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Article

Viral Transfer and Inactivation through Zooplankton Trophic Interactions

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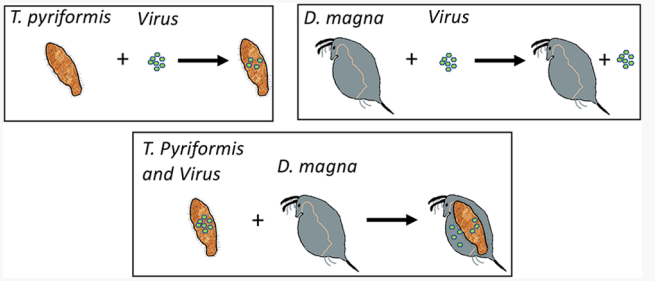
ABSTRACT: Waterborne viruses are responsible for numerous diseases and are abundant in aquatic systems. Understanding the fate of viruses in natural systems has important implications for human health. This research quantifies the uptake of the bacteriophage T4 and the enteric virus echovirus 11 when exposed to the filter feeders *Tetrahymena pyriformis* and *Daphnia magna*, and also examines the potential of viral transfer due to trophic interactions. Experiments co-incubating each species with the viruses over 72−96 h showed up to a 4 log virus removal for *T. pyriformis*, while direct viral uptake by *D. magna* was not observed. However, viral uptake by *D. magna* occurred indirectly by viral transfer from prey to predator, through *D. magna* feeding on virus-loaded *T. pyriformis*. This prey−predator interaction resulted in a 1 log additional virus removal compared to removal by *T. pyriformis* alone. Incomplete viral inactivation by *D. magna* was observed through recovery of infective viruses from the daphnid tissue. This research furthers our understanding of the impacts of zooplankton filter feeding on viral inactivation and shows the potential for viral transfer through the food chain. The viral−zooplankton interactions observed in these studies indicate that zooplankton may improve water quality through viral uptake or may serve as vectors for infection by accumulating viruses.



INTRODUCTION

Impaired freshwater due to microbial pollution is a global water quality concern. Human enteric viruses can be transmitted through contact of contaminated water resulting in negative human health effects, such as respiratory tract infections and gastroenteritis.[1] Human enteric viruses can be discharged into the environment through numerous routes, including raw and treated wastewater and aerosolization, and have been found in fresh surface water and groundwater.[2−4] To date, efforts to understand the fate of viruses in the environment have focused on abiotic mechanisms, such as adsorption to particles, thermal inactivation, or photoinactivation.[1,5−8] Less is known about the contribution of biotic mechanisms, such as predation by filter feeding organisms.

Filter feeding zooplankton, such as ciliated protozoa, rotifers, and cladocerans, feed by removing particulate matter from water and can dominate natural environments, as well as engineered treatment systems. For example, in wastewater treatment activated sludge, ciliates can occur at a density of 50 million cells per liter and over 175 species have been reported.[9−11] In shallow eutrophic and oligomesotrophic water, ciliates have been documented upwards of 10^3 cells/mL and can contribute more than half of the total zooplankton biomass.[12−14] While ciliates are important members of the microbial loop as grazers of bacteria, algae, and nanoflagellates, they also serve as a food source for various metazooplankton.[15,16] Metazooplankton, including cladocerans, have been shown to regulate the ciliate community,[15] and predatory actions by zooplankton can cascade down to bacterial levels.[17] Studies have not shown how trophic interactions can cascade down to virus levels.

In the study presented here, we focus on the microzooplankton ciliated protozoa *Tetrahymena pyriformis* ranging from 50 to 100 μm in size and the metazooplankton cladoceran *Daphnia magna* ranging from 2 to 5 mm in size. Both these organisms play critical roles in natural systems and engineered treatment systems. *T. pyriformis* has been shown to contribute to viral uptake in numerous laboratory studies[9,18−21] and is even considered to have a direct impact on the reduction of viral infectivity in wastewater treatment.[22] *D. magna* is a keystone zooplankton grazer in its role as both a predator of bacteria, algae, and microzooplankton and a major prey item for fish[23,24] and some birds.[25,26]

The aim of this research was to examine the potential for viral transfer and inactivation via virus−*T. pyriformis*−*D. magna*

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food chain interactions. We hypothesized that predator−prey interactions facilitate virus uptake by higher trophic organisms and lead to the transfer of infective viruses through the food chain. We tested this hypothesis using the bacteriophage T4 and enterovirus echovirus 11 (E11) as model viruses. Bacteriophages, such as T4, are ubiquitous in aquatic environments and play a critical role in microbial ecology.[19,27−29] E11 is a pathogenic enteric virus infecting the gastrointestinal tract. It is a viral contaminant frequently detected in aquatic systems and is responsible for water-transmitted disease outbreaks.[28] We first quantified the potential of *T. pyriformis* and *D. magna* to remove T4 and E11 through co-incubation studies. Subsequently, we examined viral transfer between these organisms. The results from this study provide data to help understand trophic transfer of viruses in the food chain. The study findings have important implications for disinfection effectiveness during biological wastewater treatment processes, as well as inactivation models predicting viral decay in natural systems.

MATERIALS AND METHODS

***Daphnia magna* Culture and Preparation for Laboratory Experiments.** *D. magna* clones (kindly provided by Dr Piet Spaak, EAWAG) were maintained in moderately hard synthetic freshwater (MHSFW)[30] using a 16 h photoperiod and were fed *Nannochloropsis* sp. algae *ad libitum* (4−6 μm diameter, Florida Aquafarms, Dade City, FL). Prior to experimental use, daphnids were removed from culture tanks by transferring with a pipette and placing them in filter-sterilized MHSFW (hereafter referred to as sterile MHSFW) without algae for 15−20 h.

***Tetrahymena pyriformis* Culture and Preparation for Laboratory Experiments.** Axenic cultures of *T. pyriformis* ciliates were supplied by The Culture Collection of Algae and Protozoa (CCAP no. 1630/1W). Ciliates were cultured in 75 cm^2 cell culture flasks (TTP, Milian) containing proteose peptone yeast extract medium (PPYE). PPYE was prepared following the CCAP recommendations, by dissolving 20.0 g of proteose peptone (Bacto peptone, Difco) and 2.5 g of yeast extract (BioChemika) in 1 L of deionized water, which was then autoclaved and stored at 4 °C. The cultures were maintained at room temperature under sterile conditions, and the medium was renewed every week. On the day prior to the experiment, *T. pyriformis* were washed following an adapted protocol from Pinheiro et al.[19] Briefly, 15 mL of *T. pyriformis* + PPYE were centrifuged at 400*g* for 10 min to form a pellet; the overlying PPYE supernatant was removed and 5 mL of phosphate buffer saline (PBS) was added. The pellet was gently resuspended and recentrifuged at 400*g* for 6 min. The procedure of resuspension in PBS and centrifugation was completed twice, and then the *T. pyriformis* were resuspended in sterile MHSFW. The ciliates were left in sterile MHSFW without food at room temperature for 15−20 h before experimental use. *T. pyriformis* ciliates enumerated using a hemocytometer (Bright-line, Hausser-Scientific) after resuspension and immediately before experimental use.

***Tetrahymena pyriformis* Enumeration Using Flow Cytometry.** The concentration of *T. pyriformis* in prey−predator feeding experiments was determined by flow cytometry (Novocyte Flow cytometer). Experimental samples (250 μL) were fixed with 25 μL of paraformaldehyde fixative solution (abcr GmbH) and stored at 4 °C for up to 7 days before analysis. Immediately prior to analysis, 0.5 μL of a 10-fold diluted LysoTracker Green DND-26 solution (original stock solution: 1 mM solution in DMSO, Thermo Fisher Scientific) was added to each sample followed by incubation in the dark at room temperature for 10 min. The samples were then run on a Novocyte Flow cytometer, and the Novo express software was used for enumeration. The acquisition stop conditions were set to 100 μL and/or 30 s, and the FITC channel threshold was set to 10. The gating was defined on the cytogram obtained for the negative control (sterile MHSFW samples containing paraformaldehyde and lysotracker; free of *T. pyriformis*) and consistently applied to all samples. A calibration curve was created using known concentrations of *T. pyriformis* solutions (as counted with a hemocytometer) and correlating these values to the flow cytometer counts. A limit of blank (LOB), which is the highest signal detected in control samples, of 1.12×10^3 cells/mL was measured by flow cytometry. Only samples above the LOB were reported in our data.

Virus Propagation and Preparation. Echovirus 11 (E11) (Gregory strain, ATCC VR37) was produced by infecting subconfluent monolayers of Buffalo green monkey kidney (BGMK) cells (kindly provided by the Spiez Laboratory, Switzerland) using a previously published protocol.[31] Stock solutions were stored in the freezer at −20 °C and thawed immediately before experimental use. Infectious viral concentrations were determined by incubating 10-fold diluted sample series for 5 days with 95% confluent BGMK cells, as detailed in a previously published protocol.[31] Concentrations were reported as the most probable number of cytopathic units per milliliter (MPNCU/mL). Rstudio software (Version 1.2.1335) was used to calculate the MPNCU/mL for E11 infectivity assay. The LOQ for E11 was 60 MPNCU/mL. The LOQ reflects the lowest E11 concentration resulting in a 95% probability of detecting at least one well with a positive cytopathic effect (1 MPNCU/mL) under the experimental conditions used.

T4 bacteriophage was replicated in *Escherichia coli* B1 host (kindly provided by Professor Petr Leiman, EPFL). Phage propagation and purification were completed using an adapted previously published protocol.[32] Briefly, the bacterial debris was removed via centrifugation at 4000*g* for 15 min followed by vacuum filtration through a 0.22 μm nitrocellulose filter. The T4 was further purified through 100 kDa centrifugal filters (MilliporeSigma) by transferring 50 mL of phage solution to the filters and centrifuging at 3000*g* for 30 min. An equal volume of PBS (50 mL) was added followed by centrifugation to obtain a further purified viral stock. The purified viral stock solution was then eluted at 1000*g* for 2 min. T4 was quantified using the double agar overlay method as previously detailed,[27,33] and infective concentrations were reported as plaque-forming units per milliliter (PFU/mL). Samples were serially diluted as necessary using PBS to achieve plate counts in a 30−300 PFU range. The LOQ for T4 was 300 PFU/mL, which is calculated based on detection of a minimum of 30 PFU for a 100 μL sample using the double agar overlay method.

(RT)qPCR Procedures. Viral nucleic acids for T4 (dsDNA) and E11 (ssRNA+) were extracted from 140 μL by using the QIAamp Viral RNA Mini Kit (Qiagen) following the manufacturer's instructions. Nucleic acids (NA) were eluted in 60 μL of AVE buffer and stored at −20 °C until analysis. Briefly, quantitative PCR (qPCR) for T4 or reverse transcription qPCR (RT-qPCR) for E11 was performed on a

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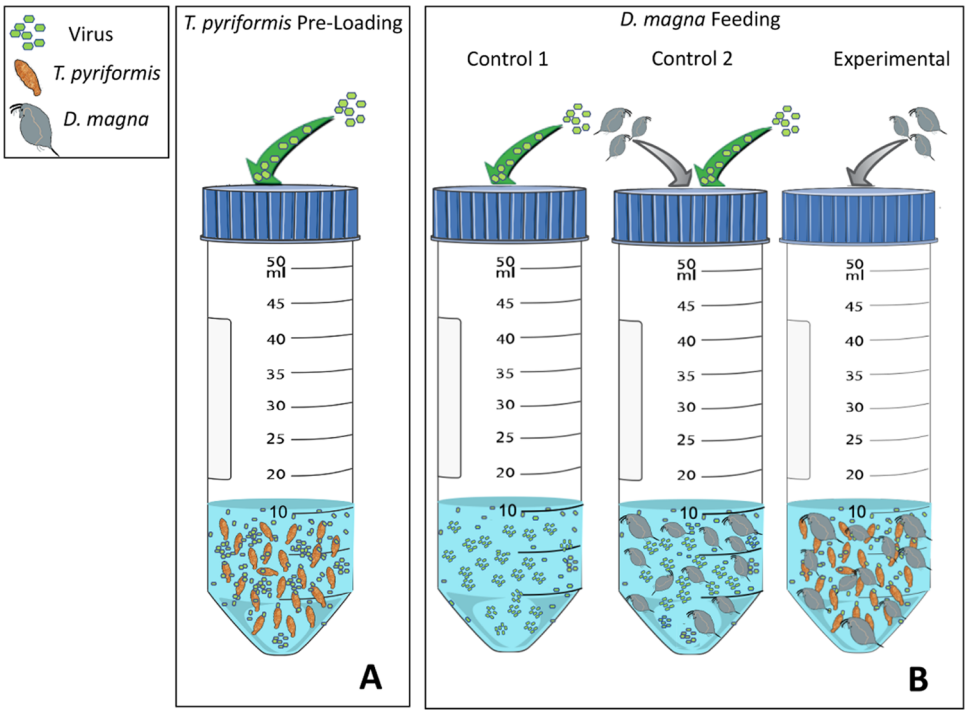

Figure 1. Experimental protocol for examining prey−predator interactions for the virus (T4 or E11)-exposed *T. pyriformis* fed to *D. magna*. (A) *T. pyriformis* were placed in sterile MHSFW spiked with virus (T4 or E11) and were co-incubated for 48 h (T4) or 72 h (E11). The exposure was completed in triplicate. (B) For experimental treatments, *D. magna* (30) were added to the pre-exposed *T. pyriformis* from (A) and were allowed to feed for 2 h in triplicate. Control 1 contained virus without zooplankton and Control 2 contained virus and *D. magna* without *T. pyriformis*.

magnetic induction cycle (MIC) qPCR machine (Biomolecular Systems). The LOQ, calculated based on the methodology by Hougs et al.,[34] for T4 was 50 GC/reaction (corresponding to 4285 GC/mL), and the efficiency was 79%. The LOQ for E11 was 25 GC/reaction (corresponding to 8571 GC/mL), and the efficiency was 89%. Further details are in the Supporting Information (SI) for primers, (RT)qPCR cycle information, and LOQ calculations.

***T. pyriformis*−Virus Co-incubation Experiments.** Laboratory studies were conducted to determine the removal of viruses (T4 or E11) by *T. pyriformis* ciliates. Batch experiments were performed with ciliates co-incubated with viruses in 10 mL of sterile MHSFW. Experimental treatments were completed in triplicate. A control containing the virus in sterile MHSFW without zooplankton was used to determine viral die-off due to abiotic factors. The initial spike concentration was 10^6 PFU/mL for T4 and 10^3 or 10^5 MPNCU/mL for E11. The initial experimental *T. pyriformis* concentration was 10^4 ciliates/mL. Kinetic uptake experiments were conducted over 96 h, and water was sampled every 24 (T4) or 48 h (E11). Water samples taken from *T. pyriformis* experimental treatments were filtered through a 0.22 μm syringe filter (polyethersulfone membrane; Millipore Millex GP syringe filter unit) to retain the ciliates on the filter but allow viruses to remain in the media for enumeration. Filtration did not impact the final viral concentration. The viral concentration was determined using appropriate culture-based techniques as described above. In addition, selected samples were analyzed by (RT)qPCR.

***D. magna*−Virus Co-incubation Experiments.** To determine virus removal kinetics by *D. magna*, a similar approach as described above was used, with the following modifications to account for differences in organisms. The initial experimental concentration was 3 daphnids/mL. Water samples were taken with a micropipette, ensuring that the tip did not come in contact with or inadvertently remove a daphnid from the system. Water samples were not filtered prior to enumeration. After taking a water sample for enumeration, daphnids were fed 20 μL of concentrated (10^6 cells/mL) *Nannochloropsis* sp. algae to provide ample food to maintain the experimental population for the duration of the 72−96 h of exposure. A control containing sterile MHSFW with *Nannochloropsis* sp. algae and the spiked virus was used to determine the impacts of algae on the viral concentration. The viral concentration was determined using culture-based techniques as previously described.

Virus−Prey−Predator Feeding Experiments. To determine the potential of viral transfer between predator (*D. magna*) and prey (*T. pyriformis*) (Figure 1), *T. pyriformis* were exposed to T4 or E11 viruses as described above for 48 or 72 h, respectively, in triplicate. Following viral loading, 30 unexposed *D. magna* (approximate concentration of 3 daphnids/mL) were added to each experimental treatment. *D. magna* were allowed to feed on *T. pyriformis* for 2 h (Figure 1). One control, containing the virus in sterile MHSFW, was used to determine the changes in viral concentration without zooplankton exposure. A second control containing the virus with *D. magna* in sterile MHSFW was used to determine any changes in viral concentration with exposure to *D. magna* but in the absence of *T. pyriformis*. Unfiltered water samples were taken at the start and end of the feeding period and were enumerated using culture-based techniques and (RT)qPCR. *T. pyriformis* were enumerated using flow cytometry at the experimental start and end.

At the end of the 2 h feeding experiment, *D. magna* were collected. Isolation and rinsing procedures were implemented

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to remove virus particles that may be bound to the carapace. *D. magna* were first collected by retaining organisms on a 100 μm sterile nylon cell strainer (Corning) and then rinsed with 50 mL of PBS. The *D. magna* were then individually transferred with a sterile cell-inoculating loop (1 μL, VWR) to a new sterile cell strainer and rinsed with 50 mL of PBS. The transfer and rinse processes were repeated two times, and then daphnids were transferred to a preweighed 1.5 mL centrifuge tube to obtain a wet weight. Sterile pellet pestles attached to a motor (Sigma-Aldrich) were used for 1 min to blend the daphnids into a uniform mixture in 500 μL of PBS. The blended tissues were then used for cell culture or (RT)qPCR.

Prey−predator feeding experiments were also completed with viral-unexposed *T. pyriformis*, to differentiate between the feeding of *D. magna* on viral-exposed *T. pyriformis* (preloaded with virus) versus *T. pyriformis* not exposed to virus prior to the experiment. The experimental treatments contained unexposed *T. pyriformis*, unexposed *D. magna*, and spiked virus. The *D. magna* were allowed to feed for 2 h, and water samples were taken for viral enumeration and flow cytometry.

Quality Assurance. *D. magna* tissue (1 g, 30 daphnids) unexposed to viruses was homogenized as per the protocol described above to test for interference with infectivity or (RT)qPCR assays. Unspiked *T. pyriformis* were also tested for interference with infectivity or (RT)qPCR assays. Unspiked *D. magna* blended tissues or *T. pyriformis* whole organisms did not result in false positives or assay interferences. *D. magna* (30 daphnids) were spiked with a known concentration of virus, blended as previously described, and the virus concentration was enumerated using infectivity or (RT)qPCR assays. The blended samples did not result in interference, and the measured concentrations were not significantly different from the original spike amount. To test the effectiveness of the rinsing procedure used to remove attached viruses to *D. magna*, 30 daphnids were euthanized by placing them at −80 °C for 20 min, then allowed to thaw, and finally spiked with the virus. The rinsing procedure as described above resulted in a 3−4 log reduction in infective viral concentrations.

Data Analysis. The viral removal rate constants (k, h^{-1}) as a result of *T. pyriformis* or *D. magna* uptake were calculated by fitting the entire experimental time series to a log-linear model

$$C_t = (C_0)e^{-kt} \quad (1)$$

where C_0 is the viral concentration at $t = 0$, C_t is the viral concentration at a given time point, t is the time in hours, k is the viral removal rate constant in h^{-1}. Also, k-values were obtained for the control beakers ($k_{control}$) to account for changes in virus concentration due to processes other than filter feeding, such as thermal virus inactivation or adsorption to vessel surfaces. The final k-value attributed to the filter feeding action of *T. pyriformis* or *D. magna* ($k_{organism}$) was corrected for decay measured in the control treatments

$$k_{organism} = k_{experimental} - k_{control} \quad (2)$$

All removal rate constants were determined for pooled replicates and are reported with the associated standard errors.

The specific infectivity (SpI), used to compare data from infectivity assays with data from (RT)qPCR, was calculated as follows

$$SpI = \frac{PFU/mL}{GC/mL} \text{ or } \frac{MPNCU/mL}{GC/mL} \quad (3)$$

Values for SpI are reported with the associated standard errors.

Statistical analysis of results was completed using SPSS (IBM v 26), and results were considered significant for $p < 0.05$. All virus concentration data were $\log_{10}$-transformed prior to statistical analysis. Due to variability in filter feeding that can occur when working with the organisms in this study, data was only directly compared within a given set of experiments.

RESULTS AND DISCUSSION

Virus Removal in Batch Experiments by *T. pyriformis* or *D. magna* Filter Feeding. Laboratory batch co-incubation experiments were conducted to determine if virus removal occurred as a result of filter feeding by *T. pyriformis* or *D. magna*. For *T. pyriformis*, co-incubation with T4 resulted in significant exponential virus decay ($R^2 = 0.995$, $p < 0.005$) (Figure 2A). The calculated k-value for the experimental treatments was significantly greater than the control beaker containing MHSFW (t-test, $p < 0.005$). The mean $k_{organism}$-value (eq 2) was 0.12 ± 0.01 h^{-1}. Similarly, E11 virus removal was observed after co-incubation with *T. pyriformis* (Figure 2B). The $k_{experimental}$-value was significantly greater than the $k_{control}$-value (t-test, $p < 0.05$). The mean $k_{organism}$-value (eq 2) for the three experimental replicates was 0.06 ± 0.02 h^{-1}.

In addition to using culture-based techniques, (RT)qPCR was used to determine the changes in viral genome concentration for T4 and E11 when co-incubated with *T. pyriformis*. The mean $k_{organism}$-values as quantified by (RT)-qPCR were 0.09 ± 0.02 h^{-1} for T4 and 0.04 ± 0.004 h^{-1} for E11. The time series data used to calculate these values are shown in the SI (Figures S1 and S2). The $k_{organism}$-values obtained for genome data for both T4 and E11 are of similar magnitude. Considering the similar decline in both genomic copies and infectivity, *T. pyriformis* filter feeding resulted either in the physical removal of viruses by ingestion and/or sorption, or in inactivation that involved the digestion of the nucleic acid. Further research is necessary to determine the relative contributions of true inactivation but is outside the scope of the current study.

In contrast to *T. pyriformis*, filter feeding of *D. magna* during co-incubation experiments did not result in a significant decline in viral infectivity of T4 or E11 in comparison to that of the *D. magna*—free control ($p > 0.1$) (Figure 2C,D). For T4, the concentration in the media for the control and experimental replicates remained stable with a negligible reduction in concentration (Figure 2C). For E11, a 2 log decline in infectivity was observed (Figure 2D) in both the control and experimental treatments. This decline may be due to interactions of E11 with the algae spiked into the water that was necessary to sustain the *D. magna* population.

Our results of *T. pyriformis* co-incubation with T4 and E11 align with other studies that have exposed *Tetrahymena* sp. to various viruses and showed the uptake of the virus by these ciliates.[9,11,19−21] A wide range of viruses have been co-incubated with *Tetrahymena* sp., including bacteriophage T4 and MS2, enteroviruses (echovirus, poliovirus, coxsackievirus), adenovirus, and rotavirus. While uptake was observed, the infectivity of these viruses after exposure varied among these studies.[9,11,19−21] For example, in a study using *Trichorhina thermophila*, T4 was inactivated and located in the digestive vacuoles, indicating ingestion with inactivation rather than sorption.[19] A study with poliovirus and echovirus showed that both these viruses were associated with *T. pyriformis* via ingestion or adsorption but not fully inactivated by *T.*

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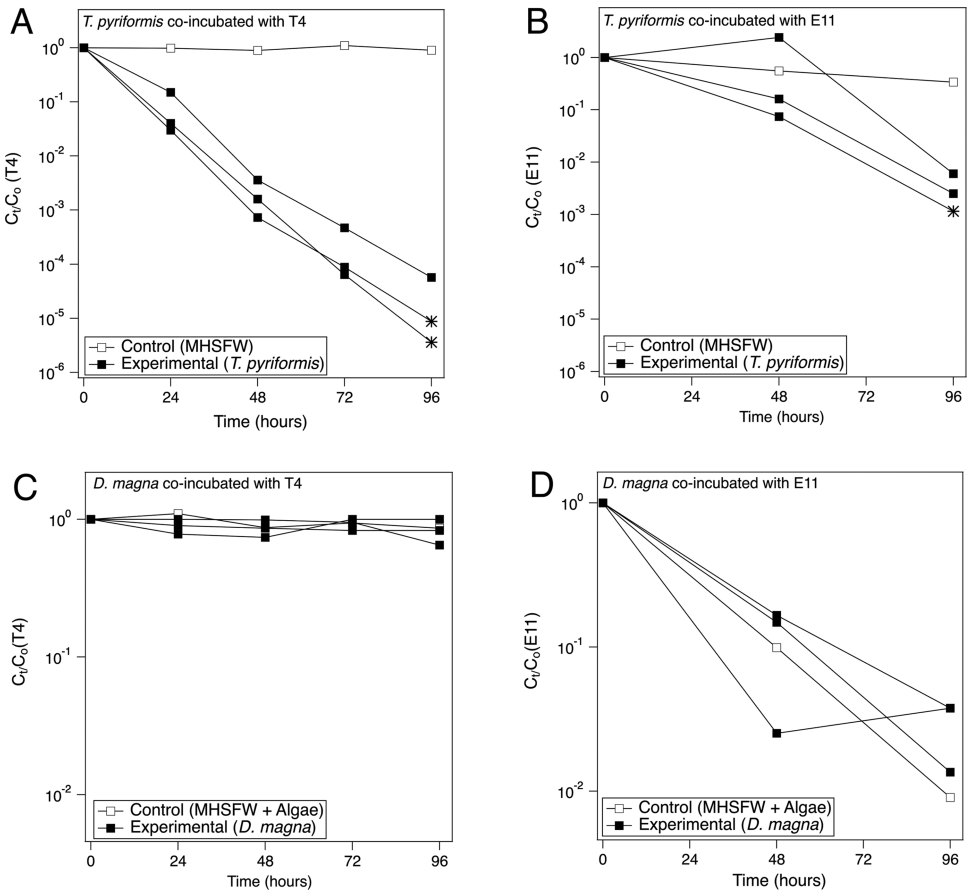

Figure 2. Viral removal by *T. pyriformis* or *D. magna* exposed to MHSFW in batch laboratory experiments quantified by culture-based techniques. (A) and (B) show the removal of T4 and E11 by *T. pyriformis*, respectively. (C) and (D) show the removal of T4 and E11 by *D. magna*, respectively. Experimental treatments were completed in triplicate. The starred symbols indicate infective virus concentrations below the LOQ. The data in the figure were used to calculate removal rate constants (*k*-values) reported in the main text. Note: The solid lines connect data points for each replicate to assist with figure interpretation and do not represent the model fit used to calculate the *k*-values reported in the text.

pyriformis.[9] Our studies show that *T. pyriformis* removed both T4 and E11 from culture, but the percent infectivity of the viruses within the organism was not directly studied and is an area of future research.

Limited information is available on the uptake of viruses from water by *D. magna*. Two studies have observed the uptake of the avian influenza virus (AIV) by *D. magna*.[25,26] Another study showed inactivation of the ranavirus by *Daphnia pulex*.[35] The uptake of enterovirus or phages from water by *D. magna* has not been previously studied. Our results show that the *D. magna* do not directly uptake or accumulate T4 and E11 from water. The mechanisms governing the uptake of viruses are unclear for *D. magna*, but uptake is likely incidental and passive since the size of viruses is smaller than the typical size range of food ingested by *D. magna* (0.5−5 μm range), and studies varying the daphnid density or food concentration available did not significantly impact viral uptake rates.[35,36] Published research showing uptake of viruses by *Daphnia* sp. used larger enveloped viruses with the diameter of spherical AIV ranging 80−120 nm[37] and icosahedral nonenveloped ranavirus ranging 120−200 nm.[38] Uptake directly from water was not observed for the two viruses used in the study presented here, which may be due to the size, shape, and structure of the viral particles used. E11 is spherical with a diameter of 25−30 nm,[39] and T4 contains an icosahedral head of 47 nm and a 142 nm long tail.[40]

Viral Transfer and Uptake via Prey−Predator Interactions. Laboratory batch experiments were conducted using *T. pyriformis* that were pre-exposed to viruses as a food source for *D. magna*. The viral pre-exposure time period of *T. pyriformis* to the viruses was determined from the previously described co-incubation experiments. The time period was chosen with a goal of approximately 1 log reduction of viral infectivity in the media after exposure to *T. pyriformis*. A 2 h feeding window was chosen to allow sufficient time for interaction between *D. magna* and *T. pyriformis* while minimizing the effects of defecation, which could result in reintroduction of viral material back into the media through fecal inputs. This feeding window was also based on a previously published filtration rate of 3 mL h^{-1} $daphnid^{-1}$ for *D. magna* feeding on *T. pyriformis*.[41] Feeding was confirmed during each exposure with an average of 1.1 ± 0.2 log removal of *T. pyriformis* by *D. magna*.

For T4, an exposure of *T. pyriformis* for 48 h resulted in a 0.94 ± 0.03 log reduction of T4 infectivity in the media. After 2 h of *D. magna* feeding on the viral-loaded *T. pyriformis*, the T4 infectivity in the media was further reduced by 1.3 ± 0.1 log resulting in a final mean amount of 2.5×10^5 PFU infective T4 (Figure 3A). Similar trends were observed for E11. To ensure sufficient E11 uptake, *T. pyriformis* was exposed to a high (10^5 MPNCU/mL) concentration of the virus for 72 h, resulting in a 1.9 ± 0.1 log reduction of E11 in the media

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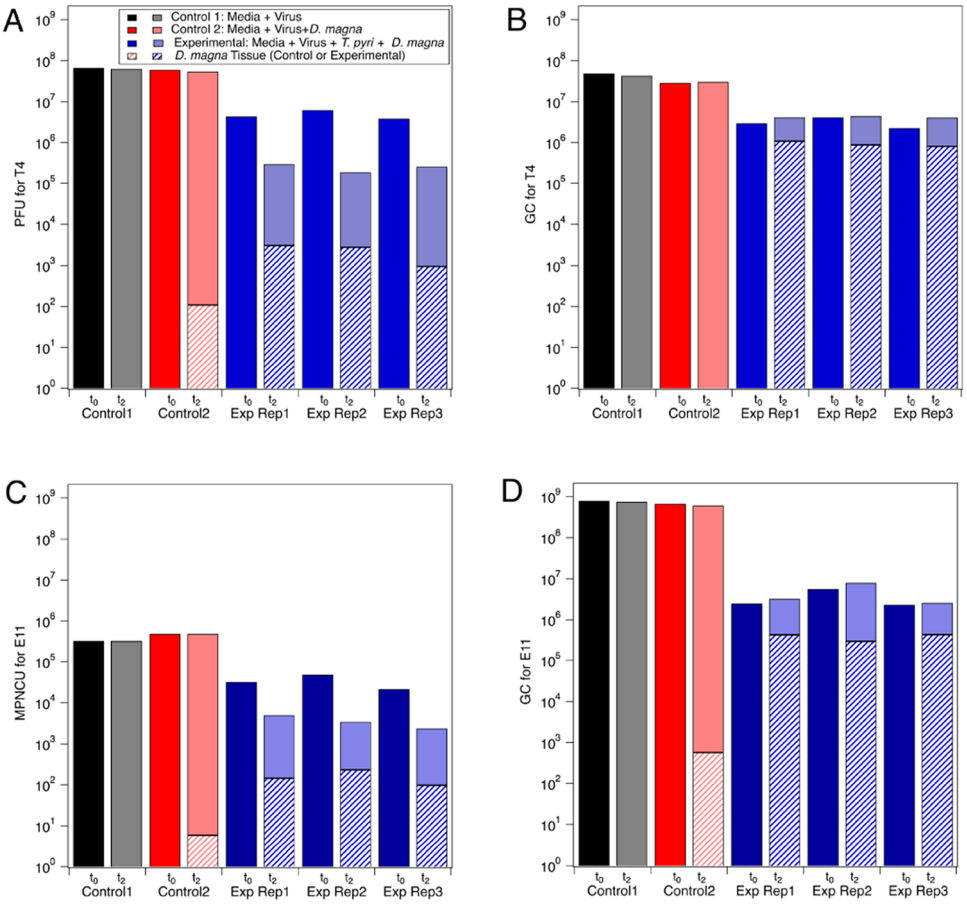

Figure 3. Material balance from a 2 h feeding of viral-preloaded *T. pyriformis* to *D. magna*, where t_0 is equal to the initial time point and t_2 is equal to the final sample time point at 2 h. (A) and (B) show the values from the infectivity assay (PFU) and qPCR (GC) for T4, respectively. (C) and (D) show the values from the infectivity assay (MPNCU) and (RT)qPCR for E11, respectively. The tissue of Control 2 in panel (B) showed a positive qPCR signal at t_2 for T4, but the signal was below the LOQ.

(Figure S3). Subsequent feeding of the viral-loaded *T. pyriformis* to *D. magna* further reduced the infective E11 in the media by 1.0 ± 0.1 log, resulting in a final mean amount of 3.8×10^3 MPNCU infective E11 in the media (Figure 3C).

Interestingly, and in contrast to virus co-incubation with *T. pyriformis* alone, the grazing of virus-loaded *T. pyriformis* by *D. magna* only resulted in a decrease of infectivity but not genome copies (Figures 2 and 3). For the experimental treatments of both viruses tested, the SpI (eq 3) in the media decreased by an order of magnitude or more after 2 h (1.3 ± 0.1 log decrease for T4, 1.0 ± 0.1 log decrease for E11), as a result of *D. magna* feeding activity. In contrast, the SpI in control samples stayed constant over the duration of the experiment. This indicates that feeding by *D. magna* caused virus inactivation that did not primarily involve the physical removal of the virus from solution or digestion of the genome.

Analysis of homogenized *D. magna* tissue revealed the presence of infective viruses in the organisms. For T4, a mean amount of 2.3×10^3 PFU was detected in the tissue of the experimental *D. magna* population, which is 1.3 log more compared to the tissue of the *T. pyriformis*-free control (Figure 3A). For E11, 1.6×10^2 MPNCU of the infective virus was recovered from the *D. magna* tissue, which also corresponds to 1.4 log surplus compared to that of the control (Figure 3C). The viral amounts detected in the control (Figure 3, Control 2, t_2) represent residual viruses that may be sorbed to the carapace after rinsing. *D. magna* tissue also accumulated the genomic material. Amounts of 9.3×10^5 and 3.9×10^5 genomic copies were detected for T4 and E11, respectively, in the tissue (Figure 3B,D), far in excess of the amounts detected in the *T. pyriformis*-free controls.

An overall material balance on infective units (PFU or MPNCU) shows an average 1.3 log (T4) and 1.0 log (E11) differences between the initial value in the media and final value in the media and tissue (Figure 3A,C). In contrast, no measurable difference was observed between the initial and final genomic copy values for the media and tissue (Figure 3B,D), confirming a reduction in infectivity without changes to the nucleic acid content. While *D. magna* feeding on *T. pyriformis* did result in a reduction of viral infectivity in the media, the recovery of infective viruses from the *D. magna* tissue indicates that inactivation of infectious viruses by *D. magna* is incomplete.

When prey−predator feeding experiments were conducted without pre-exposing *T. pyriformis* to viruses, virus removal by *D. magna* was not observed. During these experiments, *D. magna* still consumed *T. pyriformis*, resulting in a comparable reduction in organisms as quantified by flow cytometry. However, viral concentrations remained stable during the 2 h of feeding. These experiments provided support to our findings that E11 and T4 uptakes by *D. magna* were mediated by the consumption of *T. pyriformis* containing E11 and T4. Prey−predator interactions can thus enhance viral grazing by zooplankton compared to grazing by a single species alone.

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Previous studies have not examined the potential of viral transfer due to the trophic interaction between microzooplankton and metazooplankton, such as *T. pyriformis* and *D. magna*. Studies have considered the role of *D. magna* as a vector of pathogens to higher trophic organisms. For example, one study examined tadpole feeding on *D. magna* that consumed *Batrachochytrium dendrobatidis* zoospores. The *D. magna* predation on *B. dendrobatidis* zoospores resulted in reduced number of zoospores and subsequently reduced the infection of tadpoles.[42] Another study considered trophic interactions between *Listeria* sp. fed to *T. pyriformis*, which was then fed to *Amoeba proteus*. The outcome of the bacterial trophic transfer varied depending on the strain of *Listeria*. For *L. monocytogenes* delivered via *T. pyriformis* to *A. proteus*, the bacteria were able to replicate within the amoeba resulting in growth restriction, while for *Lycosa innocua*−*T. pyriformis* fed to *A. proteus*, the bacteria did not replicate and amoeba growth was improved.[43] Our results show that viral transfer does occur from ciliates to daphnids, but further work is needed to consider if the transfer would continue to predators of daphnids, such as fish, amphibians, and birds, and if viral transfer can impact the health of the carriers. Our study showed that *D. magna* did not fully inactivate the E11 or T4 taken up through the *T. pyriformis* consumption; hence, the potential to act as a vector to higher organisms may exist.

Implications of Viral Transfer via Prey−Predator Interactions. Both ciliates, such as *T. pyriformis*, and cladocerans, such as *D. magna*, are ubiquitous in the environment and play critical roles as filter feeders, regulating the microbial loop and serving as prey items for higher trophic organisms.[12−15] In addition to their role in natural systems, these organisms are also found in wastewater treatment and drinking water treatment facilities. The filter feeding ciliate, *T. pyriformis*, in our study caused a 2−4 log viral removal over a period of 48−72 h based on a density of 10^4 ciliates/mL, which is comparable to the densities of 10^3−10^4 ciliates/mL in wastewater treatment sludge or eutrophic ponds.[10,11,13,14] An additional 1 log viral removal was observed by *D. magna* feeding on viral-exposed *T. pyriformis*. Previously published work quantified the *D. magna* feeding rate on *T. pyriformis* of 3 mL h^{-1} $daphnid^{-1}$,[41] and densities of daphnids in the environment can range from 100 to 700 daphnids L^{-1} during peak population booms.[44−46] Based on our study results, the *T. pyriformis*−*D. magna* food chain can have an impact on viral loads in environmental systems and prey−predator interactions change the viral fate in the environment. *D. magna* may be contributing to further inactivation or, in contrast, may protect viruses from inactivation while acting as a viral vector with infective viruses available for transfer to higher trophic levels.

Although *D. magna* protection effects against disinfection treatment with viruses have not been previously studied, *D. magna* has exhibited protection of *E. coli* for chloramine disinfection after ingestion.[47] Numerous studies with amoebas have shown protection of viruses against inactivation, including chemical disinfection. For example, the enterovirus coxsackievirus B5 (CVB5) persisted within the amoeba *Vermamoeba vermiformis*, which can be a means of viral protection in environmental waters. CVB5 was also associated with expelled vesicles that could serve as another route of environmental dispersion and protection.[48] Similarly, amoebas were shown to internalize human adenovirus type 5 (HAdV 5), and infective HAdV 5 were found in amoebas after sodium hypochlorite disinfection.[49] Similar work examining chemical disinfection protection of viruses by metazooplankton, such as *D. magna*, is needed.

To date, the potential of trophic transfer and subsequent protection effect of viruses by microzooplankton and metazooplankton, such as *D. magna*, have not been considered in natural systems. The results presented in this paper demonstrate that viral transfer within a food chain is possible. These findings have implications regarding persistence of viruses in surface water, which is of importance to both human and ecosystem health. Further research is necessary to understand the extent that viral uptake, trophic transfer, and protection may have on disinfection in water treatment. In addition, research is needed to understand the impacts of environmental variables on filter feeding activity and subsequent viral concentrations in natural systems.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.0c02545.

Detailed (RT)qPCR procedures, and additional figures and tables referenced in text (PDF)

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N.S.I. contributions: overall study conceptualization and investigation, formal analysis, and writing of the original manuscript draft. M.O. contributions: study investigation, formal analysis, and reviewing and editing the manuscript. X.F.-C. contributions: study investigation, methodology development, and reviewing and editing the manuscript. V.B. contribution: study investigation. T.K. contributions: project resources, overall project support, and reviewing and editing the manuscript. All authors have given approval to the final version of the manuscript.

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Notes

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