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Widespread detection of circular replication initiator protein (*rep*)-encoding ssDNA viral genomes in estuarine, coastal and open ocean net plankton

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ABSTRACT: Circular replication initiator protein (*rep*)-encoding ssDNA (CRESS-DNA) viruses have been widely reported in viral metagenomic surveys and in association with invertebrate zooplankton in freshwater and marine habitats. However, there have been no systematic or quantitative studies of their distribution in marine waters. We investigated the distribution of CRESS-DNA viruses in net plankton (>64 µm) collected from geographically widespread locations, using quantitative PCR that targets viral genotypes previously recovered from soil, freshwater and estuarine free-living viruses, and viruses associated with arthropod tissues. We detected CRESS-DNA viruses in most net plankton samples except for a sample containing only the cyanobacterium *Trichodesmium*. Soil and freshwater plankton CRESS-DNA viruses were detected only at sites with substantial freshwater and runoff effects, while 2 CRESS-DNA viruses recovered from plankton of the Chesapeake Bay were detected in most net plankton tested. CRESS-DNA viruses recovered from marine copepods, the freshwater cladoceran *Daphnia* and the freshwater amphipod *Diporeia* were primarily detected in habitats where similar hosts were observed in zooplankton counts. Our data suggest that CRESS-DNA viruses previously recovered from invertebrate tissues and from virioplankton may be widely distributed in plankton >64 µm, providing evidence for a zooplankton origin of this viral group.

KEY WORDS: Virioplankton · Circovirus · Zooplankton · Copepods · Amphipods · *Daphnia*

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INTRODUCTION

Viruses are the most abundant biological entities on the planet, typically comprising 10^6 to 10^8 virus-like particles per milliliter of fresh or seawater (Proctor et al. 1988, Bergh et al. 1989). Viral lysis is a significant component of mortality across marine habitats (Weinbauer 2004), where viruses infect diverse organisms from bacteria to whales (Suttle 2005). Their activities strongly influence biogeochemical cycles in marine plankton, releasing dissolved organic matter (DOM) from lysed cells, and thereby causing enhanced growth rates of co-occurring microorganisms (Middelboe et al. 1996, Middelboe & Lyck 2002). Viral communities in the oceans are incredibly diverse, comprising many thousands of viral genotypes per liter (Breitbart et al. 2002, 2004). Most viruses are believed to be bacteriophage or viruses infecting eukaryotic microorganisms (Weinbauer 2004). Recently, viral metagenomic studies have elucidated a wide diversity of circular, replication initiator protein (*rep*)-encoding single-stranded DNA (CRESS-DNA) viruses, which have only been found infecting eukaryotic hosts, across a range of habitats and associated with tissues of vertebrates and invertebrates (Rosario et al. 2009, 2012b, Dayaram et al. 2013a,b,c). However, the environmental distribution and ecological significance of CRESS-DNA viruses in estuarine and marine plankton remains poorly understood.

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Eukaryotic viruses with circular ssDNA genomes are well studied in vertebrate hosts, where they cause wasting disease in pigs (porcine circovirus 1 and 2; Tischer et al. 1982), and disease in chickens and other avian taxa (Todd et al. 1990). CRESS-DNA viruses, which include circoviruses, cycloviruses, and other taxa with multipartite genomes (e.g. nanoviruses, geminiviruses, mastreviruses) replicate using rolling circle replication (Krakowka et al. 2012). They are characterized by the presence of a conserved genome architecture including a stem loop bearing a nonanucleotide motif and origin of replication. CRESS-DNA viral genomes generally have 2 or more open reading frames (although satellite viruses, nanoviruses and subviral particles may have only 1) encoding for a capsid protein and a replication initiator protein gene (Rosario et al. 2012b). CRESS-DNA viral genes experience high mutation rates (1.2×10^{-3} substitutions site^{-1} yr^{-1} (Duffy & Holmes 2008, Firth et al. 2009, Harkins et al. 2009, Grigoras et al. 2010), resulting in little nucleotide similarity between viruses observed either in culture or from metagenomic surveys. CRESS-DNA viral replication initiation protein genes are more conserved than capsid genes. Two recent surveys discovered CRESS-DNA viral genomes bearing a capsid gene with amino acid similarity with ssRNA viruses, suggesting recombination between CRESS-DNA and ssRNA viruses in ancestral hosts (Diemer & Stedman 2012, Rosario et al. 2012a, Hewson et al. in 2013b).

Environmental CRESS-DNA viruses have been recovered in metagenomes from a freshwater Antarctic lake (López-Bueno et al. 2009), the meromictic Fayetteville Green Lake and soils in upstate New York (Hewson et al. 2012) and the Sargasso Sea and Chesapeake Bay (Rosario et al. 2009). The detection of CRESS-DNA viruses in metagenomes is influenced by the common use of strand displacement amplification, which significantly enriches short, circular DNA over longer linear genome fragments (Kim et al. 2008, Ng et al. 2009). A quantitative study of putative ssDNA virus genotypes in Fayetteville Green Lake and Cayuga Lake indicated very low abundances relative to total virus-like particle abundances, where the genotypes decayed at rates similar to total virioplankton in aquatic habitats (Hewson et al. 2012). These data further confirm that ssDNA viral genotypes do not likely represent a significant fraction of total viral abundance in plankton. Clues of host origin for environmental CRESS-DNA viruses come from study of viral communities associated with invertebrate tissues, notably arthropods. CRESS-DNA viruses have been described in whiteflies (Ng et al. 2011a), mosquitoes (Ng et al. 2011b), and dragonflies (Rosario et al. 2011), and most recently have been described in association with aquatic crustaceans *Labidocera aestiva*, *Acartia tonsa* (Dunlap et al. 2013), *Daphnia mendotae* (Hewson et al. 2013b), and *Diporeia* spp. (Hewson et al. 2013a).

Net plankton (operationally defined here as material collected in the >64 µm size fraction) comprises primarily large inorganic and non-living organic particles, large phytoplankton, zooplankton, and microorganisms associated with these components. The aim of this study was to examine net plankton from several marine habitats for the presence and abundance of CRESS-DNA viruses, using quantitative PCR that targets several previously recovered genotypes from environmental and tissue metagenomes. Here we show that these CRESS-DNA viruses, which share substantial nucleotide identity with CRESS-DNA viral genes that are amplified with our primer sets, are present in net plankton collected from widespread locations.

MATERIALS AND METHODS

Net plankton samples were collected from 12 locations in the Central Atlantic Ocean, East Tropical South Pacific Ocean, Gulf of Maine, and Chesapeake Bay and tributaries, from summer 2009 to spring 2012 (Table 1, Fig. 1). Samples were collected by oblique or vertical net tow using a 30 cm diameter 64 µm nitex plankton net (with the excep-

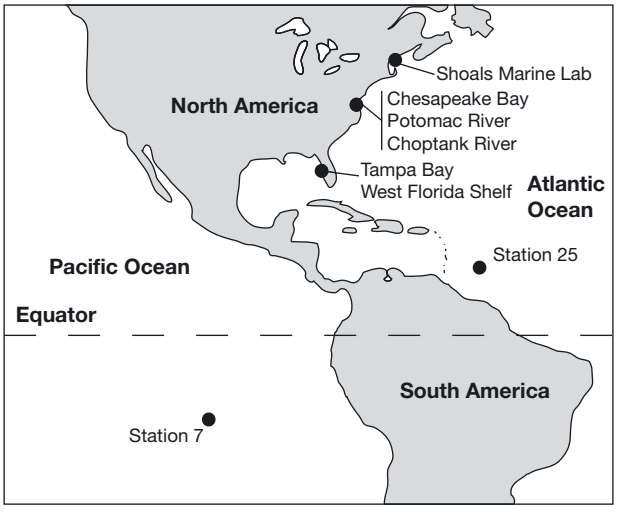

Fig. 1. Locations of sample collections in the Gulf of Maine, Cheseapeake Bay, West Florida Shelf and mid-ocean samples

Table 1. Latitude, longitude and site characteristics of samples studied for eukaryotic ssDNA detection. CB: Chesapeake Bay; GME: Gulf of Maine; GMX: Gulf of Mexico; AO: Atlantic Ocean; PO: Pacific Ocean

Origin	Geographic area	Date	Habitat type	Latitude	Longitude
Potomac River	CB	4/28/2012	Estuary	38° 15′ 45″ N	76° 56′ 18″ W
Choptank River	CB	3/22/2010	Estuary	38° 32′ 55″ N	76° 15′ 38″ W
Chesapeake Bay	CB	4/28/2010	Estuary	38° 31′ 39″ N	76° 25′ 08″ W
Chesapeake Bay	CB	5/17/2010	Estuary	38° 31′ 39″ N	76° 25′ 08″ W
Bayboro Harbor, Tampa Bay	GMX	4/27/2009	Estuary	27° 45′ 39″ N	82° 37′ 52″ W
Tampa Bay	GMX	10/8/2009	Estuary	27° 36′ 28″ N	82° 42′ 29″ W
Shoals Marine Lab Time Series Stn	GME	6/26/2009	Coastal water	43° 00′ 35″ N	70° 39′ 04″ W
Babb's Cove, Shoals Marine Lab	GME	5/18/2009	Coastal water	42° 59′ 18″ N	70° 36′ 58″ W
Babb's Cove, Shoals Marine Lab	GME	6/7/2009	Coastal water	42° 59′ 18″ N	70° 36′ 58″ W
Babb's Cove, Shoals Marine Lab	GME	7/6/2009	Coastal water	42° 59′ 18″ N	70° 36′ 58″ W
Babb's Cove, Shoals Marine Lab	GME	6/30/2010	Coastal water	42° 59′ 18″ N	70° 36′ 58″ W
Star Island, Shoals Marine Lab	GME	8/26/2010	Coastal water	42° 58′ 26″ N	70° 36′ 33″ W
West Florida Shelf Station 8G	GMX	10/9/2009	Coastal water	26° 16′ 15″ N	82° 37′ 17″ W
West Florida Shelf Station 9A	GMX	10/10/2009	Coastal water	26° 25′ 45″ N	82° 30′ 57″ W
West Florida Shelf Station 10B	GMX	10/11/2009	Coastal water	26° 24′ 44″ N	82° 27′ 03″ W
Atlantic Station 25	AO	6/19/2010	Open ocean	11° 18′ 14″ N	56° 38′ 36″ W
East Tropical Pacific Station 7	PO	2/14/2010	Open ocean	10° 00′ 00″ S	100° 00′ 00″ W

tion of the Station 7 and 25 samples, which were collected with 1 m diameter 125 µm nets). Plankton tow material was decanted from the plankton net cod end and placed into either sterile whirlpak bags or 50 ml conical tubes before being frozen at −80°C until analysis. A subsample of plankton tow material was preserved in 10% formaldehyde for examination of zooplankton composition.

Nucleic acid was extracted from net plankton subsamples (0.35 g plankton wet weight) using the Zymo Research Insect and Tissue DNA Midiprep kit following manufacturer's recommendations. Subsamples were obtained by thawing the plankton tows, allowing particulate matter in tows to settle to the bottom of Whirlpak bags or conical tubes, and then removing concentrated plankton in the tows using a sterile transfer pipette. DNA was eluted in 150 µl of nuclease free water (Ambion). Extracted DNA was quantified using PicoGreen DNA fluorescence (1 µl extracted DNA in 30 µl assay, fluorescence measured using an ABI 7500 Real-Time PCR machine) and all samples diluted to 0.5 µg ml^{-1} to standardize template amounts in PCR reactions.

We conducted our analysis of CRESS-DNA distribution using viral genotypes that had been discovered as part of previous metagenomic efforts (Table S1 in the Supplement at www.int-res.com/articles/suppl/m494p065_supp.pdf; Fig. 2). These included the copepod circoviral genotypes from *Labidocera aestiva* (LACopCV) and *Acartia tonsa* (ATCopCV) (Dunlap et al. 2013); both positive sense and negative sense strands of the putative RNA-DNA hybrid virus from *Daphnia mendotae* (DMClaHV) (Hewson et al. 2013b); a virus discovered associated with the amphipod *Diporeia* spp. in Lake Michigan (LM29173) (Hewson et al. 2013a); circovirus-like genome fragments from soil of Fayetteville Green Lake (FGL_c7) and from the water column of the Cayuga Lake catchment (CL_c973) (Hewson et al. 2012); and 2 circovirus-like genomes from Chesapeake Bay (CB-A and CB-B; Rosario et al. 2009). The quantitative primer and probe sets used in this survey were developed as part of these previous studies (Hewson et al. 2012, 2013a,b, Dunlap et al. 2013).

Quantitative PCR was performed in 25 µl reactions containing 1× Taqman MasterMix (Applied Biosystems), 200 pmol each of forward and reverse primer and probes (Supplemental Table 1), and 2 ng template DNA as described previously (Hewson et al. 2012). Reactions were run in duplicate, with duplicate oligonucleotide standards over 8 orders of magnitude (from 10^8 to 10^1 copies $µl^{-1}$) and 2 negative controls. An additional sample replicate, which was spiked with 10^4 copies of the oligonucleotide standard, was used to determine PCR inhibition by material in the DNA extracts. Samples were subject to thermal cycling in an Applied Biosystems 7300 machine. Thermal cycling comprised 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, and 60 cycles at 95°C for 15 s and at 60°C for 1 min.

The cycle threshold (Ct) and baseline for each run was calculated automatically by the Applied Biosystems 7300 system software. Regressions for each standard curve were calculated using Microsoft Excel 2010. If the Ct for any well was within 1 ther-

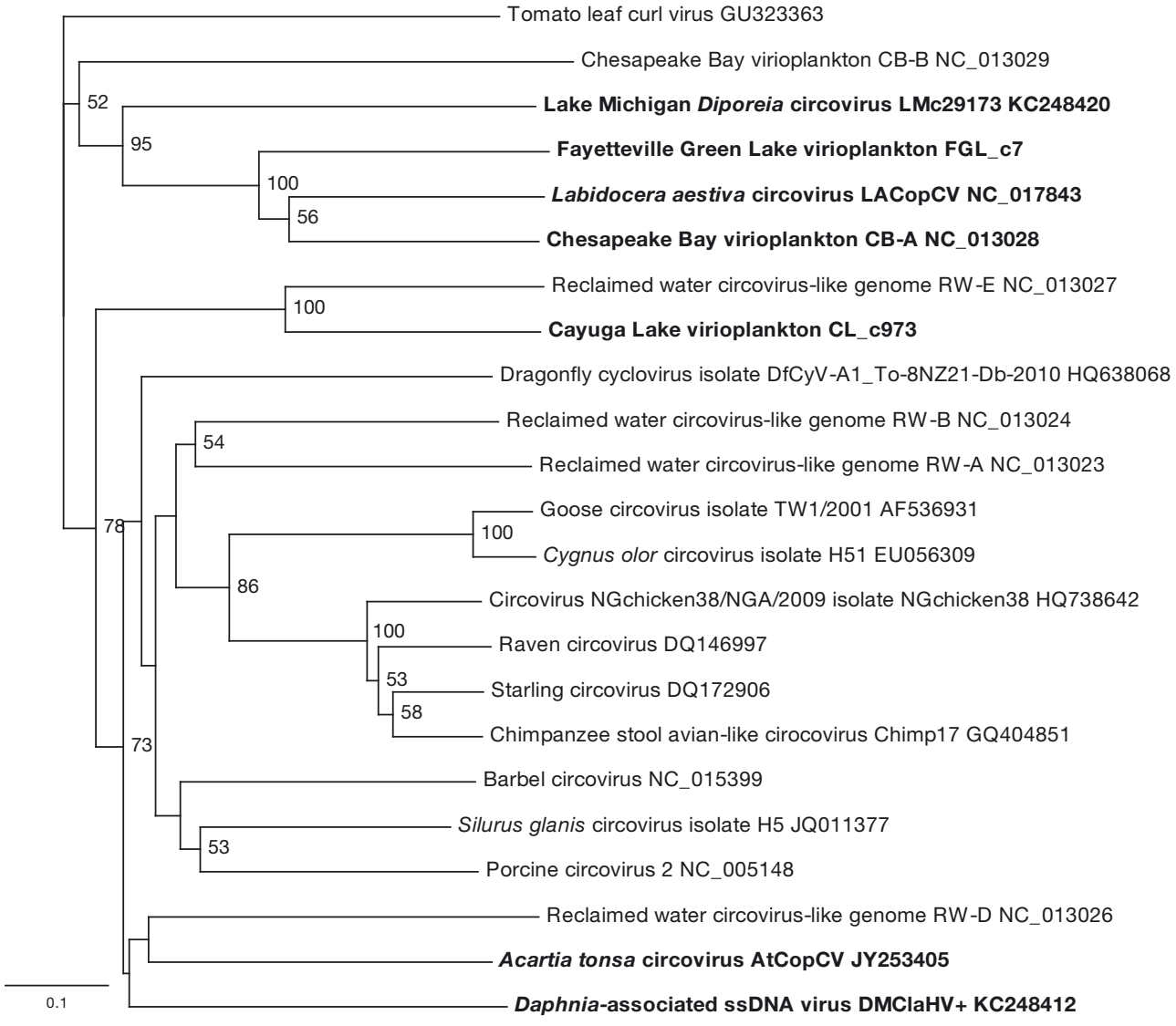

Fig. 2. Phylogenetic representation of CRESS-DNA replication-initiator protein sequences for viral genotypes targeted in this study (**bold**) and representative circovirus, cyclovirus and geminivirus sequences. The neighbor-joining tree was created using unweighted pair-group mean average, and was based on an amino acid alignment created in MUSCLE (www.ebi.ac.uk/Tools/msa/muscle/). Numbers above branch nodes indicate bootstrap values of 1000 iterations. Scale bar = 0.1 substitutions site^{-1}

mal cycle of the average Ct for the NTCs, amplification was considered not detected. If a replicate for any sample failed to amplify (and the second technical replicate amplified successfully), amplification was considered to not have been detected. To assess inhibition by co-extracted organic matter, the difference between the Ct of each inhibition assay sample and the average Ct of the 10^4 standards was calculated (we assume that inhibition has a linear effect across all ranges of target abundance). The difference was subtracted from the Ct of the other replicates for that sample. These corrected Ct values were then used to calculate average number of copies 2 ng^{-1} (Short & Zehr 2007).

To assess composition of zooplankton communities in net plankton samples, 10 ml subsamples of preserved material was examined under a dissecting scope at 10× magnification. Because we did not standardize the time of plankton tow, nor have information on total volume filtered, analysis of composition was relative between samples and independent from differences in total zooplankton abundance. Zooplankton were classified to order according to conventional descriptions.

RESULTS AND DISCUSSION

Our results demonstrate the widespread distribution of CRESS-DNA viruses in estuarine, coastal and open ocean net plankton; however, there were differences in distribution patterns between viral genotypes examined. CRESS-DNA viruses were detected in all samples tested except a sample from Tampa Bay on 8 October 2010 (Table 2). The Tampa Bay sample was taken at a station in a large surface aggregation of the cyanobacterium *Trichodesmium* spp. that was devoid of zooplankton (Fig. 3). The detection of CRESS-DNA viral genotypes in all other samples which harbored zooplankton suggests that they are likely the source of viral nucleic acids.

The most widely detected CRESS-DNA genotype, CB-A, was initially recovered from the Chesapeake Bay in a metagenomic survey of virioplankton (i.e. Environmental CRESS-DNA virus; Rosario et al. 2009). The host of CB-A was not identified in its initial description (Rosario et al. 2009); in our survey, CB-A co-occurred primarily with sites with a large proportion of larval and adult copepods, amphipods and chaetognaths. In contrast, a second Chesapeake Bay circovirus-like genome (CB-B) described by Rosario et al. (2009) was detected at fewer locations, and was mainly detected in samples from the Chesapeake Bay estuary when and where CB-A was not detected. The remaining 2 environmental CRESS-DNA viral genotypes, which were retrieved from soil and water columns of freshwater lake catchments in upstate New York (Hewson et al. 2012) were detected in very few samples and generally at abundances of $<10^2$ copies per 2 ng DNA. The exception was detection of both upstate New York genotypes in net plankton from Babb's Cove in May 2009, which corresponded with an intense rainfall event (1 cm in the preceding 24 h, National Weather Service). The detection of putatively freshwater and soil-borne environmental CRESS-DNA genotypes in these samples may have been due to runoff and the presence of sediment particles at the Shoals Marine Laboratory dock in Babb's Cove.

Tissue-derived CRESS-DNA viruses (i.e. those viruses which were initially recovered from animal tissues) were also recovered from a variety of habitats, although the distribution varied with viral genotype. The most widely distributed genotype, LaCopCV, recovered from the estuarine copepod *Labidocera aestiva* (Dunlap et al. 2013), was detected on multiple dates from Babb's Cove, Shoals Marine Laboratory, and at one station on the West Florida Shelf. In contrast, ATCopCV, recovered from the cosmopolitan

Table 2. Results of quantitative PCR detection of CRESS-DNA genomes in net plankton. Quantities in all cases are copies 2 ng extracted DNA^{-1} (±1 SE). –: not detected; *: samples for which one analytical replicate indicated <10 copies of the viral genotype, but a duplicate indicated no copies were present (i.e. false positive). Dates given as mm/dd/yr

Origin	Date	Approx. salinity	ATCopCV	LACopCV	DMCla HV+	DMCla HV–	LM c29173	CB-A	CB-B	FGLc7	CLc 937
Potomac River	4/28/12	2	–	*	297000 ± 42000	1680 ± 410	31000 ± 17000	–	231 ± 37	36 ± 8	–
Choptank River	3/22/10	8	–	–	*	73 ± 40	–	*	31 ± 22	–	–
Chesapeake Bay	4/28/10	22	–	*	–	–	*	*	155 ± 94	–	–
Chesapeake Bay	5/17/10	22	–	*	–	*	–	330 ± 196	–	*	–
Tampa Bay	10/8/09	35	–	–	–	–	–	–	–	–	–
SML Time Series Station	6/26/09	30	*	–	–	2110 ± 140	–	135000 ± 19000	–	–	*
Babb's Cove, SML	5/18/09	30	–	*	102000 ± 38000	–	–	*	–	210 ± 78	3080 ± 190
Babb's Cove, SML	6/7/09	30	–	7500 ± 5900	–	–	–	*	–	44 ± 10	*
Babb's Cove, SML	6/30/10	30	–	680 ± 260	–	*	*	850 ± 630	–	–	–
Star Island, SML	8/26/10	31	11 ± 4	5100 ± 3500	–	*	*	920 ± 550	16 ± 8	–	–
Station 8G	10/9/09	32	–	8100 ± 5500	–	*	2900 ± 1600	850 ± 660	9 ± 4	–	*
Station 9A	10/10/09	35	–	*	–	*	10300 ± 3100	470 ± 150	–	–	–
Station 10B	10/11/09	35	205 ± 62	*	–	*	*	–	–	–	–
Station 25	6/19/10	35	107 ± 1	*	–	*	–	1240 ± 770	157 ± 13	–	–
Station 7	2/14/10	35	–	*	–	–	*	330 ± 100	–	–	–

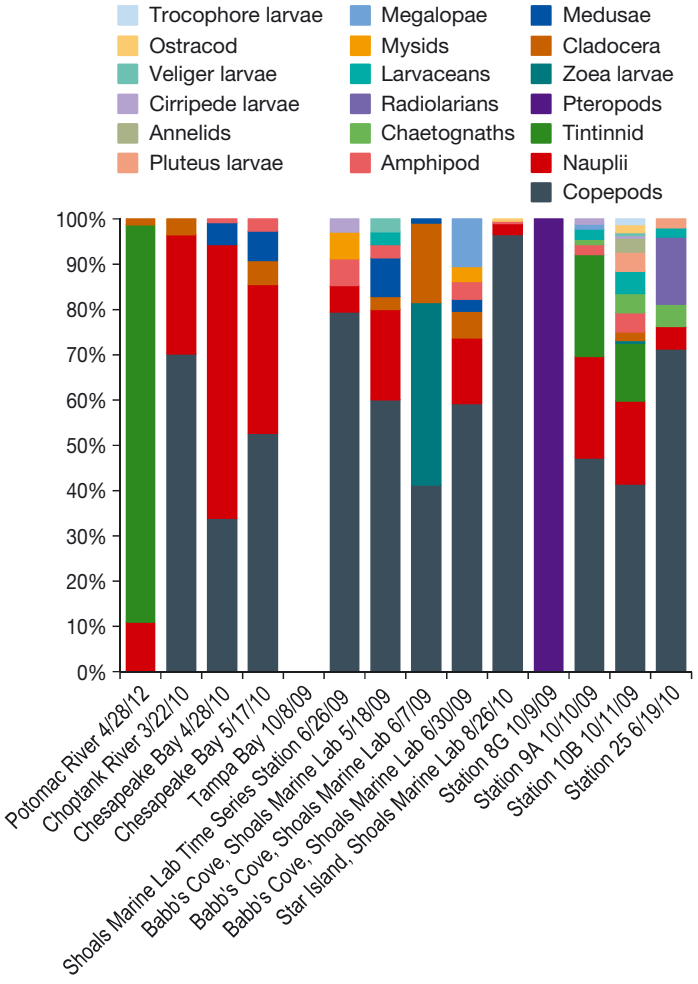

Fig. 3. Composition of zooplankton communities (as percentage of individuals counted) determined by light microscopy. The sample from Tampa Bay (8 October 2009) was comprised entirely of *Trichodesmium* filaments, i.e. devoid of zooplankton

copepod *Acartia tonsa* (Dunlap et al. 2013), was detected in fewer samples and at lower total abundances per sample. The *Daphnia*-associated putative DNA-RNA hybrid virus (DMClaCV) (Hewson et al. 2013b) was detected only in samples from the Chesapeake Bay, waters between the Shoals Marine Lab and mainland (within the plume of the Piscatacqua River), and at Babb's Cove. Finally, LMc29173 (from freshwater *Diporeia* spp. amphipods; Hewson et al. 2013a) was detected in the Potomac River and on the West Florida Shelf. There was no clear relationship between the pattern of invertebrate-associated CRESS-DNA virus distribution and order-level phylogeny patterns between samples, which may be due to variable species- or genera-level differences in zooplankton composition between locations, weather-related events, or lack of similarity between the organisms from which CRESS-DNA were retrieved and those sampled as part of our survey.

The sample in which most viral genotypes were detected was the Potomac River sample, where 3 environmental CRESS-DNA viruses and 3 freshwater invertebrate-associated viruses were recovered. The salinity at the Potomac River Site was lower than other samples from the Chesapeake Bay, suggesting it was influenced the most by freshwater and catchment-associated viruses. Almost all other stations from which samples were obtained had some terrestrial influence — except Station 7 in the South Pacific Ocean. The only virus recovered from this station was the environmental circovirus-like genome CB-A (also the most widely detected virus) further emphasizing the widespread distribution of this genotype.

Our use of TaqMan© quantitative PCR primers and probes demands specificity over ≥90 nt, since reporting of detection only occurs upon annealing of internal hybridization probe to complementary DNA and amplification of the annealed product (which implies hybridization of forward and reverse primers). Hence, we are confident that detection of viral genotypes reflects the presence of CRESS-DNA genotypes bearing regions of DNA that are highly similar to the genotypes detected as part of previous surveys (Hewson et al. 2012, 2013a,b, Dunlap et al. 2013). It is possible that unrelated CRESS-DNA viruses bearing the same region of DNA sequence as targeted by our qPCR assays were detected. However, given the high mutation rate of CRESS-DNA viruses (Rosario et al. 2012b), we are confident that our detection strategy is specific to very similar viruses to those detected in our previous work. It is possible that the CRESS-DNA viral sequences we detected in net zooplankton were originally associated with organisms on which the zooplankton feed, which were present in the digestive tracts of the zooplankton when their DNA was extracted. However, the viral sequences from which primers were designed were isolated from environmental sources or from organisms (>64 µm) with digestive systems removed or cleared prior to viral DNA extraction (Hewson et al. 2012, 2013a,b, Dunlap et al. 2013).

Our detection of CRESS-DNA viruses in widespread net plankton samples is amongst the first investigations of viruses associated with particles in the zooplankton size range, and provides new insight into their distribution in environmental settings. Our data also provide evidence for the origin of CRESS-DNA viruses detected in environmental metagenomes, since previous work has focused on free-living virio-

plankton. We found that environmental CRESS-DNA viruses could be detected in almost all net plankton samples, while invertebrate-associated CRESS-DNA viruses were more restricted in their distribution and were present in some samples at very low quantities. Since we found the greatest diversity of tested CRESS-DNA viruses at sites which had a confluence of terrestrial (and freshwater) and marine conditions, we speculate that CRESS-DNA viruses may either be transported with their host organisms between habitats or may be present as free particles and transported in virioplankton, which then attach to net plankton at their new site. Previous investigation of CRESS-DNA viral decay rates in freshwater lakes have indicated that they are typically on par or slower than native virioplankton. Moreover, our study raises interesting questions about the ecological role of CRESS-DNA viruses in marine ecosystems. If current observations of viruses associated with important zooplankton groups, like marine copepods, and our observation of CRESS-DNA associated with net plankton extend to effects on host metabolism, feeding rates, or fecundity of mesozooplankton, then they may influence food web dynamics in ways that are currently not appreciated.

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