Three-year survey of abundance, prevalence and genetic diversity of chlorovirus populations in a small urban lake

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Abstract

Inland water environments cover about 2.5 percent of our planet and harbor huge numbers of known and still unknown microorganisms. In this report, we examined water samples for the abundance, prevalence, and genetic diversity of a group of infectious viruses (chloroviruses) that infect symbiotic chlorella-like green algae. Samples were collected on a weekly basis for a period of 24 to 36 months from a recreational freshwater lake in Lincoln, Nebraska, and assayed for infectious viruses by plaque assay. The numbers of infectious virus particles were both host- and site-dependent. The consistent fluctuations in numbers of viruses suggest their impact as key factors in shaping microbial community structures in the water surface. Even in low-viral-abundance months, infectious chlorovirus populations were maintained, suggesting either that the viruses are very stable or that there is ongoing viral production in natural hosts.

Introduction

Viruses are ubiquitous members of the biosphere that are found in essentially every ecosystem on Earth [e.g., 1, 2]. For example, microscopic and metagenomic analyses of aquatic environmental samples indicate that high concentrations of viruses (10^5 to 10^6 particles/ml) that infect microorganisms, primarily bacteria, are present [e.g., 1, 3–5]. The virus number typically exceeds that of cellular organisms by at least an order of magnitude; thus, the number of different viruses within a community is huge. Their functions of predation and gene transfer make viruses key drivers in the dynamics of microbial ecosystems [2, 6]. Furthermore, viruses play important roles in the global biogeochemical cycling of carbon and nutrients [5, 7–9]. Although most studies have been conducted on marine environments, large numbers of viruses infecting microorganisms also exist in freshwater environments [e.g., 10–12]. In addition to bacterial viruses, viruses that infect eukaryotic algae are also common in both terrestrial and marine waters throughout the world, including members of the family Phycodnaviridae [13]. Phycodnaviruses are a genetically diverse, yet morphologically similar, group of large dsDNA-containing viruses (160 to 560 kb) that infect eukaryotic algae [14, 15]. Currently phycodnaviruses are classified into six genera. Members of one genus, Chlorovirus, are common in freshwater, occasionally reaching titers as high as thousands of plaque-forming units (PFU) per ml [e.g., 16–21]. Chloroviruses infect certain unicellular, eukaryotic, symbiotic chlorella-like green algae, often referred to as zoochlorellae. However, when the zoochlorellae are in the symbiotic phase, they are resistant to virus infection. Known zoochlorellae that serve as virus hosts are associated with either the protozoan Paramecium bursaria, the coelenterate Hydra viridis or the helizoon Acanthocystis turfeacea [22]. Four such zoochlorellae
isolates can be cultured axenically and are susceptible to lytic virus infections, allowing for plaque assays. These zoochlorellae (recently renamed [23, 24]) are *Chlorella variabilis* NC64A [25], *Chlorella variabilis* Syngen 2-3 [25; Quispe et al., manuscript in preparation], *Chlorella heliozoae* SAG 3.83 [26] and *Micractinium conductrix* Pbi [27]. Viruses infecting these four zoochlorellae are referred to as NC64A, Syngen, SAG, and Pbi viruses, respectively. The Syngen viruses were discovered during the course of the experiments reported in this manuscript (Quispe et al., manuscript in preparation).

The chloroviruses have been isolated from many geographic locations worldwide, including North and South America, Europe, Asia and Australia [e.g., 16–21, 28]. Although several recent metagenomic studies have been conducted on viruses in fresh water that suggest that chloroviruses have a seasonality [e.g., 29–32], a systematic long-term analysis of infectious chlorovirus populations in inland waters has not been conducted. As noted above, chloroviruses infect zoochlorella isolates grown in the laboratory, but there is no evidence that these symbiotic algae grow independently of their hosts in indigenous waters. Thus, the chloroviruses probably play dynamic, albeit largely undocumented, roles in regulating microbial communities in the ecosystem. Intriguingly, it was recently reported that DNA sequences similar to those found in the chlorovirus ATCV-1 were present in human throat swabs and that their presence was associated with a modest, but statistically significant, decrease in certain cognitive behaviors in humans. Furthermore, mice fed ATCV-1-infected algae also exhibited statistically significant decreases in performance in certain cognitive tests [33].

The current report describes the first systematic, culture-dependent, 3-year study to monitor the dynamics and diversity of infectious chlorovirus populations weekly in a freshwater environment. The chloroviruses were ubiquitous throughout the year, and they were both host- and site-dependent as well as seasonal. Furthermore, chlorovirus populations present in the same sample exhibited heterogeneity in plaque size and shape, indicating that they are dynamic and genetically diverse in nature. Finally, attempts to grow the zoochlorellae hosts for the viruses in indigenous sterilized water were unsuccessful unless the water was supplemented with organic nitrogen sources. Therefore, the mechanism(s) in nature that allows long-term chlorovirus persistence and distribution in indigenous freshwater is still unknown.

**Materials and methods**

**Collection site**

Holmes Lake is a 0.4-square-kilometer inland lake located in an urban, recreational 13.5-square-kilometer watershed in Lancaster County, Nebraska, USA. The lake was created primarily for flood control and it is managed by the City of Lincoln. The lake is fed by two drainages that consist of approximately 32 kilometers of open channels. Most of the stream network includes urban residential, rural residential and commercial property, so sediments and nutrients from the watershed are constantly flowing into the lake. During the winter months, the lake and surrounding areas are used for ice-skating, fishing, hockey and sledding. In the warmer months, recreational activities include boating, picnicking, swimming and fishing.

**Sampling**

Water samples were collected once per week from two sites in the lake (Fig. 1). Weekly samples were analyzed from May 2011 to May 2014 for site 1 and from June 2012 to May 2014 for site 2. Most samples (~150 ml each) were collected during the daytime (6-7 am) on Tuesdays. Samples were taken within one meter of the shore near the surface of the lake, using autoclaved 250-ml bottles. After collection, the samples were transported immediately to the laboratory and filtered through a sterile cellulose acetate 0.45-μm-pore-size filter (FJ25ASC004FL01, GVS, Fisher Scientific) prior to assay (Fig. 2). Occasionally, we were unable to collect samples due to extreme weather conditions in the winter.

![Fig. 1 Water samples were collected weekly from two sites in Holmes Lake, located in Lincoln, Nebraska, USA. Site 1 is a sandy bank that lacks natural vegetation and has more apparent anthropological disturbance. Site 2 is characterized by more stagnant water and more natural vegetation. Numbers around the lake indicate the plaque-forming units/ml (PFU/ml) of NC64A and SAG viruses in samples collected around the lake on one day in July 2011. Note that there were over 2 log differences between some of the sites.](image-url)
Cell cultures and plaque assays

C. variabilis NC64A, C. variabilis Syngen 2-3 and C. heliozoae SAG 3.83 strains were grown on Bold’s basal medium (BBM) containing 5 % (W/V) sucrose and 1 % (W/V) peptone (Modified Bold’s basal medium, MBBM) [34]. M. conductrix Pbi was grown on FES medium [27]. All strains were grown to early log phase (4-7 × 10^6 cells/ml) and concentrated tenfold (4-7 × 10^7 cells/ml) by centrifugation for the plaque assays. Cell cultures were kept under constant shaking (200 rpm) and light at 26 °C.

Each water sample was analyzed by plaque assay with the four zoochlorellae strains. Plaque assays were performed as described previously [25] with minor modifications. Three ml of MBBM or FES top agar (7 g/L agar) was mixed with 300 µl of actively growing cells (4-7 × 10^7 cells/ml) and the water sample. Freshly filtered water samples of 100 to 1500 µl were plated to produce significant counts (25-120 plaques/plate) when possible. The samples were poured over solidified agar (15 g/L) containing the appropriate growth medium. Plates were incubated for one week in constant light and temperature, and weekly plaque averages were determined from four plates per sample/strain. A few high-titer water samples were diluted up to tenfold in 50 mM Tris buffer, pH 7.5, to produce plaque numbers within the desired range.

Cell cultures on native water

A NalgeneTM Rapid-FlowTM sterile disposable 500-ml bottle top filter with polyethersulfone membrane (cat# 295-4545) was used to filter 400 ml of indigenous water, followed by an autoclave cycle at 15 psi for 20 min. Then, 30 ml of water with or without the addition of organic nitrogen sources was inoculated with 1-5 × 10^6 cells/ml of actively growing algae cells. One molar sodium nitrate (Sigma), 1 M urea (Sigma) or 0.2 M asparagine (Sigma) stock solutions were prepared and added as the sole nitrogen source to a final concentration of 10 mM to either water samples or BBM (control). Before inoculation, cells were washed three times with BBM. Cell cultures were kept under constant shaking (200 rpm) and light at 26 °C. Visual evaluations and the photographs in Fig. 6 were recorded after 12-15 days.

Results and discussion

Chloroviruses are ubiquitous throughout the year

To understand how spatial-temporal and ecological processes might interact to shape chlorovirus richness in nature, the seasonal variation and genetic diversity of chloroviruses were determined by analyzing indigenous water samples from Holmes Lake using a culture-dependent plaque assay method that specifically detects the four chlorovirus types referred to as NC64A, SAG, Syngen and Pbi viruses.

Three out of the four chlorovirus types (NC64A, SAG, and Syngen viruses) were present throughout the year in Holmes Lake. Syngen viruses were the most abundant viruses in the urban lake; in contrast, no Pbi viruses were detected during the surveillance period. These results contrast with previous studies on the distribution of chloroviruses in inland waters in England, in which infectious SAG and Pbi viruses were present, but NC64A viruses were absent [35],
suggesting a non-uniform worldwide distribution of chloroviruses in inland waters. Other studies that focused only on NC64A viruses indicated that they were common in various locations within the United States [16, 17], China [18], Japan [20], Korea [21] and Australia (Van Etten, unpublished results) but absent in northern Europe (Van Etten, unpublished results). Likewise, Pbi viruses were common in various locations in Germany [27], Russia [36] and Canada (Van Etten, unpublished results). Taken together, these results indicate that chloroviruses are widely dispersed, and local environmental conditions enrich for certain viral types, which depends on the environmental distribution of their natural host(s).

Seasonal spatio-temporal pattern of chlorovirus populations

Typical chlorovirus titers in freshwater range from 1 to 100 PFU/ml [16, 17]. However, titers as high as 100,000 and 40,000 PFU/ml of NC64A viruses were detected in single samples from Montana (Nelson, unpublished results) and South Carolina [17], respectively. These observations suggest that titers in the thousands of PFU/ml do occur and that the abundance, ubiquity and potentially high diversity of these viruses might play important roles in freshwater environments. To determine if virus titers were similar in Holmes Lake, samples were collected on a single day (July, 2011) from different locations around the lake. The number of SAG plaque-forming viruses varied from 1 to 335 PFU/ml, and NC64A plaque-forming viruses, from <1 to 168 PFU/ml (Fig. 1).

For the seasonal survey, two sites at Holmes Lake were selected that represent contrasting ecological and chlorovirus abundance patterns; site 1 was a sandy bank that lacked natural vegetation and had more apparent anthropological disturbance (Fig. 1). It consistently had lower chlorovirus titers (combined 3-year average of 26 PFU/ml). Site 2 was characterized by more stagnant water and more natural vegetation (Fig. 1). It had relatively high chlorovirus titers throughout the year (combined 2-year average of 161 PFU/ml). The highest titers for NC64A, SAG and Syngen viruses were 58, 165 and 142 PFU/ml, respectively, in 2013 for site 1. In contrast, in 2013 the highest titers for NC64A, SAG and Syngen viruses were 584, 1,313 and 980 PFU/ml, respectively, for site 2 (Supplementary Fig. S1). As a comparison, we occasionally sampled another small pond near Lincoln, and the highest titers obtained were 3,882, 6,795, and 5,039 PFU for NC64A, SAG and Syngen viruses, respectively, in April 2012. Therefore, the virus concentrations can vary considerably within a small geographical region.

Previous investigations of inland waters suggested that the highest titer for NC64A viruses occurred during the late spring [16, 19]. Similarly, metagenomic studies of freshwater in Lake Ontario, Canada, suggested that chloroviruses varied seasonally during the year and were highest in the summer [29]. In the current 3-year study, chlorovirus populations showed two distinct peaks each year; site 1 had a peak between the second week of April to the first week of July and the second week of October to the second week of December for all three virus types. The NC64A and Syngen viruses had similar seasonal patterns that co-varied throughout the year (Fig. 3), suggesting that they might share the same or a very similar host at the sampling sites. In contrast, SAG viruses also had two peaks, but at slightly different seasonal phases, and they were more variable from year to year. This result indicates that SAG viruses probably replicated in a different host(s) than the NC64A and Syngen viruses, which agrees with laboratory results. Samples collected at site 2 had higher plaque counts throughout the year (Fig. 3). Although these samples exhibited less pronounced seasonal peaks, sporadic peaks of SAG viruses occurred between June and October. For all virus types, the seasonal patterns at site 2 were more variable from year to year than those at site 1 (Supplementary Fig. S1).

These results indicate that the abundance of infectious chloroviruses can vary substantially within the same body of water. As shown in our two representative sites, the site with low virus titers (combined 3-year average of 26 PFU/ml) consistently had seasonal patterns with two viral peaks per year and more dynamic seasonal variation, whereas the site with higher titers (combined 2-year average of 161 PFU/ml) exhibited less pronounced seasonal features but more stable virus populations over time, likely because of constant local enrichment of microorganisms that sustain virus replication. Taken together, there is a seasonal spatio-temporal pattern that is host- and site-dependent, with chloroviruses emerging during the spring, disappearing in the summer, and returning at the end of the fall and beginning of the winter. These patterns might be controlled by environmental factors such as water temperature, pH, salinity, etc., which vary considerably. Although these environmental factors were not examined at the time that water was collected for the virus studies, these factors were occasionally monitored by the Nebraska Department of Environmental Quality, and Table 1 summarizes some of their results for 2010. Clearly, the water temperature could contribute to the chlorovirus variations (Table 1).

Genetic diversity of the chlorovirus community

The morphology of the chlorovirus plaques can vary in size [16] (Fig. 4). For example, significant differences were observed when two NC64A viruses, NY-2A and the prototype chlorovirus PBCV-1, were compared at
the physiological, genomic and DNA methylation levels. NY-2A has the largest genome (370 kb) of all of the characterized chloroviruses [37, 38], and its genome is heavily methylated relative to the 330-kb genome of PBCV-1 [39]. In addition, NY-2A has a burst size that is two- to threefold lower than that of PBCV-1, as well as a longer replication cycle (6-8 hrs for PBCV-1 and ~18 hrs for NY-2A [40]). Consequently, NY-2A produces small plaques (1 mm size), whereas PBCV-1 produces medium-size plaques (3 mm size). Thus, we used plaque size to evaluate the genetic diversity of the chloroviruses in our survey. Natural samples of the chloroviruses formed plaques of several sizes on the same plate (Fig. 4); large plaques that formed under these experimental conditions were defined as having a diameter greater than 4 mm, whereas small plaques were those with a diameter smaller than 1 mm. The plaque sizes from water samples collected throughout the year on the NC64A and Syngen lawns varied, but medium size plaques (1-4 mm) were the predominant phenotype (Fig. 5). Large and small plaques were sporadic and did not exhibit an obvious seasonal pattern at either collection site. All plaques were sharply defined and clear. SAG viruses had predominantly medium and small plaque sizes (Fig. 5). Some of the SAG plaques were irregular in shape and not necessarily defined, suggesting a more versatile genetic background in these viruses. Thus, SAG viruses exhibited the highest heterogeneity in plaque size and shape compared to NC64A and Syngen viruses. Together, these results indicate that chlorovirus populations are dynamic and genetically diverse in nature.

**Table 1.** Summary of water chemistry parameters collected by the Nebraska Department of Environmental Quality at Holmes Lake in Lincoln, Nebraska. Monthly collections, taken near the dam, were evaluated during May 2010 to September 2010

<table>
<thead>
<tr>
<th>Date collected</th>
<th>Temp (°C)</th>
<th>Chlorophyll a (V)</th>
<th>ORP (mV)</th>
<th>DO (% sat)</th>
<th>pH</th>
<th>C (μmhos/cm)</th>
<th>T (NTU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>May 2010</td>
<td>14</td>
<td>0.028</td>
<td>339</td>
<td>91</td>
<td>8.6</td>
<td>566</td>
<td>1</td>
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<tr>
<td>July 2010</td>
<td>29</td>
<td>0.073</td>
<td>209</td>
<td>194</td>
<td>9.4</td>
<td>273</td>
<td>24</td>
</tr>
<tr>
<td>Aug. 2010</td>
<td>29</td>
<td>0.023</td>
<td>275</td>
<td>106</td>
<td>9.2</td>
<td>294</td>
<td>16</td>
</tr>
<tr>
<td>Sept. 2010</td>
<td>21</td>
<td>0.034</td>
<td>417</td>
<td>74</td>
<td>7.7</td>
<td>327</td>
<td>12</td>
</tr>
</tbody>
</table>

*ORD* oxygen reduction potential, *DO* dissolved oxygen, *C* specific conductance, *T* turbidity
Algal hosts of chloroviruses in native freshwater

Chloroviruses replicate in four known zoochlorella strains isolated from symbiotic interactions with protists and can be cultured axenically in the laboratory. To determine if the NC64A, Syngen and SAG zoochlorella strains possibly grow free of their symbiotic host in indigenous water from Holmes Lake, we filtered and autoclaved water collected during September, November and December 2013. None of the zoochlorella strains grew in the water alone (Fig. 6). However, all three zoochlorella isolates grew when an exogenous organic nitrogen source (urea or asparagine) was added to the sterile indigenous water samples (Fig. 6). The growth rates were visually evaluated and observed to vary among the strains after addition of the two nitrogen sources when compared to BBM plus nitrogen controls (Fig. 6). Addition of nitrate alone to the indigenous water samples did not support growth of any zoochlorella strains (results not shown). As expected, none of the three zoochlorella isolates grew in non-sterilized water, as they were probably immediately infected by residential chloroviruses (results not shown).

The inability of the three virus host zoochlorellae to grow in sterilized indigenous water is interesting because these results lead to the question: what is supporting the replication of the three groups of chloroviruses? Although very little is known about the natural history of the chloroviruses, several factors need to be considered in examining this issue. i) What is the population of green endosymbiotic protists containing zoochlorellae in nature, and do they continually shed zoochlorellae, or when they die, do they release zoochlorellae that can be infected by indigenous chloroviruses? Currently, we do not have an answer to either question. However, pertinent to these questions is the recent report that chloroviruses...
Abundance, prevalence and genetic diversity of chlorovirus populations

Tend to accumulate and attach to Paramecium bursaria cells (referred to as green paramecium) without actually infecting them [41]. Additionally, Hydra species also maintain a diverse community of eukaryotic viruses, including chloroviruses, as part of their holobiont [42]. Thus, in nature, viruses would be near the zoochlorellae if green protists release their symbionts, either by death or for some other reason. Furthermore, if there is a temporary increase in an organic nitrogen source, the liberated zoochlorellae might grow, at least for a short time. Although a systematic count of green endosymbiotic protists was not conducted in the current study, sporadic microscopic observations indicated that they were rare in the water samples. ii) In general, infectious bacterial viruses do not survive very long in natural environments because exposure to sunlight leads to UV-induced genetic mutations [42]. Equivalent stability studies have not been conducted on chloroviruses in a natural environment. It should be noted, however, that most chloroviruses encode a functional DNA repair enzyme, a pyrimidine dimer-specific glycosylase, which could aid in their survival [37, 43]. Although the DNA repair protein is not packaged in the virion, the gene is expressed early during virus infection in the laboratory [44, 45]. iii) The host/virus concentrations necessary to support bacterial virus replication in an aqueous environment have been the subject of several studies (reviewed in reference [12]). Most of these studies indicate that at least $10^5$ to $10^4$ host cells per ml are necessary to maintain a constant virus population in nature. Although similar information is lacking for the chlorovirus/zoochlorella systems, the number of host cells required would be expected to be less because the chlorella cells are substantially larger that bacterial cells (see discussion of this issue in reference [12]). However, one can make some rough calculations based on the following assumptions taken from laboratory studies with PBCV-1: a) each green paramecium harbors ~200 or more zoochlorellae [46], b) the average burst size for the chloroviruses is ~800 particles per zoochlorella [17], and c) about 25% of the released virus particles are infectious [17]. Therefore, it would require five green paramecia per ml to release 1000 zoochlorellae, which is the minimum number of cells to support bacteria phage growth. If

<table>
<thead>
<tr>
<th>Month</th>
<th>Species</th>
<th>Environment</th>
<th>Urea 10 mM</th>
<th>Asparagine 10 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sept. 2013</td>
<td>Chlorella variabilis (NC64A)</td>
<td>Indigenous water</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
</tr>
<tr>
<td></td>
<td>Chlorella heliozoae (SAG 3.83)</td>
<td>Indigenous water</td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
</tr>
<tr>
<td></td>
<td>Chlorella variabilis (Syngen 2-3)</td>
<td>Indigenous water</td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
</tr>
<tr>
<td>Nov. 2013</td>
<td>Chlorella variabilis (NC64A)</td>
<td>BBM</td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
</tr>
<tr>
<td></td>
<td>Chlorella heliozoae (SAG 3.83)</td>
<td>BBM</td>
<td><img src="image9" alt="Image" /></td>
<td><img src="image10" alt="Image" /></td>
</tr>
<tr>
<td></td>
<td>Chlorella variabilis (Syngen 2-3)</td>
<td>BBM</td>
<td><img src="image11" alt="Image" /></td>
<td><img src="image12" alt="Image" /></td>
</tr>
<tr>
<td>Dec. 2013</td>
<td>Chlorella variabilis (NC64A)</td>
<td>BBM</td>
<td><img src="image13" alt="Image" /></td>
<td><img src="image14" alt="Image" /></td>
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<tr>
<td></td>
<td>Chlorella heliozoae (SAG 3.83)</td>
<td>BBM</td>
<td><img src="image15" alt="Image" /></td>
<td><img src="image16" alt="Image" /></td>
</tr>
<tr>
<td></td>
<td>Chlorella variabilis (Syngen 2-3)</td>
<td>BBM</td>
<td><img src="image17" alt="Image" /></td>
<td><img src="image18" alt="Image" /></td>
</tr>
</tbody>
</table>

Fig. 6 In vitro flask tests of algae growth in sterilized indigenous water. Strains were grown on autoclaved indigenous water alone and/or Bold’s basal medium (BBM) supplemented with 10 mM of urea or asparagine. Pictures were taken 12-15 days post-incubation. The image of the bottom of the flask was cropped using Photoshop CS5.1.
all 200 zoochlorellae from a single green paramecium were infected with a chlorovirus, one would obtain ~160,000 virus particles per paramecium, of which ~40,000 would be infectious. However, we would expect these numbers to be much lower in natural conditions because it is very unlikely that all of the released zoochlorellae would be infected by viruses, and the average burst size would probably be less than 200. Furthermore, the specific infectivity of viral particles in nature would probably be much lower than 25%. These are some of the factors that need to be considered to explain how the chloroviruses are maintained in nature. Finally, we cannot discard the possibility that chloroviruses have another natural host, especially when thousands of infectious particles are present in a milliliter of indigenous water. Over the years we have made many attempts to infect natural free-living Chlorella or related organisms with chloroviruses; all of these attempts have been unsuccessful because the viruses do not attach to the algae tested [47, Van Etten, unpublished results]. However, if another host exists, it might not be a green alga.

Conclusions

A 3-year study of an urban lake in Lincoln, Nebraska, indicated that infectious chloroviruses infecting three zoochlorella hosts were present throughout the year. In this study, the highest titer for one of the chloroviruses reached ~1300 PFU/ml. Typically, the values were in the range of 1 to 100 PFU/ml, but they were host- and site-dependent. The viruses exhibited variations in plaque size and morphology, indicating that even viruses that infect the same host are genetically diverse in natural waters. In laboratory settings, chloroviruses infect a few zoochlorella strains; however, there is no evidence that these zoochlorellae grow free of their hosts in indigenous waters. This observation raises the question: what is supporting chlorovirus replication in native environments? Therefore, the ecological processes that enable long-term chlorovirus persistence and distribution in inland freshwaters remain to be discovered.

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

References


Supplementary Fig. S1. Plot representing the seasonal dynamics of chlorovirus populations over a 3-year period at site 1 and over a 2-year period at site 2 in Holmes Lake. Symbols represent the average values of plaque-forming units/ml (PFU/ml) for every week over each year. The x-axis indicates months, and the y-axis indicates PFU/ml of indigenous water. Each panel represents relative abundance for NC64A (A, B), Syngen (C, D) and SAG viruses (E, F) from each corresponding week and location.