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Samantha R. Coy  
*University of Tennessee, Knoxville*

Alyssa N. Alsante  
*University of Tennessee, Knoxville*

James L. Van Etten  
*University of Nebraska, Lincoln*

Steven W. Wilhelm  
*University of Tennessee, Knoxville, wilhelm@utk.edu*

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Cryopreservation of *Paramecium bursaria* Chlorella Virus-1 during an active infection cycle of its host

Samantha R. Coy, Alyssa N. Alsante, James L. Van Etten, Steven W. Wilhelm

1 Department of Microbiology, University of Tennessee, Knoxville, Tennessee, United States of America, 2 Department of Plant Pathology and Nebraska Center for Virology, University of Nebraska, Lincoln, Nebraska, United States of America

□ Current address: Department of Oceanography, Texas A&M University, College Station, Texas, United States of America

* wilhelm@utk.edu

Abstract

Best practices in laboratory culture management often include cryopreservation of microbiota, but this can be challenging with some virus particles. By preserving viral isolates researchers can mitigate genetic drift and laboratory-induced selection, thereby maintaining genetically consistent strains between experiments. To this end, we developed a method to cryopreserve the model, green-alga infecting virus, *Paramecium bursaria Chlorella virus 1* (PBCV-1). We explored cryotolerance of the infectivity of this virus particle, whereby freezing without cryoprotectants was found to maintain the highest infectivity (~2.5%). We then assessed the cryopreservation potential of PBCV-1 during an active infection cycle in its *Chlorella variabilis* NC64A host, and found that virus survivorship was highest (69.5 ± 16.5%) when the infected host is cryopreserved during mid-late stages of infection (i.e., coinciding with virion assembly). The most optimal condition for cryopreservation was observed at 240 minutes post-infection. Overall, utilizing the cell as a vehicle for viral cryopreservation resulted in 24.9–30.1 fold increases in PBCV-1 survival based on 95% confidence intervals of frozen virus particles and virus cryopreserved at 240 minutes post-infection. Given that cryoprotectants are often naturally produced by psychrophilic organisms, we suspect that cryopreservation of infected hosts may be a reliable mechanism for virus persistence in non-growth permitting circumstances in the environment, such as ancient permafrosts.

Introduction

Viruses are abundant components of all biological systems and they likely infect every lineage of eukaryotic algae. Their impact is most readily noticed following infection and lysis of abundant bloom forming algae [1–3], though lytic activity of all algal viruses contributes to significant biomass recycling via the ‘viral shunt’ [4]. To date, 65 eukaryotic algal viruses have been isolated and developed as laboratory strains [5, 6]. Most of these are maintained through serial
propagation on their respective hosts. Though this has been effective for culturing many 
strains over the last few decades [7, 8], each passage allows for genetic mutations that can accu-
mulate in a population [9], leading to a deviation from a standard 'wild-type.' Moreover, it is 
impertative to control evolution following the development of genetically tractable algal hosts 
[10] and (ultimately) virus systems. Although seed-stock systems can be developed without 
cryopreservation, many systems are not amenable to this either because the virus particles are 
degraded during purification efforts or lose their infectivity during storage. Moreover, it can 
take time to achieve axenic status with new virus isolates, thus making contaminating bacterial 
activity a significant source of degradation. Thus, a protocol for successful virus cryobiological 
preservation that is applicable to a wide variety of algae-virus systems would offer an opportu-
nity to universally improve virus management and distribution in the laboratory.

Cryopreservation is not a new concept in biological sciences. For most protocols, it involves 
controlled cooling of biota to sub-freezing temperatures to achieve biological cessation while 
preserving viability. This most often manifests as slow-cooling at a rate of 1˚C / min in the 
presence of osmoprotectant(s) (e.g., dimethylsulfoxide (DMSO), glycerol) for long-term stor-
age at -130˚C or below [11]. Too slow a cooling rate can result in higher intracellular concen-
tration of osmoprotectants, resulting in toxicity, whereas too fast a cooling rate allows the 
formation of intracellular ice crystals which can rupture cell membranes [12]. The thawing 
process is typically quick, as microbial death is commonly associated with slow thaw rates. 
Though cryopreservation is a standard method for maintaining cellular organisms, it has 
rarely been utilized for the preservation of algal viruses.

One eukaryotic algal virus cryopreservation protocol is in existence. It was developed for 
HaV, a dsDNA virus that infects the red tide forming dinoflagellate Heterosigma akashiwo 
[13]. Researchers investigated a combination of cryoprotectants and storage temperatures with 
the highest recovery (8.3% of infectious virus) employing flash freezing of HaV particles sus-
pended in 20% DMSO. This protocol has been adapted for a handful of other algal viruses 
with viable recovery ranging from < 1% to 27% [14–16]. The typical low recovery in these pro-
cedures is likely due to physiological differences between viruses and cells including differ-
ces in permeability, osmolarity tolerance, and toxicity to osmoprotectants. It is also clear 
that these protocols deviate from the standard method which controls the cooling rate; to our 
knowledge this has not been tested as a matter of improving virus particle survival. Owing to 
these complications, we decided to take a new approach by investigating cryopreservation 
recovery and stability of actively infecting, cell-associated algal viruses.

Chloroviruses are large (> 300 kb) dsDNA viruses in the family Phycodnaviridae [17]. They 
are members of the proposed order the Megavirales [18], also known as “giant” viruses, and 
remain the best characterized algal-virus system to date. Isolated in the early 1980’s [7], the 
prototype chlorovirus Paramecium bursaria Chlorella virus 1 (PBCV-1) has been maintained 
through serial propagation on its host, Chlorella variabilis NC64A. PBCV-1 is inactivated by 
freezing, though other closely related virus strains, including other chloroviruses, persist 
through freeze/thaw events [19, 20]. As a great deal of research has centered on PBCV-1, 
including genomics [21], transcriptomics [22, 23], and proteomics [21], it is important to 
develop a successful cryopreservation protocol for this strain that may serve as a model for 
preserving algal viruses. There are several reports of cryopreservation techniques for eukary-
otic algae [24–28] which might be adapted for the preservation of actively replicating 
chloroviruses.

Here, we tested the cryo-potential of chlorovirus PBCV-1 using a protocol that yielded con-
sistent recovery (~50% viable cells) of four strains of algae over 15 years: Chlorella vulgaris C-
27, Chlorella vulgaris M-207A7, Nannochloropsis oculate ST-4, and Tetraselmis tetrathlele T-
501 [29]. Owing to the close relationship between C. vulgaris and C. variabilis, as well as the
consistent results across unique algae, we elected to determine if these results could be recapitulated in PBCV-1. To test this, we attempted cryopreservation of both the virus particle as well as the virus replicating in its host.

**Materials and methods**

**Virus particle cryopreservation**

Chlorella variabilis NC64A was infected with PBCV-1 during mid-logarithmic growth at standard culturing conditions (25°C; continuous light exposure at 30μEin/m²/s) using Modified Bold’s Basal Medium [30]. Following complete lysis, the viral lysate was pre-filtered through a sterile, 0.45 μm polycarbonate syringe filter and titered by plaque assay [31, 32] for initial infectivity assessments. Cryoprotectant choice was guided by Nakanishi et al. [29], in which a combination of 5% DMSO (v/v), 5% ethylene glycol (v/v), and 5% proline (w/v) was found to consistently produce the highest algal recoveries. Stock solutions of each cryoprotectant were made at a concentration of 30% with sterilized Milli-Q water and combined in a 1:1:1 ratio to yield a final concentration of 10% for each compound. For virus particle cryopreservation, 1 mL of PBCV-1 particles (7.82x 10⁸ plaque forming units (PFUs) per ml) was added to 1 mL of ice-chilled cryoprotectant solution contained in a 2-mL cryovial. The cryovials were incubated on ice for 45 min, then transferred to a freeze-rate controlled container (Mr. Frosty, Thermo Fisher Scientific Inc., USA) filled with isopropanol for overnight incubation at -80°C. The next morning, cryovials were transferred to a -150°C freezer. At the designated recovery times, vials were removed from the freezer and set in a 40°C water bath. After thawing, the samples were serially diluted ten-fold in 50 mM Tris-HCl (pH = 7.8) and virus infectivity was determined by plaque assay [31]. Virus viability was calculated as a percentage by comparison to the initial virus particle stock titer before cryopreservation. Long-term experiments assessed the stability of virus infectivity in particles stored at -150°C.

**Infected Chlorella cryopreservation**

Chlorovirus PBCV-1 was propagated as described above and titered to obtain infectious PFUs/ml. This virus particle stock was used to infect late-logarithmically growing C. variabilis NC64A at an M.O.I. of 5, at which point infected cultures were returned to standard incubation conditions. At 1, 10, 30, 60, 120, 180, 240, 300, and 360 min post-infection (PI), 1 mL aliquots of infected cells were mixed with 1 mL of ice-chilled cryoprotectants [final concentration: 5% DMSO (v/v), 5% ethylene glycol (v/v), and 5% proline (w/v)] in duplicates. The mixture was incubated on ice for 45 min, then transferred to a freeze-rate controlled container (Mr. Frosty, Thermo Fisher Scientific Inc., USA has a -1°C/min cooling rate) filled with isopropanol for overnight incubation at -80°C. The next morning, cryovials were immediately transferred to a -150°C freezer. At the designated recovery times, vials were removed from the freezer and placed in a 40°C water bath. After thawing, the infected cells were pelleted in a Sorvall Legend RT Benchtop Centrifuge at 3,700 rpm (~3,000 rcf) for 10 min: (free virus requires higher speeds for pelleting). Cell pellets were re-suspended in 2 mL of 0.01M HEPES solution (pH = 6.5). Suspensions were immediately diluted and plaque assayed, plating late-infection treatments first. Viability was determined as a percentage of the pre-frozen cellular concentration (3.57 x 10⁶ cells/mL), as only surviving infected cells would be capable of producing plaques. Long-term experiments were conducted in the same manner, though only time points 10, 180, and 240 min PI were collected and assayed. The complete step-by-step method can be found at protocols.io [33].
Results

Following the cryopreservation procedures of other algal virus researchers [13–16], we investigated the cryo-potential of the PBCV-1 particle. Cryoprotectant alone treatments elicited a lethal effect: ~87% of the infectious virus particles were inactivated in the presence of these chemicals following 24 hr exposure at 4˚C. Given this effect, we decided to freeze PBCV-1 particles at -150˚C without any cryoprotectants. This resulted in ~2.5% recovery of the infectious virus population, which was stable for storage periods of up to one year (Fig 1). Seeing room for improvement, we tested the cryo-potential of PBCV-1 in an infected, cell-associated state.

The PBCV-1 replication cycle requires about 6–8 h to release nascent virus particles [34]. Post-infection sampling times for cryopreservation (10, 30, 60, 120, 180, 240, 300, 360 min PI) followed similar sampling strategies used in PBCV-1 transcription studies [22, 23]. Specifically, these time points were collected across distinct physiological phases in the PBCV-1 lifecycle and thus represent likely unique conditions for cryopreservation. Following 24-h storage of cryopreserved, infected cells, we found that late stages of infection were more conducive to virus survival than early stages (Fig 2). Thus, we followed cryo-stability for one year in one early (10 min PI) and two late infection stages (180 and 240 min PI) (Fig 3). Small day-to-day fluctuations in virus titers were common, but were typically consistent among treatments, suggesting human error. Despite these fluctuations, the virus particle stock control, 180-min, and 240-min PI treatment yielded an acceptable relative standard deviation (RSD) for these plate counts [35] across all recovery assessments, indicating cryo-stability (Table 1). Cryo-stability

Fig 1. Cryo-stability of the PBCV-1 particle. Viability of chlorovirus PBCV-1 was determined by plaque assaying viruses that had been stored as particles either at 4˚C or -150˚C. Green circles represent virus particles stored at 4˚C, while red squares denote virus particles stored at -150˚C. Error bars are represented as the standard deviation of biological and technical replicates.

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was not observed in the 10 min PI samples (Table 1). In comparison to virus particle cryopreservation, the cell-associated method yielded significant improvement in survivorship for the optimal 240-minute treatment (24.9–30.1 fold increases).

**Discussion**

The current maintenance strategy for chloroviruses involves serial propagation on the alga host followed by lysate particle storage at 4°C. Chloroviruses are relatively stable under these conditions, though even PBCV-1 is known to degrade after several years of storage. In any case, many algae-virus systems are less amenable to long-term storage at 4°C. For example, new algae-virus systems are not always quickly made axenic, and are thus susceptible to degradation from contaminating bacteria. On the other hand, viruses propagated on axenic hosts can still degrade. For reasons unknown, chloroviruses are more stable in lysates (bacterial-free) than in particle stocks purified by sucrose density gradients [36], but they always eventually lose their infectivity. Serial propagation of viruses is therefore often required. Even if this is done infrequently, it can still promote genetic drift and result in deviation from wild-type status. This is concerning for all virus types, though RNA viruses, which have the fastest mutation rates, would be most susceptible [9, 37]. Beyond considering spontaneous, replication-associated errors, chloroviruses encode putative enzymes involved in genomic rearrangements. For
example, GIY-YIG mobile endonucleases and an IS607 transposon may be involved in insertions/deletions and/or gene loss/duplications observed in genomic comparisons of chloroviruses [38, 39]. Thus, maintenance of wild-type strains is important for consistency between experiments. Virology labs could follow the microbial culture collection strategy, which typically uses a cryo-banking/seed-stock system for the dissemination of microbial specimens. The purpose of the seed-stock system is to minimize serial propagation of microbiota. The American Type Culture Collection (ATCC) suggests that consumers transfer their cultures no more than five-times after propagation from the thawed culture collection stock.

### Table 1. Statistical assessment of PBCV-1 infectivity across storage treatments for ~1 year.

| Treatment                                      | N  | Average | SD  | RSD | 95%CI     |
|------------------------------------------------|----|---------|-----|-----|-----------|
| Virus Particle Stock (4°C)                     | 67 | 75.1    | 16.9| 22.5| 71.1–79.2 |
| Virus Particle Stock (-150°C)                  | 124| 2.53    | 0.61| 24.0| 2.42–2.64 |
| Cell-associated virus 10 minutes PI (-150°C, +CPA) | 79 | 7.56    | 3.38| 44.7| 6.81–8.31 |
| Cell-associated virus 180 minutes PI (-150°C, +CPA) | 82 | 31.9    | 10.9| 34.2| 29.5–34.3 |
| Cell-associated virus 240 minutes PI (-150°C, +CPA) | 82 | 69.5    | 16.5| 23.8| 65.9–73.0 |

+CPA, cryoprotectants present as described in materials and methods section. Asterisks (*) denote an acceptable RSD (i.e., Coefficient of Variation) for plaque assays based on a 35% threshold used in bacterial plating standards set from chapter 1223 by the U.S. Pharmacopeia and National Formulary.

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seemingly strict standard, it is not difficult to imagine the consequences of violating this. For example, the United States Pharmacopeia and National Formulary requires test organisms to be maintained this way for routine antibiotic efficacy screens, and non-compliance can undermine therapeutic treatment [35]. Although there is no direct clinical link to maintaining algal viruses this way, the logic is consistent with any research requirements. The cryopreservation protocol described here can help researchers better set up these cryo-banking/seed stock systems.

Standard cryopreservation techniques are not designed for the unique structure and physiology of virus particles. Indeed, cryoprotectants are classified by their permeability across cell membranes, which often coincides with their molecular weight [24]. Smaller compounds, such as ethylene glycol and DMSO, are considered penetrating cryoprotectants, while larger compounds (e.g. amino acids; L-proline) are typically non-penetrating. That said, the exclusion size threshold has not been established for most viruses so it is not clear which, if any of these compounds penetrate the viral capsid. It is generally thought that virus capsids are permeable to water and ions, though the latter diffuses much slower; this mechanism has been used to osmotically rupture capsids [40, 41], including PBCV-1 [42]. The final cryoprotectant solution used for PBCV-1 particle cryopreservation has an estimated osmolarity of ~150 mOsmoles/L, which is comparable to the storage buffer used for this virus. In light of this, we propose that the lethal effect the cryoprotectants have on the PBCV-1 particle is not the result of osmotic stress, and that inactivation instead occurred by toxicity of cryoprotectants or oxidative stress. This would be consistent with viruses not being metabolically active and therefore unable to repair damage caused by this treatment. It is also consistent with the observation that Mimivirus, a giant virus relative which also contains an internal lipid membrane, is said to be inactivated by lipophilic compounds such as DMSO [43]. That said, DMSO is often used as a stabilizer for freezing of enveloped virus particles [44]. This discrepancy may be due to unique properties between external and internal membranes, or even system differences between animal and plant viruses, which imparts resistance in some cases over others. Regardless, the mechanism of inactivation may be better ascertained by looking at survivorship of virion particles via epifluorescent microscopy, flow cytometry [45–47], or using bioassays to quantify oxidative stress.

Although the algal cell is in a sub-optimal physiological state during infection, it is apparently robust enough to survive and maintain an active infection during cryopreservation. That said, fewer infectious virus were recovered when the cell was cryopreserved during early infection stages. This might be explained by differences in adsorption rates and synchronicity of infection, resulting in fewer infected cells at the start of the experiment. Most, if not all cells are infected at the later stages of infection (3–4 hr PI). Regardless of any differences in synchronicity, the algal cell will be completely arrested during cryopreservation, and will only continue the infection cycle after thawing. Internal, mature viruses that have not yet lysed their host cell might still be inactivated by cryoprotectants, thus reducing viral burst size, but our experiments did not account for this. We also did not account for inefficiencies in infection rates; though we infected at M.O.I. values based on infectious particle counts, it is possible that all the cells were not infected. Had we plated the infected cell population prior to cryoprotection we could have corrected for this in our results. In any case, accounting for infection inefficiency can only improve PBCV-1 survivorship and the success of our method.

The general classification of cryoprotectants based on membrane permeability is consistent in the infected cell treatment. Although the C. variabilis NC64A genome encodes a secondary active transporter for the uptake of proline, radio-labeled solute uptake experiments revealed that PBCV-1 infection abolishes its activity [48]. With that in mind, the toxicity of the cryoprotectant mixture would equate to ~90 mOsmoles/L, as only DMSO and ethylene glycol are
penetrating, and many of the components in the MBBM media would be spent by late-logarithmic growth. This concentration is comparable to buffers routinely used in our lab for handling *C. variabilis* (40 mOsmoles/L), so there is little concern of osmotic stress. The chances of osmotic stress were also low considering the consistent success associated with this cryopreservation formula across eukaryotic algae, including two *Chlorella* spp. [29]. Our results are likely applicable to any algal virus whose host can be cryopreserved. That said, we expect that researchers may still have to adjust their cryoprotectant mixture to account for system differences related to osmolarity tolerance and cryoprotectant toxicity. There has also been research indicating that axenicity impacts cryopreservation survival in microalgae. In this light, it is possible that the bacterial community produces secondary metabolites which promote survival [49]. In another scenario, organisms with psychrophilic tendencies might be adapted to freeze situations and cryoprotectant additives may not be necessary.

The goal of this study was to develop a long-term cryopreservation method for chlorovirus PBCV-1, but there are also interesting ecological implications of this research. Recent metagenomic and isolation efforts indicate that giant viruses of microeukaryotes (e.g., *Phycodnaviridae* and *Mimiviridae*) are widely distributed in nature [50, 51], but it is not well understood how these viruses persist in the environment. Freezing events represent a potential mechanism of inactivation for some algal viruses, though chlorovirus ATCV-1 is stable during these conditions [19]. In two other studies, a closely related giant virus of the family *Mimiviridae* [52], as well as a second giant virus in the family *Molliviridae* [53], were revived from 30,000 year old permafrost. Both of these viruses were revived using *Acanthamoeba spp.*, one of the main hosts for many giant viruses. That said, there have been questions about whether *Acanthamoeba* and other protists used for laboratory viral propagation are the natural or primary hosts of these ancient viruses [54]. Although these viruses might be able to withstand freezing temperatures on their own, the results of this study suggest that a natural host might serve as a better vehicle for surviving freezing. Indeed, many microbes produce natural cryoprotectants (e.g., L-proline, trehalose, betaine, etc.) or encode machinery to transport these osmoprotectants into the cell. Following this thought process, it is possible that environments containing frozen, infected cells might contain naturally cryopreserved algal-virus systems. These systems may be deciphered following advances in single-cell sorting and sequencing techniques. Indeed, a similar approach has been successfully utilized to identify and sequence single virus genomes in the ocean [55]. Though this latter study sorted virus particles, flow-cytometry sorting of viral infected cells may be achieved using fluorescent probes specific for viral marker genes (e.g., major capsid protein) or dyes to detect viral-induced host phenotypes (e.g., membrane blebbing). As a proof of concept, viral genetic sequences recovered from Siberian permafrost could be used to probe for still frozen viral-infected host cells, thereby testing the natural host range of these viruses.

To our knowledge, this is the first report of successful cryopreservation of a eukaryotic algal virus during its infection cycle. We expect that respective cellular hosts will provide more suitable physiological conditions for cryopreservation and storage of algal viruses that infect eukaryotic algae. We also recommend that laboratories working with algal viruses establish cryopreserved seed-stock systems to better preserve wild-type controls for future experimentation, especially in lieu of future modification of these viral systems.

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Author Contributions

Conceptualization: Samantha R. Coy, Steven W. Wilhelm.

Data curation: Samantha R. Coy, Alyssa N. Alsante.

Formal analysis: Samantha R. Coy.

Funding acquisition: Steven W. Wilhelm.

Investigation: Samantha R. Coy, Alyssa N. Alsante.

Methodology: Samantha R. Coy, Alyssa N. Alsante, James L. Van Etten.

Project administration: Steven W. Wilhelm.

Resources: James L. Van Etten.

Supervision: Steven W. Wilhelm.

Validation: Samantha R. Coy.

Visualization: Samantha R. Coy.

Writing – original draft: Samantha R. Coy, Alyssa N. Alsante, James L. Van Etten, Steven W. Wilhelm.

Writing – review & editing: Samantha R. Coy, Alyssa N. Alsante, James L. Van Etten, Steven W. Wilhelm.

References

1. Bratbak G, Egge JK, Heldal M. Viral mortality of the marine alga *Emiliania huxleyi* (*Haptophyceae*) and termination of algal blooms. Mar Ecol Prog Ser. 1993; 93(1–2):39–48. https://doi.org/10.3354/meps093039

2. Rowe JM, Dunlap JR, Gobler CJ, Anderson OR, Gastrich MD, Wilhelm SW. Isolation of a non-phage-like lytic virus infecting *Aureococcus anophagefferens*. J Phycol. 2008; 44(1):71–6. https://doi.org/10.1111/j.1529-8817.2007.00453.x PMID: 27041042

3. Nagasaki K, Tomaru Y, Nakashiki K, Hata N, Katanozaka N, Yamaguchi M. Dynamics of *Heterocapsa circularisquama* (*Dinophyceae*) and its viruses in Ago Bay, Japan. Aquat Microb Ecol. 2004; 34(3):219–26. https://doi.org/10.3354/ame034219

4. Wilhelm SW, Suttle CA. Viruses and nutrient cycles in the sea—viruses play critical roles in the structure and function of aquatic food webs. BioScience. 1999; 49(10):781–8. https://doi.org/10.2307/1313569

5. Coy SR, Gann ER, Pound HL, Short SM, Wilhelm SW. Viruses of eukaryotic algae: diversity, methods for detection, and future directions. Viruses. 2018; 10(9). Epub 2018/09/14. https://doi.org/10.3390/v10090487 PMID: 30208617.

6. Short SM, Staniewski MA, Chaban YV, Long AM, Wang D. Diversity of viruses infecting eukaryotic algae. In: H P., Abedon ST, editors. Viruses of microorganisms. Poole, UK: Caister Academic Press; 2018. p. 211–44.

7. Van Etten JL, Burbank DE, Xia Y, Meints RH. Growth-cycle of a virus, PBCV-1, that infects *Chlorella*-like algae. Virology. 1983; 126(1):117–25. https://doi.org/10.1016/0042-6822(83)90466-x PMID: 18638936

8. Castberg T, Thyrhaug R, Larsen A, Sandaa RA, Heldal M, Van Etten JL, et al. Isolation and characterization of a virus that infects *Emiliania huxleyi* (*Haptophyta*). J Phycol. 2002; 38(4):767–74.

9. Peck KM, Lauring AS. Complexities of viral mutation rates. J Virol. 2018; 92(14):8. https://doi.org/10.1128/jvi.01051-17 PMID: 29720522
10. Waller RF, Cleves PA, Rubio-Brotos M, Woods A, Bender SJ, Edgcomb V, et al. Strength in numbers: collaborative science for new experimental model systems. PLoS Biol. 2018; 16(7):10. https://doi.org/10.1371/journal.pbio.2006333 PMID: 29965960

11. Mazur P. Freezing of living cells—mechanisms and implications. Am J Physiol. 1984; 247(3):C125–C42

12. Mazur P, Leibo SP, Chu EHY. A two-factor hypothesis of freezing injury—evidence from Chinese-hamster tissue-culture cells. Exp Cell Res. 1972; 71(2):345–55. https://doi.org/10.1016/0014-4827(72)90033-5 PMID: 5045639

13. Nagasaki K, Yamaguchi M. Cryopreservation of a virus (HaV) infecting a harmful bloom causing microalga, Heterosigma akashiwo (Raphidophyceae). Fish Sci. 1999; 65(2):319–20. https://doi.org/10.2331/fishsci.65.319

14. Kim J, Kim CH, Youn SH, Choi TJ. Isolation and physiological characterization of a novel algicidal virus infecting the marine diatom Skeletonema costatum. Plant Pathol J. 2015; 31(2):186–91. https://doi.org/10.5423/PPJ.NT.03.2015.0029 PMID: 26060438

15. Kim J, Yoon SH, Choi TJ. Isolation and physiological characterization of a novel virus infecting Stephanoptyxis palmeriana (Bacillariophyta). Algae. 2015; 30(2):81–7. https://doi.org/10.4490/algaes.2015.30.2.081

16. Kim J, Kim CH, Takano Y, Jang IK, Kim SW, Choi TJ. Isolation and physiological characterization of a new algicidal virus infecting the harmful dinoflagellate Heterocapsa pygmaea. Plant Pathol J. 2012; 28(4):433–8. https://doi.org/10.5423/ppj.nt.07.2012.0093

17. Jeanniard A, Dunigan DD, Gurnon JR, Agarkova IV, Kang M, Vitek J, et al. Towards defining the chloroviruses: a genomic journey through a genus of large DNA viruses. BMC Genomics. 2013; 14. https://doi.org/10.1186/1471-2164-14-158 PMID: 23497343

18. Colson P, De Lamballerie X, Yutin N, Asgari S, Bigot Y, Bideshi DK, et al. "Megavirales", a proposed new order for eukaryotic nucleocytoplasmic large DNA viruses. Arch Virol. 2013; 158(12):2517–21. https://doi.org/10.1007/s00705-013-1768-6 PMID: 23812617

19. Long AM, Short SM. Seasonal determinations of algal virus decay rates reveal overwintering in a temperate freshwater pond. ISME J. 2016; 10(7):1602–12. https://doi.org/10.1038/ismej.2015.240 PMID: 26943625

20. Bubeck JA, Pfiltzner AJP. Isolation and characterization of a new type of chlorovirus that infects an endosymbiotic Chlorella strain of the heliozoon Acanthocystis turfacea. J Gen Virol. 2005; 86:2871–7. https://doi.org/10.1099/vir.0.81068-0 PMID: 16186243

21. Dunigan DD, Cerny RL, Bauman AT, Roach JC, Lane LC, Agarkova IV, et al. Paramecium bursaria chlorovirus 1 proteome reveals novel architectural and regulatory features of a giant virus. J Virol. 2012; 86(16):8821–34. https://doi.org/10.1128/JVI.00907-12 PMID: 22696644

22. Yanai-Balser GM, Duncan GA, Eudy JD, Wang D, Li X, Agarkova IV, et al. Microarray analysis of paramecium bursaria chlorovirus 1 transcription. J Virol. 2010; 84(1):532–42. https://doi.org/10.1128/JVI.01698-09 PMID: 19828609

23. Blanc G, Mozar M, Agarkova IV, Gurnon JR, Yanai-Balser G, Rowe JM, et al. Deep RNA sequencing reveals hidden features and dynamics of early gene transcription in paramecium bursaria chlorovirus 1. PloS One. 2014; 9(3):10. https://doi.org/10.1371/journal.pone.0090989 PMID: 24608750

24. Hubalek Z. Protectors used in the cryopreservation of microorganisms. Cryobiology. 2003; 46(3):205–29. https://doi.org/10.1016/s0011-2240(03)00046-4 PMID: 12818211

25. Benson EE. Cryopreservation of phytodiversity: A critical appraisal of theory and practice. Crit Rev Plant Sci. 2008; 27(3):141–219. https://doi.org/10.1080/07352680802202034

26. Day JG, Watanabe MM, Morris GJ, Fleck RA, McLellan MR. Long-term viability of preserved eukaryotic algae. J Appl Phycol. 1997; 9(2):121–7. https://doi.org/10.1023/a:1007991507314

27. Taylor R, Fletcher RL. Cryopreservation of eukaryotic algae—a review of methodologies. J Appl Phycol. 1998; 10(5):481–501. https://doi.org/10.1023/a:1008094622412

28. Rhodes L, Smith J, Tervit R, Roberts R, Adamson J, Adams S, et al. Cryopreservation of economically valuable marine micro-algae in the classes Bacillariophyceae, Chlorophyceae, Cyanophyceae, Dinophyceae, Haptophyceae, Prasinophyceae, and Rhodophyceae. Cryobiology. 2006; 52(1):152–6. https://doi.org/10.1016/j.cryobiol.2005.10.003 PMID: 16321370

29. Nakashishi K, Deuchi K, Kuwano K. Cryopreservation of four valuable strains of microalgae, including viability and characteristics during 15 years of cryostorage. J Appl Phycol. 2012; 24(6):1381–5. https://doi.org/10.1007/s10811-012-9790-8

30. Dunigan DD, Agarkova I. Formulation of MBBM (modified Bold's Basal medium). protocolsio. 2016: https://doi.org/10.17504/protocols.io.etwbepe
31. Van Etten JL, Burbank DE, KuczmarSKI D, Meints RH. Virus-infection of culturable Chlorella-like algae and development of a plaque assay. Science. 1983; 219(4587):994–6. https://doi.org/10.1126/science.219.4587.994 PMID: 17187937

32. Dunigan DD, Agarkova I. PBCV-1 virus plaque assay. protocolio. 2016: https://doi.org/10.17504/protocolio.estbeen

33. Coy SR, Alsante A, Wilhelm SW. Long term cryopreservation of chloroviruses by infection of Chlorella. protocolio. 2018: https://doi.org/10.17504/protocolio.wa2fage

34. Dunigan DD, Fitzgerald LA, Van Etten JL. Phycodnaviruses: a peek at genetic diversity. Virus Res. 2006; 117(1):119–32. https://doi.org/10.1016/j.viruses.2006.01.024 PMID: 16516998

35. Convention. USP. U.S. Pharmacopeia National Formulary 2018: UPS41-NF36: Nielsen bookata; 2018.

36. Agarkova I, Hertel B, Zhang XZ, Lane L, Tchourbanov A, Dunigan DD, et al. Dynamic attachment of Chlorovirus PBCV-1 to Chlorella variabilis. Virology. 2014; 466:95–102. https://doi.org/10.1016/j.virology.2014.07.002 PMID: 25240455

37. Sanjuan R, Nebot MR, Chirico N, Mansky LM, Belshaw R. Viral nutation rates. J Virol. 2010; 84(19):9733–48. https://doi.org/10.1128/JVI.00694-10 PMID: 20660197

38. Filee J, Pouget N, Chandler M. Phylogenetic evidence for extensive lateral acquisition of cellular genes by nucleocytoplasmic large DNA viruses. BMC Evol Biol. 2006; 6(320). https://doi.org/10.1186/1471-2148-8-645-1 PMID: 17109990

39. Cordova A, Desemo M, Gelbart WM, Ben-Shaul A. Osmotic shock and the strength of viral capsids. Biophys J. 2003; 85(1):70–4. https://doi.org/10.1016/S0006-3495(03)74455-5 PMID: 12829465

40. Wulfmeyer T, Polzer C, Hiepler G, Hamacher K, S Playstation R, Dunigan DD, et al. Structural organization of DNA in Chlorella viruses. PLoS One. 2012; 7(2). https://doi.org/10.1371/journal.pone.0030133 PMID: 22359540

41. Roos WH, Ivanovska IL, Evilevitch A, Hamacher K, Shoeman R, Dunigan DD, et al. Structural organization of DNA in Chlorella viruses. PLoS One. 2012; 7(2). https://doi.org/10.1371/journal.pone.0030133 PMID: 22359540

42. Claverie JM, Abergel C. Virus Taxonomy Ninth Report of the International Committee on Taxonomy of Viruses: Elsevier Inc.; 2012. p. 223–8.

43. Wallis C, Melnick JL. Stabilization of enveloped viruses by dimethyl sulfoxide. J Virol. 1968; 2(9):953–4 PMID: 4302192

44. Noble RT, Fuhrman JA. Use of SYBR Green I for rapid epifluorescence counts of marine viruses and bacteria. Aquat Microb Ecol. 1998; 14(2):113–8. https://doi.org/10.3354/ame14113

45. Brussaard CPD, Marie D, Bratbak G. Flow cytometric detection of viruses. J Virol Methods. 2000; 85(1–2):175–82. https://doi.org/10.1016/S0166-0934(99)00167-6 PMID: 10716350

46. Brussaard CPD. Optimization of procedures for counting viruses by flow cytometry. Appl Environ Microbiol. 2004; 70(3):1506–13. https://doi.org/10.1128/AEM.70.3.1506-1513.2004 PMID: 15006772

47. Agarkova I, Dunigan D, Gurnon J, Greiner T, Barres J, Thiell G, et al. Chlorovirus-mediated membrane depolarization of chlorella alters secondary active transport of solutes. J Virol. 2008; 82(24):12181–90. https://doi.org/10.1128/JVI.01687-08 PMID: 18842725

48. Amaral R, Pereira JC, Pais A, Santos LMA. Is axenicity crucial to cryopreserve microalgae? Cryobiology. 2013; 67(3):312–20. https://doi.org/10.1016/j.cryobiol.2013.09.006 PMID: 24055827

49. Wilhelm SW, Coy SR, Gann ER, Moniruzzaman M, Stough JMA. Standing on the shoulders of giant viruses: five lessons learned about large viruses infecting small eukaryotes and the opportunities they create. PLoS Pathog. 2016; 12(8). https://doi.org/10.1371/journal.ppat.1005752 PMID: 27559742

50. Kerperes C, Grolmusz V. The “giant virus finder” discovers an abundance of giant viruses in the Antarctic dry valleys. Arch Virol. 2017; 162(6):1671–6. https://doi.org/10.1007/s00705-017-3286-4 PMID: 28247094

51. Legendre M, Bartoli J, Shmakova L, Jeudy S, Labadie K, Adrait A, et al. Thirty-thousand-year-old distant relative of giant icosahedral DNA viruses with a pandoravirus morphology. Proc Natl Acad Sci USA. 2014; 111(11):4247–9. https://doi.org/10.1073/pnas.1320670111 PMID: 24591590

52. Legendre M, Lartigue A, Bertaux L, Jeudy S, Bartoli J, Lescoat M, et al. In-depth study of Mollivirus sibericum, a new 30,000-y-old giant virus infecting Acanthamoeba. Proc Natl Acad Sci USA. 2015; 112(38):E5327–E35. https://doi.org/10.1073/pnas.1510795112 PMID: 26351664
54. Wilhelm SW, Bird JT, Bonifer KS, Calfee BC, Chen T, Coy SR, et al. A student's guide to giant viruses infecting small eukaryotes: from *Acanthamoeba* to *Zooxanthellae*. Viruses. 2017; 9(3). https://doi.org/10.3390/v9030046 PMID: 28304329

55. Wilson WH, Gilg IC, Moniruzzaman M, Field EK, Koren S, LeCleir GR, et al. Genomic exploration of individual giant ocean viruses. ISME J. 2017; 11(8):1736–45. https://doi.org/10.1038/ismej.2017.61 PMID: 28498373