sea Water	66/38 (16 + 22), 57.5% (24.2% + 33.3%)	165/32 (6 + 26), 19.4% (3.6% + 15.8%)	22/10(4 + 6), 45.5% (18.2% + 27.3%)	141/21, 14.9%	108/15, 13.9%	52/14, 26.9%	18/14, 77.8%
In total, % of isolation	184/54 (30 + 24), 29.3% (16.3% + 13.0%)	214/50 (8 + 42), 23.3% (3.7% + 19.6%)	26/13(6 + 7), 50% (23.1% + 26.9%)	177/39, 22.0%	131/20, 15.3%	62/21, 33.9%	19/15, 78.9%

1967, *Stichococcus bacillaris* (Nägeli, 1849), *Dunaliella salina* (Dunal) Teodoresco, 1905, and *Chlorella vulgaris* Beyerinck [Beijerinck], 1890 were used as indicator cultures. The cultures were obtained from the collection of the Department of Ecological Physiology of Algae, IBSS and were maintained in stabilizing Goldberg medium. During the search for and isolation of algal viruses from samples of sea water, mussels and fish, as well as in the experimental study, the cultures in the intense growth (logarithmic growth) phase were used at concentrations that did not exceed 10^4 cells/mL. We studied the mantle fluid of the Black Sea mussel *Mytilus galloprovincialis* (Lamarck, 1819) and gills (a 10% suspension) in the fish. The methods of preparing of the material have been described previously [5]. To study the species-specificity of the Black Sea algal viruses, their contacts with different algal species were examined.

The morphological characters of algal viruses were examined under a TEM-1230 transmission electron microscope (Jeol, Japan) at the Kholodny Institute of Botany, NASU and the Zabolotny Institute of Microbiology and Virology, NASU, as well as under an electron microscope (EM; Sumy, Ukraine) at the Taras Shevchenko Kiev National University. The EM had no scale ruler; thus the size of the virus was determined by taking the magnification of the microscope stills into account. The samples were contrasted with a 1% aqueous solution of uranyl acetate. We investigated the viral suspension as the native suspension (after isolation) and after isolation and concentration using SW 25.2 and Sorvall AH-629 centrifuge rotors, respectively, at 5000 rpm for 30 minutes and 29000 rpm for 60 min, with the following fixation with a 2.5% glutaraldehyde solution. The concentration and purification of the PpV suspension was based on the common technique of polyethylene glycol treatment.

The existence of the viral envelope and a super capsid (a complex lipid membrane) in algal viruses was determined via the processing of 2 mL of the virus suspension for 1 hour with 0.2 mL of chloroform as a lipid solvent that destroys lipid membranes. The treated suspension was further tested for its lysis ability (the ability to cause infection) by carrying out several successive passages in an indicator culture.

To determine the size of the *E. huxleyi* Black Sea algal virus that was isolated in 2014, ultrafiltration was performed; to do this a syringe, filter holder, and nitrocellulose Sartorius filters were used. After passage of the virus suspension through filters with pore diameters of 300, 200, and 50 nm, the filtrate was added to the culture of the indicator microalgae. The size of the studied algal virus was determined via the pore diameter of the filters by taking both the maximum diameter at which the filtrate still caused the lysis of culture (the maximum size of the virus), and the minimum diameter at which the filtrate did not cause lysis (the minimum size of the virus) into account.

RESULTS AND DISCUSSION

From May 2002 to February 2015, more than 500 samples were studied in total, with more than 200 strains of isolated algal viruses of the microalgae *T. viridis* (TvV), *P. tricornutum* (PtV), *D. viridis* (DvV), *P. pusilla* (PpV), *I. galbana* (IgV), and *E. huxleyi* (EhV) (Table 1). The isolation of algal viruses from mussels was more successful than that from sea water samples, because the percentage of positive samples for algal viruses was always higher in the mussels. This is due to the accumulation of viruses from the environment by the filtering apparatus of the shellfish. However, the sampling of sea water is less complicated and therefore was used more often.

It was recorded that in 2002–2006 the percentage of isolation of TvV (16.3%) was slightly higher than that of PtV (13%) in all the samples that were studied. In 2007–2011, the situation was different: PtV was iso-

Table 2. The results of contact of the Black Sea algal viruses with the cultures of indicator and non-indicator species of microalgae

Microalgal culture	Algal viruses					
	TvV	DvV	PtV	PpV	IgV	EhV
Tetraselmis viridis	Lysis	Culture growth	Culture growth	Culture growth	Culture growth	Culture growth
Dunaliella viridis	"	Lysis	"	"	"	"
Phaeodactylum tricornutum	Culture growth	Culture growth	Lysis	"	"	"
Prorocentrum pusillum	"	"	Culture growth	Lysis	Lysis	Lysis
Isochrysis galbana	"	"	"	"	"	"
Emiliania huxleyi	"	"	"	Culture growth	Culture growth	"
Dunaliella salina	"	"	"	Not studied	Not studied	Not studied
Chlorella vulgaris	"	"	"	"	"	"
Stichococcus bacillaris	"	"	"	"	"	"

lated 5 times more often. In 2012, the total frequency of isolation of TvV and PtV from the samples increased by almost 2 times (50%) relative to those for 2007–2011 (23.3%) and 2002–2006 (29.3%); the increase was due to isolation as PtV and TvV.

The results of monitoring the algal viruses indicate changes in the structure and number of phytoplankton in the Black Sea for three time periods: 2002–2006, 2007—2011, and 2011–2012. We assume that these changes in the Seavastopol bays in different periods of time (a decline in 2007–2011 and an increase from October 2011 to December 2012 relative to the data for 2002–2006) may be caused by both regional and global environmental factors.

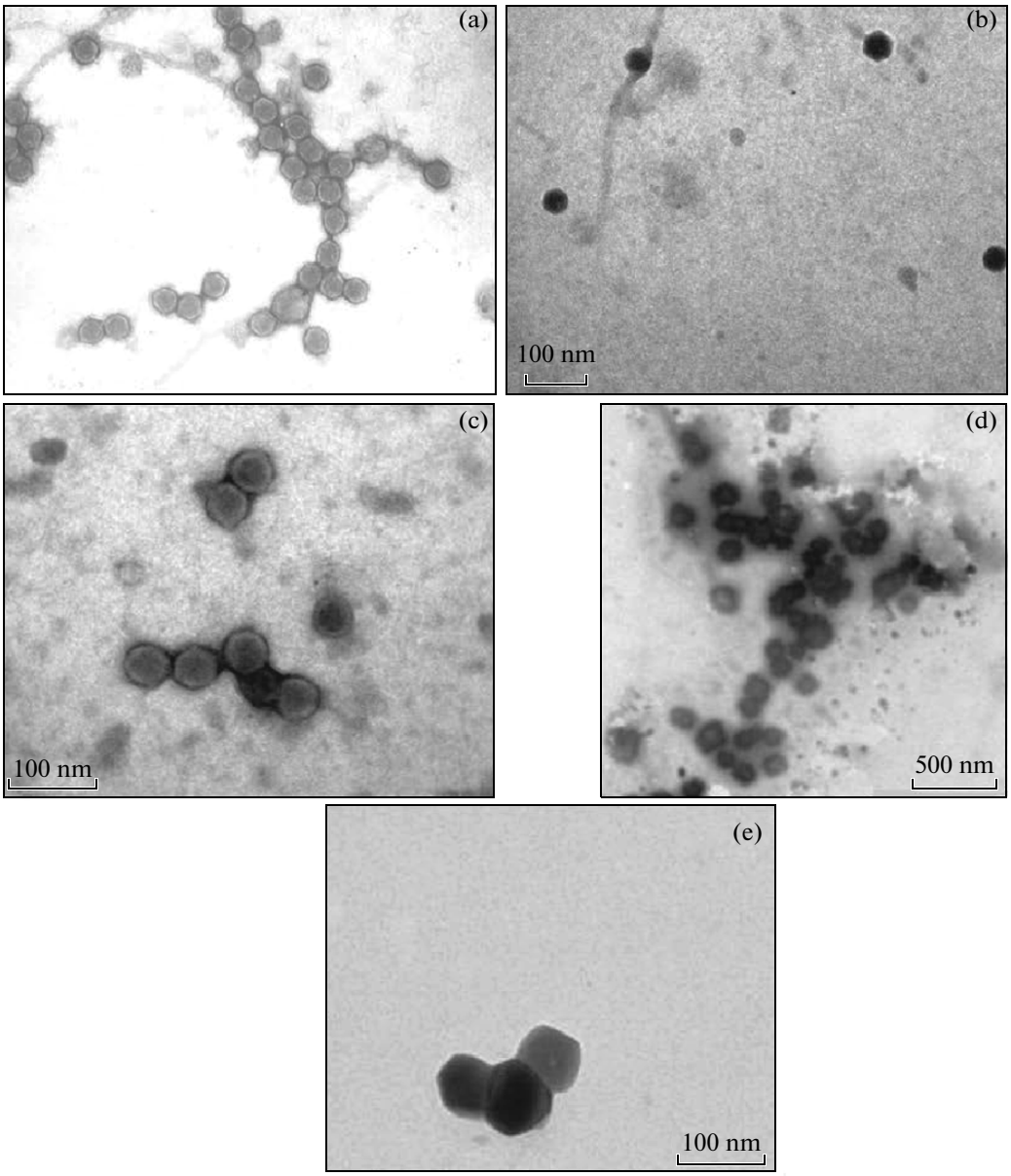
The isolation of DvV was carried out in 2008—2012; 39 strains of the virus were isolated from 177 samples. In 2010, the search for PpV began; 20 strains were isolated from 131 samples. From 2012 to 2013 we searched for the algal virus of the microalgae *I. galbana* in water samples and mussels from the Black Sea. The isolation of IgV was a surprise to algologists, as the viral host, the alga *I. galbana*, was not previously recorded in the Black Sea. The finding was the first in the Black Sea. The *I. galbana* virus is the first indirect corroboration of the circulation of the microalgae in this area. In 70% of the cases, the IgV virus was isolated from mussels. This high percentage of isolation of algal virus from the mussel was recorded for the first time.

In 2014, we began to search for the virus of the *E. huxleyi* microalgae (EhV) in samples of sea water and mussels. It was isolated from water samples (78.9%) in all seasons, but in August and September at the maximum of the air and sea water temperatures it was not recorded. In the hot season there were problems in maintaining the *culture* of *E. huxleyi,* as its growth and development required a temperature no higher than 23–24°C. These observations may indicate a decrease in the number of the *E. huxleyi* microalgae in the hottest season of the year (late summer and early autumn).

It was found that Black Sea algal viruses were isolated from water samples in accordance to the seasonal dynamics of the number of their microalgal hosts, mostly in February and April, and less in September and October, which corresponds to known spring–autumn "peaks" of phytoplankton species [3, 9]. Such a seasonal correlation was not observed in the algal viruses isolated from mussels, which can be explained by their long preservation in filtering hydrobionts.

The infectious titer of algal viruses in suspensions reached 10^{11} infectious units per 1 mL (inf. un./mL), i.e., at least 10^{11} virions in 1 mL for TvV and DvV, 10^7–10^8—for PtV, 10^4–10^5—for PpV, IgV and EhV. The viral titer of the water samples was dependent on the season. Thus, during the periods of the maximum number, the titer in water samples reached 10^5–10^6 for TvV and DvV, 10^3–10^4 for PtV, and usually did not exceed 10^2 virions (inf. un./mL) for PpV, IgV, and EhV. The titer of algal viruses in mussels usually was 2–3 orders higher than that in the sea water. Lack of sensitivity to chloroform was found for TvV, DvV, PtV, and PpV, indicating the lack of a viral envelope. The sensitivity to chloroform for IgV and EhV implies the existence of a viral envelope, i.e., a more complex morphological structure.

By studying the character of the contacts of Black Sea algal viruses with non-indicator algae we obtained results that show the absence of strict species-specificity for some viruses. In other words, other hosts were revealed for the Black Sea algal viruses. It was revealed that TvV also lyses the microalgae *D. viridis,* while DvV lysed only the indicator algae (Table 1). IgV is also



The Black Sea algal viruses TvV (a), PtV (b), DvV (c), PpV (d) and IgV (e).

capable of lysing the microalgae *P. pusillum,* while PpV, in turn, lysed *I. galbana.* EhV caused lysis of cultures of the microalgae *I. galbana* and *P. pusilla,* while these viruses do not lyse a culture of microalgae *E. huxleyi* (Table 2). Thus, in four of the six species of Black Sea algal viruses, additional hosts were revealed beyond their indicator hosts. The wide range of hosts increases the prospects for the survival and distribution in nature of the viruses, but does not point to their morphologic and genetic similarity, as shown by the data from electron microscopy and genetic analysis [7].

According to the data from electron microscopy (figure), the Black Sea algal viruses TvV, PtV, DvV, PpV and IgV, which are new to science, have an icosahedral form; their sizes are 56–60, 45–48, 50–53, 88–92, and 128–132 nm, respectively. The morphologies of some forms of TvV, PtV, and DvV have been described previously [1, 5, 6, 8]. Using the method of filtration through nitrocellulose filters we revealed that the sizes of the virions of the EhV Black Sea algal virus varies within 50–200 nm. The size of the virions of the EhV algal virus from the Norwegian Sea varied from 160 to 180 nm [10].

Despite the fact that we observed lysis of the *D. viridis* culture by TvV, lysis of *I. galbana* cultures during contact with PpV, and lysis of *P. pusilla* cultures with IgV, these viruses differed in size. The sensitivity of the viruses to chloroform revealed that only IgV and EhV

have viral envelopes (a super capsid). The analysis of the sequenced genomes of TvV and DvV displayed a complete lack of homology in their genes [7]. Taking their morphology into account and based on the analysis of their genomes, the Black Sea algal viruses were attributed to the Phycodnaeviridae Family [5, 7].

Thus, the monitoring of the Black Sea algal viruses that has been performed since 2002 using the author's patented methods enabled us to isolate more than 200 strains of algal viruses of the microalgae *T. viridis, D. viridis, P. tricornutum, P. pusillum, I. galbana*, and *E. huxleyi* from samples of sea water and hydrobionts taken off the coast of Sevastopol City and the Crimean Peninsula. It was determined that the seasonality of Black Sea algal viruses is characteristic of the spring and autumn peaks of abundance, which is typical for their host species from the phytoplankton. The virus titer in the mussels typically was 2–3 orders of magnitude higher than their concentrations in sea water. The lack of strict species specificity of the Black Sea algal viruses for their hosts was revealed. A wider range of hosts was defined for four viruses (TvV, PpV, IgV, and EhV), which gives them a greater opportunity for survival and circulation in nature.

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