LIMNOLOGY and OCEANOGRAPHY

ASLO

Limnol. Oceanogr. 00, 2017, 00–00

doi: 10.1002/lno.10734

Viruses on the menu: The appendicularian *Oikopleura dioica* efficiently removes viruses from seawater

Janice Lawrence,[1] Joachim Töpper,[2] Elżbieta Petelenz-Kurdziel,[3] Gunnar Bratbak,[3] Aud Larsen,[3,4] Eric Thompson,[3,4,5] Christofer Troedsson,[4] Jessica Louise Ray [4]*

[1]Department of Biology, University of New Brunswick, Fredericton, New Brunswick, Canada
[2]Norwegian Institute for Nature Research, Bergen, Norway
[3]Department of Biology, University of Bergen, Bergen, Norway
[4]Uni Research Environment, Uni Research AS, Bergen, Norway
[5]Sars International Centre for Marine Molecular Biology, University of Bergen, Bergen, Norway

Abstract

Appendicularians are planktonic marine tunicates with elaborate filter-feeding houses that can efficiently trap particles as small as 0.2 μm. While marine viruses are seldom considered outside their role in disease transmission, we conducted a controlled laboratory experiment to determine if the appendicularian *Oikopleura dioica* can trap and ingest the *Emiliania huxleyi* virus (EhV; 160–180 nm diameter). Removal and retention of EhV during 2.5 h and overnight incubations at 15°C were measured using flow cytometry and quantitative polymerase chain reaction specific for the *mcp* gene of EhV. The fate of retained EhV was tested by quantifying EhV DNA in three biological compartments: house-trapping, ingestion/digestion, and defecation. Clearance rates for EhV varied from approximately 2 mL ind^{-1} d^{-1} to 50 mL ind^{-1} d^{-1}, with highest rates for 4–5 d-old animals. EhV particles were cleared by *O. dioica* at rates similar to those reported for larger food particles, with mean clearance rates in the 2.5 h incubations ranging from approximately 2 mL ind^{-1} d^{-1} to 50 mL ind^{-1} d^{-1}. This demonstrates efficient virus removal by *O. dioica* and a previously overlooked link between the microbial loop and the classical marine food web. EhV DNA was readily detectable above background levels in *O. dioica* houses, gut contents, and faecal pellets, suggesting that appendicularian houses and faecal pellets may contribute to the dispersal of viruses. Furthermore, clearance of EhV and presumably other viruses by *O. dioica* may be a significant sink for viruses and thus an important factor in regulating the population dynamics of viruses and their hosts.

Appendicularians (Chordata, Tunicata) are an abundant and diverse group of gelatinous zooplankton found in marine environments across the globe (Gorsky et al. 1999). This unique group of planktonic tunicates secretes elaborate filter-feeding house structures that enable efficient capture of food particles as small as 0.2 μm in diameter (Bedo et al. 1993; Flood et al. 1998; Acuña and Kiefer 2000). Thus, appendicularians form a crucial conduit by which pico- and nanoplanktonic biomass bypass the microbial loop and enter the pelagic food web (Flood et al. 1992). With clearance rates of up to $\sim$ 30 mL h^{-1} per animal (Troedsson et al. 2007), appendicularians filter water more rapidly than similarly-sized copepods and may have significant impacts on food concentration when present in high abundance (Alldredge 1981). In addition, appendicularian houses are constantly replaced throughout the organism's life to minimize the reduction in filtration efficiency that occurs as trapped particles clog houses (Troedsson et al. 2007). These discarded houses contribute significantly to the particulate organic matter (POM) pool in marine environments (Alldredge 2005), and can serve as particle traps that ferry trapped particles to deeper water during sinking (Hansen et al. 1996).

While the efficiency of appendicularian feeding on marine pico- and nanoplanktonic organisms has been thoroughly examined (Gorsky et al. 1999; Scheinberg et al. 2005), a few authors have suggested that appendicularians might trap even smaller particles ($<$ 0.2 μm) (Flood 1978; Flood et al. 1992; Lombard et al. 2011). To our knowledge, however, no one has yet assessed the feeding potential of appendicularians on femtoplankton (e.g., viruses), as previous examinations were conducted using femtoplankton-sized

*Correspondence: jessicalouiseray@gmail.com

Additional Supporting Information may be found in the online version of this article.

J_ID: LNO Customer A_ID: LNO10734 Cadmus Art: LNO10734 Ed. Ref. No.: LO-17-0061.R1 Date: 12-October-17 Stage: Page: 2

colloids (Flood et al. 1992), or plastic beads (Fernández et al. 2004, as analyzed by Lombard et al. 2011). Viruses are found globally at titers up to 10^8 viruses mL^{-1} of seawater (Bergh et al. 1989) and range in diameter from tens (Nagasaki 2008) to several hundred nanometers (Arslan et al. 2011), making the largest marine viruses similar in size to the smallest known bacteria (Raoult et al. 2007). Research over the last few decades has established an indisputable role of viruses in driving nutrient cycling (Bratbak et al. 1994; Wilhelm and Suttle 1999) and regulating host diversity (Avrani et al. 2011 and references therein), and therefore in shaping the structure and function of marine ecosystems (Rohwer and Thurber 2009; Avrani et al. 2012). Less clear, however, is how viral abundance, stability, and persistence are regulated in marine environments.

The objective of this study was to determine if a cosmopolitan appendicularian, *Oikopleura dioica*, can trap and feed on a large marine virus (160–180 nm, Wilson et al. 2005) that infects the globally important phytoplankton, *Emiliania huxleyi* (EhV). This was achieved using controlled laboratory experiments to quantify viruses in different feeding compartments (house trapped, ingested, and defecated) after short (2.5 h) and long (overnight) incubations. The results demonstrate a previously overlooked interaction that has implications for both the fate of marine viruses and the organisms they infect.

Methods

Animal culturing

Healthy individuals of *O. dioica* (Tunicata, Appendicularia) were collected from Raunefjorden in Western Norway (60°16′N, 05°15′E) during the summer of 2015 and maintained in culture at 13–15°C as previously described (Bouquet et al. 2009). One culture of *O. dioica*, designated "Esp9," was synchronized to produce a generation of animals and maintained for nine generations prior to the experiment. Esp9 cultures were fed twice daily with a combination of *Isochrysis galbana*, *Chaetoceros calcitrans*, *Synechococcus* sp., and *Rhinomonas reticulata* depending on *O. dioica* age, according to established methods (Bouquet et al. 2009). The same culture conditions and food mixtures were used to maintain the animals during experiments. Additional culturing information can be obtained from http://www.sars.no/facilities/appendic.php. Animals were cultured at approximately 15°C, which yielded a generation time of 6 d. Experiments were therefore conducted with animals ranging in age from 1 to 5 d-old (Day 1 to Day 5) in 1-d increments (*see* Table 1 for animal size). Days 1–5 are therefore referred to as "developmental stages" as the single day-time increments are arbitrary, temperature-dependent indicators of animal development. Day 6 animals were reproductively mature and could not be used for experiments due to spawning on that day.

Table 1. Numbers of healthy *O. dioica* individuals "Incubated" and "Sampled" per replicate for each developmental stage (Age) and sample type in 7 min, 2.5 h, and overnight incubations at 15°C. All samplings were performed for each of three biological replicates, with the exception of faecal pellet samples, which consisted of pooled faecal pellets from three biological replicates. Trunk lengths are provided as mean ± SD of three replicates. ns, not sampled.

		Clearance	*Ingestion*		*House-trapping*		*House-trapping + ingestion*		*Defecation*
		2.5 h and overnight	Animals (Days 3–5)		Houses (Days 3–5)		Animal-houses (Days 1–2)		Faecal pellets (Days 3–5)
			7 min		2.5 h		2.5 h		Overnight
Age	Trunk length (μm)	Incubated	Incubated	Sampled	Incubated	Sampled	Incubated	Sampled	Sampled
Day 1	153 ± 13	150	ns	ns	ns	ns	150	100	ns
Day 2	210 ± 60	150	ns	ns	ns	ns	150	100	ns
Day 3	340 ± 35	120	50	40	120	100	ns	ns	150
Day 4	540 ± 90	80	50	40	80	60	ns	ns	300
Day 5	990 ± 160	40	25	20	40	30	ns	ns	500

ID: jwaa3b2server Time: 18:56 I Path: //chenas03/Cenpro/ApplicationFiles/Journals/Wiley/LNO#/Vol00000/170190/Comp/APPFile/JW-LNO#170190

J_ID: LNO Customer A_ID: LNO10734 Cadmus Art: LNO10734 Ed. Ref. No.: LO-17-0061.R1 Date: 12-October-17 Stage: Page: 3

Preparation of virus lysate

E. huxleyi virus strain 99B1 (EhV-99B1) was maintained on *E. huxleyi* BOF92 as described previously (Castberg et al. 2002). To generate virus for experiments, a late-exponential-phase culture of *E. huxleyi* BOF92 in 2 L of 1/2 IMR medium (Castberg et al. 2002) was infected with 20 mL EhV lysate (multiplicity of infection $\sim$ 1 : 100) and incubated at 16°C under 180 μm photons m^{-2} s^{-1} with a light : dark regime of 14 : 10 until lysis was visually apparent (4 d post infection). The lysate was centrifuged at 6400 $\times$ *g* at 4°C in a Beckman JA-10 rotor to pellet cell debris and stored at 4°C. A final virus titer of $\sim$ 10^6 EhV mL^{-1} was used for all experiments as this concentration represents natural post-*E. huxleyi* bloom concentrations of EhV in coastal Norwegian ecosystems (Bratbak et al. 1995).

Clearance experiments

The rate of removal of EhV by *O. dioica* was determined at each developmental stage in separate experiments lasting 2.5 h or overnight. For the 2.5 h incubations, six 1-liter beakers were filled with 0.2-μm-filtered seawater and food phytoplankton, and a set number of randomly selected animals was added (*see* Table 1 for numbers of animals). For overnight experiments, 2-L incubations were used. For both 2.5 h and overnight experiments, an additional six beakers were filled with 0.2-μm-filtered seawater and phytoplankton to serve as no-animal controls. Three randomly selected animal and no-animal beakers received EhV (final concentration $\sim$ 10^6 EhV mL^{-1}), while the remaining three animal and no-animal beakers served as no-virus controls. This 2 $\times$ 2 factorial design ($\pm$ animal, $\pm$ virus) with three biological replicates was used in all subsequent experiments. After treatment additions, the beakers were gently mixed to ensure homogeneous distribution of EhV and beakers containing *O. dioica* were incubated with constant gentle stirring (Bouquet et al. 2009). Incubation water was sampled at the start and end of incubations for flow cytometry (FCM) and quantitative polymerase chain reaction (qPCR) (0.5 mL each). The duration of overnight experiments was 19.25 h, 21.0 h, 18.25 h, 15.5 h, and 17.5 h for Day 1–5 animals, respectively. Samples for FCM were fixed with 0.5% (v/v) glutaraldehyde at 4°C in the dark prior to snap-freezing in liquid nitrogen and storage at −80°C. Samples for qPCR were immediately processed for DNA extraction.

House-trapping experiment

The rate of virus-trapping by houses of Day 3, 4, and 5 animals was determined using individuals from the 2.5 h clearance incubations described above. Animals were removed from their houses and the houses pooled in 1.5-mL Eppendorf tubes, briefly centrifuged, and excess water removed. Excess water removal was complete for all days except Day 4, where $\sim$ 100 μL of incubation water remained. The sampled houses (Table 1) were then rinsed three times with 0.2-μm-filtered seawater and processed for DNA extraction and qPCR. A 10-μL sample from the last rinse step was collected in order to estimate the number of EhV present in the final rinse water, which may indicate loosely adhered EhV particles and/or EhV particles that were dislodged from the house during the rinsing process (Supporting Information Fig. S1A).

The results from house-trapping experiments are based on the assumption that EhV detected by qPCR represent filtered particles rather than particles that randomly adhered to inflated *O. dioica* houses, and that any adsorbed EhV particles were removed by three consecutive washes during sample processing. In order to assess the presence of loosely associated EhV particles present in the wash water, we collected 10 μL of wash water after the third and final rinsing step, and processed these aliquots for DNA extraction and qPCR. EhV signal in incubations without EhV inoculum demonstrated only sporadic EhV signal (black symbols in Supporting Information Fig. S1A). We detected EhV *mcp* gene copies in the wash water from the +EhV animal-houses (Day 1 and Day 2) and houses (Day 3 and Day 5) (gray symbols in Supporting Information Fig. S1A), indicating either EhV particles loosely adhered to *O. dioica* houses during incubation and/or that the brief centrifugations and wash steps dislodged EhV particles that were inside the food-concentrating filter. The present study is unable to distinguish between these two possibilities, although the potential consequences for downward vertical transport of EhV to marine sediments are relevant for both. Nonetheless, we demonstrated the presence of EhV *mcp* gene copies in both the animals themselves and in their faecal pellets. House-trapping wash water controls were not collected for Day 4 houses.

Ingestion experiment

Very short (7 min) experiments were conducted to quantify EhV ingestion for Day 3, 4, and 5 animals in order to accommodate the rapid (< 10 min) gut passage time of *O. dioica* (Bedo et al. 1993; López-Urrutia and Acuña 1999). Triplicate 1-liter beakers were set up for each treatment as described above ($\pm$ animal, $\pm$ virus) (*see* Table 1 for numbers of animals). Animals were retained in their houses to ensure that we sampled animals with differently aged houses, as it has been shown that filtration efficiencies vary over house age (Acuña and Kiefer 2000). Therefore, measured ingestion rates represent averages. After incubation, animals were collected, removed from their houses, anesthetized in 0.25 mg mL^{-1} 3-aminobenzoic acid ethyl ester (MS-222), and rinsed three times with 0.2-μm-filtered seawater. Animals sampled from each replicate (Table 1) were pooled in 1.5-mL Eppendorf tubes and briefly centrifuged to remove excess water. Samples were then processed for DNA extraction and qPCR.

The ingestion analysis is based on the assumption that EhV particles not removed by three consecutive washes represent ingested virus. To test this, we collected an additional

ID: jwaa3b2server Time: 18:56 I Path: //chenas03/Cenpro/ApplicationFiles/Journals/Wiley/LNO#/Vol00000/170190/Comp/APPFile/JW-LNO#170190

J_ID: LNO Customer A_ID: LNO10734 Cadmus Art: LNO10734 Ed. Ref. No.: LO-17-0061.R1 Date: 12-October-17 Stage: Page: 4

set of animals from the incubations without added EhV, exposed these animals to incubation water from the +EhV treatment, then washed three times and processed for DNA extraction and qPCR. These gut content controls demonstrated only low-level stochastic EhV signal (Supporting Information Fig. S1B), thus supporting our assumption that measurements of gut content EhV signal represents ingestion of viral particles.

Combined house-trapping/ingestion experiment

House-trapping and ingestion by Day 1 and 2 animals could not be determined separately by the above methods because the animals were too small to separate from their houses. For these developmental stages, animal-houses were collected at the end of the incubation period, anesthetized with MS-222, and rinsed three times with 0.2-μm-filtered seawater. The samples were then processed for DNA extraction and qPCR. A 10-μL-sample of rinse water from the last rinse step was collected to provide an indication of EhV carryover through the rinsing protocol (Supporting Information Fig. S1A).

Faecal pellet assessment

To determine if viral DNA was present in *O. dioica* faecal pellets, faeces from overnight incubations of Day 3, 4, and 5 animals were collected. Pellets from the three biological replicates per developmental stage were pooled in 1.5-mL Eppendorf tubes (*see* Table 1 for numbers of faecal pellets), centrifuged to remove excess water, and rinsed three times in 0.2-μm-filtered seawater. The pellets were then processed for DNA extraction and qPCR.

Sample analysis

For FCM, appropriate dilutions of glutaraldehyde-fixed water samples were prepared and counted on a FACS-Calibur flow cytometer as described previously (Larsen et al. 2004; Vardi et al. 2012). For qPCR, EhV particles in water, houses, animals, and faecal pellet samples were lysed by adding 180 μL of 56°C Buffer ATL (Qiagen, Hamburg, Germany) and 20 μL of 10 mg mL^{-1} Proteinase K (Qiagen) and incubating overnight at 56°C. Lysed samples were stored at −20°C until analysis. DNA was extracted using a QIAsymphony SP instrument (Qiagen) with the DSP DNA Mini kit (Qiagen) and Tissue_LC_200_V7_DSP protocol (Qiagen). This protocol utilizes alkaline lysis of cells and particles followed by successive washes with chaotropic salts while DNA molecules are immobilized on magnetic beads. Purified DNA was eluted in 50 μL and stored at −20°C until qPCR analysis (see below).

Quantitative PCR

The major capsid protein (*mcp*) gene from EhV was targeted for qPCR detection according to Pagarete et al. (2009). The best dilution level was assessed by serially diluting and testing a few samples. Results from these tests indicated that a 10-fold dilution of template DNA represented the best compromise between PCR efficiency and sensitivity (data not shown). Tenfold dilutions of all DNA samples were therefore prepared in 10 mM Tris-Cl pH 8.0 buffer using a Hamilton Microlab STARlet automated liquid handling platform. Primers targeting the *mcp* gene of EhV (Pagarete et al. 2009) were used for 20-μL qPCR reactions containing 1X SsoAdvanced Universal SYBR Green supermix (Bio-Rad, Carlsbad, California), 5 μL of 10-fold diluted template, and 10 pmol of each primer. All qPCR reactions were set up using a Hamilton Microlab STARlet automated liquid handling platform and run on a CFX96 Real-Time PCR Detection System (Bio-Rad). Cycling parameters consisted of an initial 95°C denaturation for 2 min followed by 40 cycles of 95°C for 5 s followed by 60°C for 10 s with a plate read after every cycle. Melt curve analysis from 65–95°C in 0.3°C increments for 0.3 s holds followed by a plate read after every increment was performed to ensure qPCR specificity. qPCR reactions always generated only a single expected product peak (data not shown). A 10-fold dilution series of a cloned qPCR product from EhV-99B1 lysate (full-length *mcp* gene GenBank accession number EU006629) was used to generate a standard curve for every qPCR plate.

Statistical analysis and clearance rate calculation

We ran hierarchical linear models with normal errors under Bayesian inference on the FCM and qPCR data (using R-package "rjags"; Plummer 2016) to estimate the mean number of virus-like particles per mL (FCM data) or gene copies per mL (qPCR data) for every treatment at both experimental time steps, i.e., at start and end ("means parameterization" sensu Kéry 2010). This analysis is analogous to "analysis of variance (ANOVA) without intercept." To account for the hierarchical structure of the data, i.e., technical replicates for each biological replicate, the models also included "biological replicate" as a random effect. The models were performed separately for each developmental stage as the number of *O. dioica* individuals varied. Instead of single parameter estimates, models under Bayesian inference yield posterior distributions for the specified parameters, in our case a distribution for the mean number of virus-like particles (VLP) per mL (FCM) and gene copies per mL (qPCR) in every treatment at both start and end of the experiment. Using the posterior distributions also yields distributions for EhV removal and clearance rates, and permits a simple derivation of uncertainty as follows:

1. In every treatment, the posterior for the start of the experiment was subtracted from the posterior for the end of the experiment giving a distribution for the mean change during the experiment; this difference usually is a negative value due to virus decay and/or EhV clearance/removal by *O. dioica* feeding.
2. To separate the effective *O. dioica* clearance/removal of experimentally-added EhV at each developmental stage from other background processes such as virus decay of experimentally added EhV, virus decay of naturally-

ID: jwaa3b2server Time: 18:56 I Path: //chenas03/Cenpro/ApplicationFiles/Journals/Wiley/LNO#/Vol00000/170190/Comp/APPFile/JW-LNO#170190

J_ID: LNO Customer A_ID: LNO10734 Cadmus Art: LNO10734 Ed. Ref. No.: LO-17-0061.R1 Date: 12-October-17 Stage: Page: 5

occurring EhV, and *O. dioica* clearance/removal of naturally-occurring EhV, we subtracted the differences in the "EhV only" treatments and the "*O. dioica* only" treatments from the differences the "EhV + *O. dioica*" treatments.

3. Since both of the single treatments (*O. dioica* only and EhV only) include the decay of background EhV particles in incubation seawater, we added the posterior for the control treatment (neither EhV nor *O. dioica* added, showing virus decay of natural EhV only) to avoid removing that effect twice.
4. From these removal rates (in particles/gene copies per mL), we then calculated daily clearance rates (in mL cleared per animal and day) for water with a starting concentration of 10^6 EhV mL^{-1} by accounting for animal concentration in the experimental water and for the experiment duration.

From the obtained distributions of removal and clearance rate, we then assessed the certainty of the mean being different from zero by calculating the ratio of values below zero. Negative means of removal/clearance rates with ratios > 0.95 and positive means of removal/clearance rates with ratios < 0.05 were considered significantly different from zero. This corresponds to a significance level of $p < 0.05$ in frequentist statistics, e.g., ANOVA (see above). Given the "repeated measurements" at the start and end of each treatment, this constitutes a conservative significance level. Further details about the parametrization of the Bayesian analysis are documented in the Supporting Information.

Results and discussion

We conducted feeding experiments to investigate potential trophic interactions between the pelagic tunicate, *O. dioica* (Tunicata, Appendicularia), and a naturally co-occurring marine microalgal virus, *E. huxleyi* virus (EhV) (Castberg et al. 2002). Clearance of EhV by *O. dioica* occurred in all experiments, but varied according to detection method, incubation period length, and animal age. Using FCM to enumerate EhV in incubation water, we observed significant EhV removal by Day 4 and 5 animals in 2.5 h incubations, and by Day 2 through 5 animals in overnight incubations (Fig. 1). Day 4 animals netted the highest removal with a reduction of $\sim 4 \times 10^5$ EhV mL^{-1} by 0.08 animals mL^{-1}, or $\sim 7 \times 10^5$ EhV mL^{-1} by 0.04 animals mL^{-1} in the 2.5 h and overnight incubations, respectively (Fig. 1). This constitutes the removal of approximately half of the $\sim 10^6$ EhV mL^{-1} starting abundance, which is a typical post-*E. huxleyi* bloom abundance in situ (Bratbak et al. 1995).

In contrast, qPCR of the EhV major capsid protein (*mcp*) gene did not reveal significant removal of EhV during 2.5 h incubations (Fig. 1). In overnight incubations, however, qPCR detected a $\sim$ 20-fold reduction in *mcp* gene copies by Day 3 and 4 animals, which were at densities of 0.06 animals mL^{-1} and 0.04 animals mL^{-1}, respectively (Fig. 1). The Day 5 overnight incubation of *O. dioica* with EhV contained $\sim$ 10-fold lower EhV inoculum than expected ($\sim 10^5$ VLP mL^{-1} or $\sim 2 \times 10^5$ *mcp* gene copies mL^{-1}), resulting in undetectable EhV loss. Because all +EhV treatments were inoculated identically from the same virus lysate, we suspect this unusual data point is due to incomplete mixing before sampling at T_0 and is therefore not representative of removal by Day 5 animals. Both FCM and qPCR (Fig. 1) measurements demonstrated that cumulative EhV clearance in the overnight incubations increased with increasing *O. dioica* age.

Using a starting abundance of 10^6 EhV particles mL^{-1}, we calculated clearance rate (mL ind^{-1} d^{-1}; Deibel 1988) intervals from model-generated posterior clearance distributions obtained from the FCM data for each developmental stage of *O. dioica*. Mean clearance rates in the 2.5 h incubations ranged from $\sim$ 2 mL ind^{-1} d^{-1} to 50 mL ind^{-1} d^{-1}, and increased with *O. dioica* age (Fig. 2). Overnight incubations provided lower estimates, ranging from negative clearance rates for Day 1 animals to $\sim$ 45 mL ind^{-1} d^{-1} for Day 5 animals (Fig. 2). These estimates are in agreement with published clearance rates for the microalga *I. galbana* (Acuña and Kiefer 2000) by *O. dioica* cultured at 13.5°C (Bedo et al. 1993) or 15°C (Broms and Tiselius 2003; Troedsson et al. 2007). This suggests the efficiency of EhV removal by *O. dioica* is comparable to that of larger food particles, and implies the potential for a significant impact of oikopleurid feeding on planktonic viral abundances.

Clearance rates estimated from FCM data were higher for 2.5 h incubations relative to overnight incubations (Fig. 2). We suggest this is due to decreasing EhV titers during *O. dioica* feeding, which would be more pronounced in the overnight incubations. However, the longer incubation period in the overnight experiments resulted in higher total removal of EhV particles by *O. dioica*, as incubation water was "recycled" by *O. dioica* feeding (Fig. 2). Furthermore, virus removal was more pronounced when measured by FCM than qPCR (Fig. 1), possibly because qPCR detects viral DNA rather than intact particles. Animals regularly defecate during feeding (gut passage time < 10 min; Bedo et al. 1993; López-Urrutia and Acuña 1999) potentially releasing free EhV DNA from digested particles that would be measured by qPCR and not FCM. This may also explain why we were able to detect significant clearance by qPCR in overnight incubations, as free DNA would have considerable time to degrade (15.5–21 h, Table 1), whereas we did not detect clearance in 2.5 h incubations.

Previous studies have revealed that 0.2 μm synthetic particles are readily trapped by filter-feeding houses, ingested by animals, and incorporated into *O. dioica* faecal pellets (Bedo et al. 1993; Fernández et al. 2004). We therefore wished to determine whether EhV particles pass through the *O. dioica* digestive system. We used qPCR to determine the fate of EhV DNA in three different *O. dioica* biological

ID: jwaa3b2server Time: 18:56 I Path: //chenas03/Cenpro/ApplicationFiles/Journals/Wiley/LNO#/Vol00000/170190/Comp/APPFile/JW-LNO#170190

J_ID: LNO Customer A_ID: LNO10734 Cadmus Art: LNO10734 Ed. Ref. No.: LO-17-0061.R1 Date: 12-October-17 Stage: Page: 6

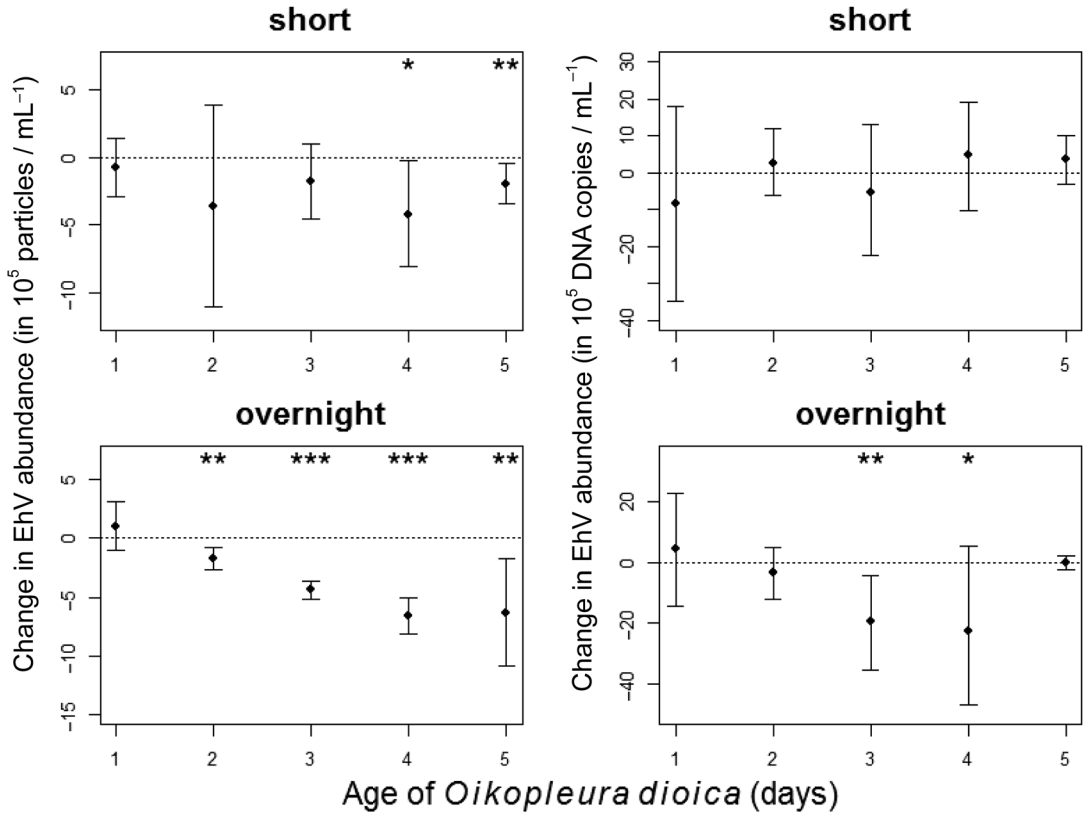

Fig. 1. Mean cumulative EhV removal of *O. dioica* measured by FCM (left panels) and qPCR (right panels) in 2.5 h (upper panels) and overnight (lower panels) incubations. "Change in EhV abundance" reflects the number of particles or gene copies removed per mL by all animals during the incubation period. The number of animals per 1 L (2.5 h) or 2 L (overnight) incubation were: Day 1: 150 animals, Day 2: 150 animals, Day 3: 120 animals, Day 4: 80 animals, Day 5: 40 animals. Error bars show the 95% credible (Bayesian counterpart to confidence) intervals (0.025 and 0.975 quantiles) and stars indicate the level of certainty for a mean being different from zero (* > 95%, ** > 99%, *** > 99.9%).

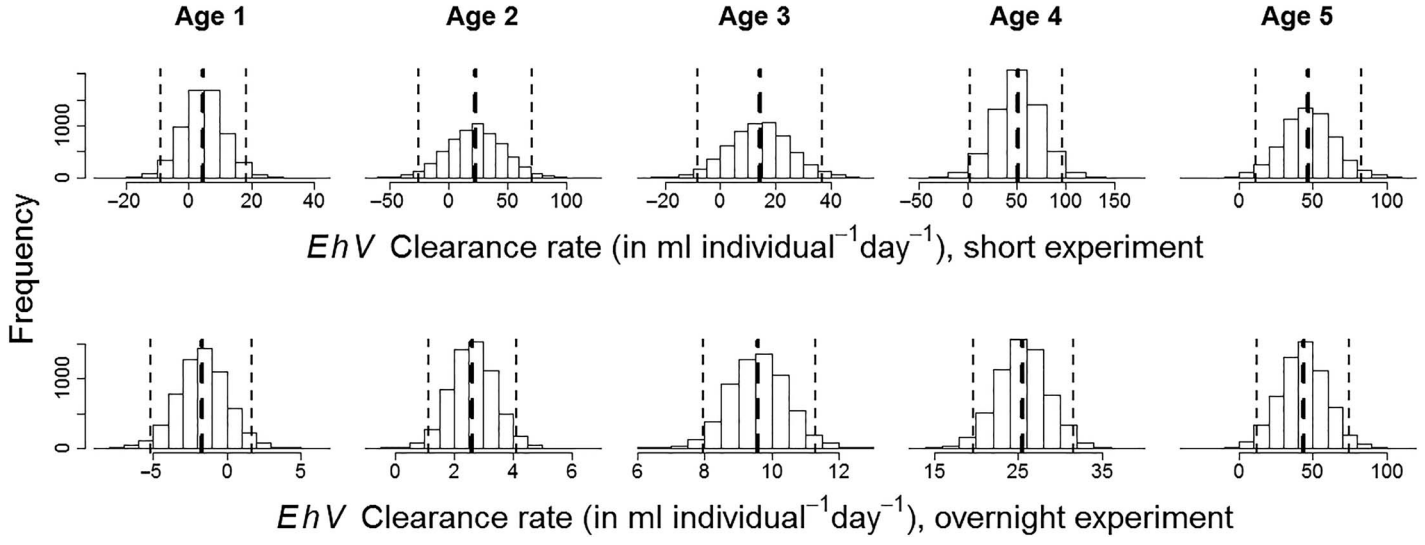

Fig. 2. Clearance rate estimates (mL ind^{-1} d^{-1}) for different developmental stages of *O. dioica* during 15°C feeding incubations. Histograms show posterior distributions for clearance rates based on FCM counts of EhV removal during 2.5 h (top row) and overnight (bottom row) incubations. Thick vertical dashed lines indicate the arithmetic clearance rate mean, while thin vertical dashed lines delimit the 95% credible intervals.

ID: jwaa3b2server Time: 18:56 I Path: //chenas03/Cenpro/ApplicationFiles/Journals/Wiley/LNO#/Vol00000/170190/Comp/APPFile/JW-LNO#170190

J_ID: LNO Customer A_ID: LNO10734 Cadmus Art: LNO10734 Ed. Ref. No.: LO-17-0061.R1 Date: 12-October-17 Stage: Page: 7

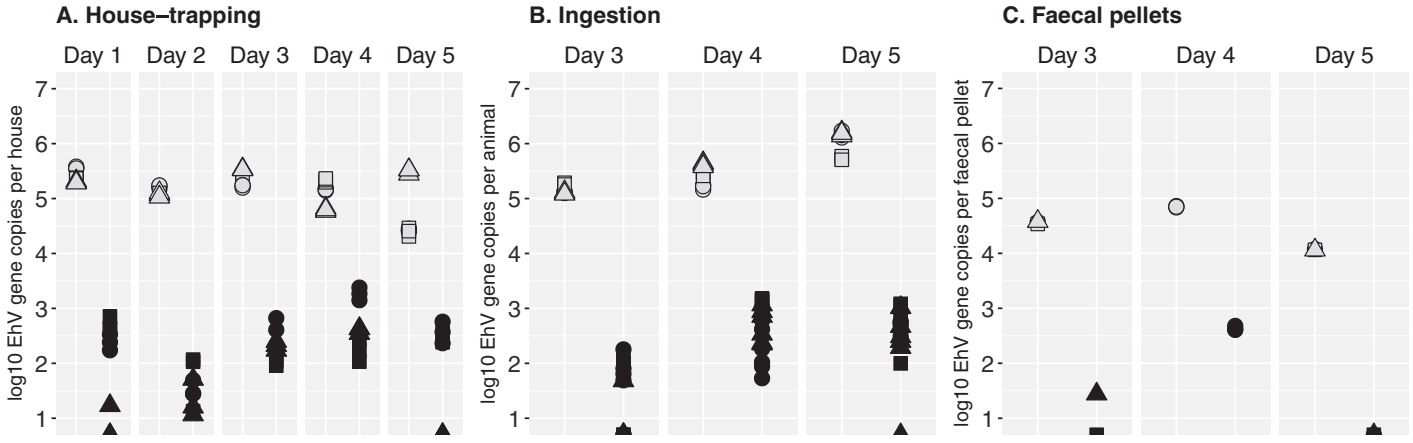

Fig. 3. Detection of EhV DNA in *O. dioica* after feeding incubations. (**A**) House-trapped after 2.5 h incubations, (**B**) ingested after 7 min incubations, and (**C**) incorporated in faecal pellets during overnight incubations. Figures show EhV *mcp* gene copies per house, per animal, and per faecal pellet, respectively, in the presence (gray symbols) or absence (black symbols) of EhV inoculum ($\sim 10^6$ EhV mL^{-1}). Ingestion results for Day 1 and Day 2 developmental stages of *O. dioica* are shown as composite house-trapping and ingestion of EhV in animal-houses (**A**), as the small size of *O. dioica* at these early stages hindered separate sampling of animals and houses. Symbol shapes distinguish biological replicates for each treatment ($n = 3$). Note logarithmic *y*-axis.

compartments: house-trapping, ingestion/digestion, and defecation as follows.

House-trapping

Results from 2.5 h incubations of Day 1–2 animal-houses and Day 3–4 houses revealed $\sim 10^5$ EhV *mcp* gene copies ind^{-1} (Fig. 3A). Abundance of house-trapped viruses by Day 5 animals exhibited higher variability between biological replicates, ranging from approximately 10^4 to 10^5 EhV *mcp* gene copies $house^{-1}$ (Fig. 3A). We also detected EhV *mcp* genes in non-virus-exposed houses, although these quantities were 2–3 orders of magnitude lower than in virus-exposed houses (Fig. 3A). Viral DNA associated with houses may derive from EhV particles that were trapped as a consequence of animal feeding, or represent virus particles that randomly adhered to houses. To understand the impacts of appendicularians on the fate of virioplankton, both possibilities are relevant. It should be noted that our results cannot distinguish between direct trapping of EhV particles and the indirect trapping of EhV particles randomly adsorbed to algal food particles, therefore the house-trapping results shown in Fig. 3 have not been corrected to account for the presence of EhV *mcp* gene copies in the wash water (Supporting Information Fig. S1A). However, as we included three consecutive wash steps to remove virus particles not trapped within the food-concentrating filter, our methods likely underestimate the total number of EhV particles trapped during *O. dioica* feeding.

Ingestion

To track ingestion of virus particles, we quantified EhV *mcp* gene copies within *O. dioica* that had been exposed to EhV for 7 min during feeding. This short incubation time is necessary to maximize EhV detection as *O. dioica* has a gut passage time < 10 min (Bedo et al. 1993; López-Urrutia and Acuña 1999). In animal-houses (Day 1 and 2) or animals alone (Day 3, 4, and 5), we could detect $> 10^5$ EhV *mcp* gene copies ind^{-1} (Fig. 3B), with a 10-fold increase occurring from Day 3 to 5 (10^5–10^6 copies $animal^{-1}$) (Fig. 3B). Background qPCR signal for non-virus-exposed animal-houses or animals was $< 10^3$ *mcp* gene copies $animal^{-1}$ for all developmental stages (Fig. 3B). These results indicate that either intact EhV particles or free EhV DNA was ingested by *O. dioica*. Our analysis is based on the assumption that EhV particles not removed by three consecutive washes represent ingested virus. Animals were anaesthetized during collection to reduce digestion and gut passage (Troedsson et al. 2007), and only occasional faecal pellets were observed. Faecal pellets were not included in the analysis, and therefore result in a potential underestimation of viral ingestion.

Faecalia

To assess the presence of EhV DNA in *O. dioica* faecal pellets, we collected and pooled sedimented faecalia from overnight incubations. EhV DNA was detected in thrice-washed pools from Day 3, 4, and 5 animals (Fig. 3C), with each faecal pellet containing approximately $1–7 \times 10^4$ *mcp* gene copies. Viral DNA in faeces from non-virus-exposed overnight incubations ranged from ~ 500 *mcp* copies $pellet^{-1}$ (Day 4) to below detection limits (Day 3 and 5) (Fig. 3C).

Ecological implications

In summary, our results demonstrate that *O. dioica* efficiently clears EhV viruses from seawater, with clearance rates similar to those reported for feeding on algae or sub-micron fluorescent microspheres (Bedo et al. 1993; Acuña and Kiefer 2000; Broms and Tiselius 2003; Fernández et al. 2004; Sato et al. 2005; Troedsson et al. 2007). EhV DNA was readily

ID: jwaa3b2server Time: 18:56 I Path: //chenas03/Cenpro/ApplicationFiles/Journals/Wiley/LNO#/Vol00000/170190/Comp/APPFile/JW-LNO#170190

J_ID: LNO Customer A_ID: LNO10734 Cadmus Art: LNO10734 Ed. Ref. No.: LO-17-0061.R1 Date: 12-October-17 Stage: Page: 8

detectable in houses, *O. dioica* animals, and faecal pellets, further indicative that EhV may be dispersed in discarded appendicularian houses and/or faecal pellets. This is the first empirical demonstration of the capture and ingestion of a biological nanoparticle, a marine algal virus, by an appendicularian. The discovery of a trophic link between *O. dioica* and EhV raises a number of questions about the diversity, magnitude and consequences of appendicularian-virus interactions on processes in the ocean, and the fates of these cleared virus particles are currently unknown but have the potential to impart significant impacts on viral trajectories in marine ecosystems.

Fate of house-trapped virus particles

Appendicularians regularly discard filter-feeding houses (every 4 h at 15°C; Fernández et al. 2004) in order to maintain high filtration efficiency (Sato et al. 2001). Up to 30% of material trapped in appendicularian houses is discarded together with filter-feeding houses (Gorsky and Fenaux 1998), thus providing a mechanism by which trapped viruses may be vertically dispersed during house sinking (Flood et al. 1998). Discarded houses sink at rates of up to 120 m d^{-1} (Hansen et al. 1996; Robison et al. 2005), potentially resulting in the deposition of high abundances of house-trapped viruses in shallow coastal sediments. Resuspension, such as during winter and spring mixing (Garstecki et al. 2002), could lead to persistence and/or dispersal of sedimented viruses (Lawrence et al. 2002). Alternatively, burial would result in virus loss and a previously unaccounted for component of downward carbon transport.

Fate of ingested virus particles

We do not know if trapped and ingested virus particles are digested during gut passage through *O. dioica*. As shown by Frada et al. (2014), EhV particles indirectly consumed by copepods grazing on EhV-infected *E. huxleyi* survived gut passage and retained infectivity. As the gut passage time of *O. dioica* is significantly shorter (< 10 min; Bedo et al. 1993; López-Urrutia and Acuña 1999) than that of *Calanus* copepods (Nejstgaard et al. 2003), we anticipate some viruses would retain infectivity. This may present a novel dispersal mechanism for infectious viruses as *O. dioica* faecal pellets sink at rapid rates (Dagg and Brown 2005). Burial in shallow sediments may then result in either resuspension or loss, as described above. In addition, as viruses and their potential hosts co-occur and therefore may be co-trapped by *O. dioica*, it will also be interesting to determine if *O. dioica* feeding increases host-virus contact rates, thus altering rates of infection.

Impact on virus abundance and diversity

According to Uye and Ichino (1995), *O. dioica* can reach densities of 53 individuals L^{-1}. Assuming post-bloom virus titers of 10^6 EhV mL^{-1} (Bratbak et al. 1995) and applying an average population-wide clearance rate of 20 mL ind^{-1} d^{-1} for EhV, an actively feeding *O. dioica* patch could remove up to 10^6 EhV mL^{-1} d^{-1}. This suggests the presence/absence of *O. dioica* populations have significant, but currently overlooked, impacts on the transmission of EhV and thus the dynamics of *E. huxleyi* populations. The implications for other viruses and the hosts they infect are unknown. Our study demonstrated ingestion of a large, enveloped, icosahedral algal virus, and the ability of appendicularians to clear other viruses warrants further investigation.

Impact on appendicularian nutrition

Assuming a carbon content of 0.055–0.2 fg C per virus particle (Jover et al. 2014, and references therein) and starting virus titer of approximately 10^6 EhV mL^{-1}, younger stages of *O. dioica* can clear 0.1–0.4 ng EhV C d^{-1}, and older animals 2.75–10 ng EhV C d^{-1}. This represents a novel trophic linkage between the microbial loop and the classical marine food web, and adds to the growing body of evidence that gelatinous zooplankton may derive nutrition from the sub-micron particle range (Bedo et al. 1993; Fernández et al. 2004; Sutherland et al. 2010). During our experiments, *O. dioica* was presented with a standard food regime to promote normal feeding activity (Bouquet et al. 2009), thus ensuring that nutritional requirements of *O. dioica* were met independent of EhV. Further experiments are required to assess whether viruses alone are able to stimulate appendicularian feeding or contribute to appendicularian nutrition.

References

Acuña, J. L., and M. Kiefer. 2000. Functional response of the appendicularian *Oikopleura dioica*. Limnol. Oceanogr. **45**: 608–618. doi:10.4319/lo.2000.45.3.0608

Alldredge, A. L. 1981. The impact of appendicularian grazing on natural food concentrations in situ. Limnol. Oceanogr. **26**: 247–257. doi:10.4319/lo.1981.26.2.0247

Alldredge, A. L. 2005. The contribution of discarded appendicularian houses to the flux of particulate organic carbon from oceanic surface waters, p. 309–326. *In* G. Gorsky, M. J. Youngbluth, and D. Deibel [eds.], Response of marine ecosystems to global change: Ecological impact of appendicularians. Éditions Scientifiques. doi:10.1093/plankt/fbi133

Arslan, D. L., M. Legendre, V. Seltzer, C. Abergel, and J.-M. Claverie. 2011. Distant Mimivirus relative with a larger genome highlights the fundamental features of Megaviridae. Proc. Nat. Acad. Sci. USA. **108**: 17486–17491. doi:10.1073/pnas.1110889108

Avrani, S., O. Wurtzel, I. Sharon, R. Sorek, and D. Lindell. 2011. Genomic island variability facilitates Prochlorococcus-virus coexistence. Nature **474**: 604–608. doi:10.1038/nature10172

Avrani, S., D. A. Schwartz, and D. Lindell. 2012. Virus-host swinging party in the oceans: Incorporating biological complexity into paradigms of antagonistic coexistence. Mob. Genet. Elem. **2**: 88–95. doi:10.4161/mge.20031

ID: jwaa3b2server Time: 18:56 I Path: //chenas03/Cenpro/ApplicationFiles/Journals/Wiley/LNO#/Vol00000/170190/Comp/APPFile/JW-LNO#170190

Bedo, A. W., J. L. Acuña, D. Robins, and R. P. Harris. 1993. Grazing in the micron and the sub-micron particle size range: The case of *Oikopleura dioica* (Appendicularia). Bull. Mar. Sci. **53**: 2–14.

Bergh, O., K. Y. Børsheim, G. Bratbak, and M. Heldal. 1989. High abundance of viruses found in aquatic environments. Nature **340**: 467–468. doi:10.1038/340467a0

Bouquet, J. M., E. Spriet, C. Troedsson, H. Otterå, D. Chourrout, and E. M. Thompson. 2009. Culture optimization for the emergent zooplanktonic model organism *Oikopleura dioica*. J. Plankton Res. **31**: 359–370. doi:10.1093/plankt/fbn132

Bratbak, G., T. F. Thingstad, and M. Heldal. 1994. Viruses and the microbial loop. Microb. Ecol. **28**: 209–221. doi:10.1007/BF00166811

Bratbak, G., M. Levasseur, S. Michaud, G. Cantin, E. Fernández, B. R. Heimdal, and M. Heldal. 1995. Viral activity in relation to *Emiliania huxleyi* blooms: A mechanism of DMSP release? Mar. Ecol. Prog. Ser. **128**: 133–142. doi:10.3354/meps128133

Broms, F., and P. Tiselius. 2003. Effects of temperature and body size on the clearance rate of *Oikopleura dioica*. J. Plankton Res. **25**: 573–577. doi:10.1093/plankt/25.5.573

Castberg, T., R. Thyrhaug, A. Larsen, R. A. Sandaa, M. Heldal, J. L. Van Etten, and B. Bratbak. 2002. Isolation and characterization of a virus that infects *Emiliania huxleyi* (Haptophyta). J. Phycol. **38**: 767–774. doi:10.1046/j.1529-8817.2002.02015.x

Dagg, M. J., and S. L. Brown. 2005. The potential contribution of fecal pellets from the larvacean *Oikopleura dioica* to vertical flux of carbon in a river dominated coastal margin, p. 293–307. *In* G. Gorsky, M. J. Youngbluth, and D. Deibel [eds.], Response of marine ecosystems to global change: Ecological impact of appendicularians. Éditions Scientifiques.

Deibel, D. 1988. Filter feeding by *Oikopleura vanhoeffeni*: Grazing impact on suspended particles in cold ocean waters. Mar. Biol. **99**: 177–186. doi:10.1007/BF00391979

Fernández, D., A. López-Urrutia, J. L. Acuña, and R. Harris. 2004. Retention efficiency of 0.2 to 0.6 μm particles by the appendicularians *Oikopleura dioica* and *Fritillaria borealis*. Mar. Ecol. Prog. Ser. **266**: 89–101. doi:10.3354/meps266089

Flood, P. R. 1978. Filter characteristics of appendicularian food catching nets. Experientia **34**: 173–175. doi:10.1007/BF01944659

Flood, P. R., D. Deibel, and C. C. Morris. 1992. Filtration of colloidal melanin from sea water by planktonic tunicates. Nature **355**: 630–632. doi:10.1038/355630a0

Flood, P. R., D. Deibel, and C. Morris. 1998. The appendicularian house, p. 105–124. *In* Q. Bone [ed.], The biology of pelagic tunicates. Oxford Univ. Press.

Frada, M. J., D. Schatz, V. Farstey, J. E. Ossolinski, H. Sabanay, S. Ben-Dor, I. Koren, and A. Vardi. 2014. Zooplankton may serve as transmission vectors for viruses infecting algal blooms in the ocean. Curr. Biol. **24**: 2592–2597. doi:10.1016/j.cub.2014.09.031

Garstecki, T., S. A. Wickham, and H. Arndt. 2002. Effects of experimental sediment resuspension on a coastal planktonic microbial food web. Estuar. Coast. Shelf Sci. **55**: 751–762. doi:10.1006/ecss.2001.0937

Gorsky, G., and R. Fenaux. 1998. The role of Appendicularia in marine food webs, p. 161–169. *In* Q. Bone [ed.], The biology of pelagic tunicates. Oxford Univ. Press.

Gorsky, G., M. J. Chrétiennot-Dinet, J. Blanchot, and I. Palazzoli. 1999. Picoplankton and nanoplankton aggregation by appendicularians: Fecal pellet contents of *Megalocercus huxleyi* in the equatorial Pacific. J. Geophys. Res. Oceans **104**: 3381–3390. doi:10.1029/98JC01850

Hansen, J. L. S., T. Kiørboe, and A. L. Alldredge. 1996. Marine snow derived from abandoned larvacean houses: Sinking rates, particle content and mechanisms of aggregate formation. Mar. Ecol. Prog. Ser. **141**: 205–215. doi:10.3354/meps141205

Jover, L. F., T. C. Effler, A. Cuhan, S. W. Wilhelm, and J. S. Weitz. 2014. The elemental composition of virus particles: Implications for marine biogeochemical cycles. Nat. Rev. Microbiol. **12**: 519–528. doi:10.1038/nrmicro3289

Kéry, M. 2010. Introduction to WinBUGS for ecologists. Academic Press.

Larsen, A., A. F. F. Gro, R. A. Sandaa, T. Castberg, R. Thyrhaug, S. R. Erga, S. Jacquet, and G. Bratbak. 2004. Spring phytoplankton bloom dynamics in Norwegian coastal waters: Microbial community succession and diversity. Limnol. Oceanogr. **49**: 180–190. doi:10.4319/lo.2004.49.1.0180

Lawrence, J. E., A. M. Chan, and C. A. Suttle. 2002. Viruses causing lysis of the toxic bloom-forming alga *Heterosigma akashiwo* (Raphidophyceae) are widespread in coastal sediments of British Columbia, Canada. Limnol. Oceanogr. **47**: 545–550. doi:10.4319/lo.2002.47.2.0545

Lombard, F., E. Selander, and T. Kiørboe. 2011. Active prey rejection in the filter?feeding appendicularian *Oikopleura dioica*. Limnol. Oceanogr. **56**: 1504–1512. doi:10.4319/lo.2011.56.4.1504

López-Urrutia, A., and J. L. Acuña. 1999. Gut throughput dynamics in the appendicularian *Oikopleura dioica*. Mar. Ecol. Prog. Ser. **191**: 195–205. doi:10.3354/meps191195

Nagasaki, K. 2008. Dinoflagellates, diatoms, and their viruses. J. Microbiol. **46**: 235–243. doi:10.1007/s12275-008-0098-y

Nejstgaard, J. C., M. E. Frischer, C. L. Raule, R. Gruebel, K. E. Kohlberg, and P. G. Verity. 2003. Molecular detection of algal prey in copepod guts and faecal pellets. Limnol. Oceanogr.: Methods **1**: 29–38. doi:10.4319/lom.2003.1.29

Pagarete, A., M. J. Allen, W. H. Wilson, S. A. Kimmance, and C. D. Vargas. 2009. Host–virus shift of the sphingolipid pathway along an *Emiliania huxleyi* bloom: Survival of the fattest. Environ. Microbiol. **11**: 2840–2848. doi:10.1111/j.1462-2920.2009.02006.x

Plummer, M. 2016. rjags: Bayesian graphical models using MCMC. R package version 4–6. Available from http://CRAN.R-project.org/package=rjags

Raoult, D., B. La Scola, and R. Birtles. 2007. The discovery and characterization of Mimivirus, the largest known virus and putative pneumonia agent. Clin. Infect. Dis. **45**: 95–102. doi:10.1086/518608

Robison, B. H., K. R. Reisenbichler, and R. E. Sherlock. 2005. Giant larvacean houses: Rapid carbon transport to the deep sea floor. Science **308**: 1609–1611. doi:10.1126/science.1109104

Rohwer, F., and R. V. Thurber. 2009. Viruses manipulate the marine environment. Nature **459**: 207–212. doi:10.1038/nature08060

Sato, R., Y. Tanaka, and T. Ishimaru. 2001. House production by *Oikopleura dioica* (Tunicata, Appendicularia) under laboratory conditions. J. Plankton Res. **23**: 415–423. doi:10.1093/plankt/23.4.415

Sato, R., Y. Tanaka, and T. Ishimaru. 2005. Clearance and ingestion rates of three appendicularian species, *Oikopleura longicauda*, *O. rufscens* and *O. fusiformis*, p. 227–254. *In* G. Gorsky, M. J. Youngbluth, and D. Deibel [eds.], Response of marine ecosystems to global change: Ecological impact of appendicularians. Éditions Scientifiques.

Scheinberg, R. D., M. R. Landry, and A. Calbet. 2005. Grazing of two common appendicularians on the natural prey assemblage of a tropical coastal ecosystem. Mar. Ecol. Prog. Ser. **294**: 201–212. doi:10.3354/meps294201

Sutherland, K. R., L. P. Madin, and R. Stocker. 2010. Filtration of submicrometer particles by pelagic tunicates. Proc. Natl. Acad. Sci. USA. **107**: 15129–15134. doi:10.1073/pnas.1003599107

Troedsson, C., M. E. Frischer, J. C. Nejstgaard, and E. M. Thompson. 2007. Molecular quantification of differential ingestion and particle trapping rates by the appendicularian *Oikopleura dioica* as a function of prey size and shape. Limnol. Oceanogr. **52**: 416–427. doi:10.4319/lo.2007.52.1.0416

Uye, S., and S. Ichino. 1995. Seasonal variations in abundance, size composition, biomass and production rate of *Oikopleura dioica* (Fol) (Tunicata: Appendicularia) in a temperate eutrophic inlet. J. Exp. Mar. Biol. Ecol. **189**: 1–11. doi:10.1016/0022-0981(95)00004-B

Vardi, A., L. Haramaty, B. A. S. Van Mooy, H. F. Fredricks, S. A. Kimmance, A. Larsen, and K. D. Bidle. 2012. Host–virus dynamics and subcellular controls of cell fate in a natural coccolithophore population. Proc. Natl. Acad. Sci. USA. **109**: 19327–19332. doi:10.1073/pnas.1208895109

Wilhelm, S. W., and C. A. Suttle. 1999. Viruses and nutrient cycles in the sea: Viruses play critical roles in the structure and function of aquatic food webs. BioScience **49**: 781–788. doi:10.2307/1313569

Wilson, W. H., D. C. Schroeder, M. J. Allen, M. T. Holden, J. Parkhill, B. G. Barrell, C. Churcher, N. Hamlin, K. Mungall, H. Norbertczak, and M. A. Quail. 2005. Complete genome sequence and lytic phase transcription profile of a Coccolithovirus. Science **309**: 1090–1092. doi:10.1126/science.1113109

Acknowledgments

Virus consumption by appendicularians was first proposed to us by Don Deibel, and we are indebted to him for his curiosity, insight, and inspiration. We also thank Anne Aasjord and Anders Olsen at the Sars Centre Appendicularian Facility for assistance with *O. dioica* and phytoplankton cultures. Funds for sample analysis and travel were provided by an NSERC grant to JL. Additional funding was provided by the Research Council of Norway project entitled "Processes and players in Arctic marine pelagic food webs - biogeochemistry, environment and climate change" (MicroPolar, project number 225956/E10).

Conflict of Interest

None declared.

Submitted 16 February 2017
Revised 14 July 2017
Accepted 14 September 2017

Associate editor: Leila Hamdan