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Isolation and preliminary characterisation of a small nuclear inclusion virus infecting the diatom *Chaetoceros* cf. *gracilis*

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ABSTRACT: A novel virus (*Chaetoceros* nuclear inclusion virus: CspNIV) causing lysis of a culture of the diatom *Chaetoceros* cf. *gracilis* was isolated from the Chesapeake Bay, USA, in April 2003. Transmission electron microscopy of ultrathin sections of infected *C.* cf. *gracilis* revealed that CspNIV proliferates within the nucleus and forms paracrystalline arrays corresponding to the alignment of icosahedral viral particles of about 25 nm diameter. CspNIV shows some strong similarities with *Heterosigma akashiwo* nuclear inclusion virus (HaNIV) (cf. Lawrence et al. 2001; J Phycol 37: 216–222). The latent period of CspNIV is <24 h. The most widespread occurrence of *Chaetoceros* viruses in Chesapeake Bay was recorded in April 2003, ca. 1 mo after the winter-spring *Chaetoceros* bloom. However, results indicate that CspNIV remains infectious in surface water of the bay no longer than 1 mo after the disappearance of its host. Thus, our results reinforce the idea that microalgae are also sensitive to viruses other than those belonging to the family *Phycodnaviridae*. Furthermore, discovery and initial description of the infection process and ecology of CspNIV expands the breadth of phytoplankton shown to be susceptible to viral attack to include a ubiquitous diatom genera.

KEY WORDS: Virus · Diatom · Phytoplankton · Infectivity · Chesapeake Bay

INTRODUCTION

Over the last 15 yr, views on aquatic food webs have changed considerably with the discovery of high viral abundance and activity in a wide range of marine environments. With concentrations ranging from 10^4 to 10^8 particles ml^{-1}, viruses are now recognized to represent the most abundant biological entities occurring in both marine and freshwater systems (Bergh et al. 1989, Proctor & Fuhrman 1990), regardless of latitude, salinity, temperature, depth or ecosystem productivity (Fuhrman 1999, Wommack & Colwell 2000). Although the majority of virioplankton are believed to infect bacterial hosts (so-called bacteriophages), we now know that viruses infecting eukaryotic microalgae can also be of quantitative and functional significance within aquatic microbial food webs. Indeed, viruses have been reported to infect a wide range of phytoplankton species, including bloom-forming species (harmful or not), free-living organisms, and symbionts (see Brussaard 2004 for review). For example, susceptibility to viral infection has been identified for species belonging to microalgal families as diverse as Prymnesiophyceae (Suttle & Chan 1995, Jacobsen et al. 1996, Brussaard et al. 1999, Sandaa et al. 2001, Schroeder et al. 2002), Prasinophyceae (Wilson et al. 2002, Evans et al. 2003), Raphidophycae (Nagasaki & Yamaguchi 1997, Lawrence et al. 2001), Dinophyceae (Tarutani et al. 2001), and Bacillariophyceae (Nagasaki et al. 2004). Evidence is accumulating that viruses, in conjunction

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with eukaryotic parasites, bacteria, and grazers, are involved in regulating the occurrence, progression, and demise of monospecific phytoplankton blooms (Brussaard 2004).

Until the early 2000s, viruses that infect unicellular algae were consistently and repeatedly assumed to belong to a single family, the *Phycodnavirideae* (Suttle 2000), characterized by a particle size of 100 to 220 nm in diameter and a large double-stranded (ds) DNA genome ranging from 130 to 560 kbp. This perspective collapsed, however, with the discovery of small single-stranded (ss) RNA viruses (<30 nm in diameter) infecting the raphidiophyte *Heterosigma akashiwo* (Lawrence et al. 2001, Culley et al. 2003, Tai et al. 2003, Lang et al. 2004), the dinoflagellate *Heterocapsa circularisquama* (Tomaru et al. 2004), the diatom *Rhizosolenia setigera* (Nagasaki et al. 2004), and the prasinophyte *Micromonas pusilla* (Brussaard et al. 2004).

Among the different groups of phytoplankton, diatoms are considered one of the most important and influential in the global ocean. They inhabit most bodies of fresh and marine waters in all parts of the world and account for up to 25% of all organic carbon fixation on the planet.

Chaetoceros Ehrenberg 1844 is arguably the largest and most species-rich genus of marine planktonic diatoms. *Chaetoceros* species belong to the Order Centrales, and form a group composed of about 400 described species, which are widely spread in all maritime regions, especially in cold and temperate coastal areas (Rines & Hargraves 1988). *Chaetoceros* blooms comprise as many as 25 concurrent species (Rines & Theriot 2003). They can dominate the planktonic communities for weeks at a time, and thus are an integral component of the ecology of the seas. Among the diatoms, *Chaetoceros* species are unrivalled in their complexity of cell and colony morphology, inviting numerous 'form and function' questions. A few species are known to cause the death of Atlantic and pen-reared salmon (Rensel 1993). Therefore, this genus is important within the disciplines of marine biology, marine ecology, marine geology, oceanography and aquaculture (Rines & Theriot 2003).

To date, direct evidence for viral infection of diatoms exists for only 1 species: *Rhizosolenia setigera* from the Ariake Sea, Japan (Nagasaki et al. 2004). Here, we offer additional information on the vulnerability of diatoms to viral attack by reporting infection and lysis of a *Chaetoceros* species by a small RNA-like virus. We provide information on the isolation and subsequent propagation of the virus in laboratory cultures, describe the development and morphology of the virus inside the host cell, and report the seasonal occurrence of viruses in Chesapeake Bay that are active against *C.* cf. *gracilis.*

MATERIALS AND METHODS

Sampling strategy. Nine stations along the major axis of Chesapeake Bay and 4 locations in western shore tributaries (Magothy, Severn, Patuxent, and Potomac Rivers) were sampled during 13 cruises in 2003 to 2004 (Fig. 1). The samples were collected 1 m below the surface using 10 l Niskin bottles mounted on a CTD rosette. Sampling dates in 2003 were March 2–5, April 7–11, June 2–6, July 1–3, August 22–24, and October 9–11; and in 2004 were February 12–13, March 21–23, May 7–10, June 7–9, July 12–14, August 30–31, and October 26–28. All 9 main-bay stations were sampled during each cruise, except in February 2004 when ice cover prevented ship operations at the 2 northernmost stations. One or more of the western shore tributaries were sampled during each cruise (Fig. 2). For May 2004 (not shown in Fig. 2), samples were obtained from the Magothy, Severn, and South Rivers (see Fig. 1).

Viral concentration. Viral communities from the Chesapeake Bay were concentrated on board ship using the ultrafiltration protocols described by Chen et al. (1996). Briefly, five 10 l Niskin bottles were poured into a 50 l Nalgene carboy. and water was then pre-filtered in series through a 25 µm polypropylene wound yarn filter and a Pellicon system (Millipore) with multifold 0.22 µm pore-size tangential-flow membrane. The filtrate was concentrated by ultrafiltration through a 30 000 MW cutoff Amicon S10Y30 spiral cartridge (Millipore). Both Pellicon system and S10Y30 spiral cartridge were mounted with the ProfFlux M-12 system (Millipore), at 30% pump speed and 16 to 18 kPa back pressure. The final volume of each concentrate ranged from 400 to 800 ml. This method has proven to be effective for the efficient concentration of virioplankton from Chesapeake Bay water samples (Wommack et al. 1999). Viral concentrates (VC) were then stored at 4°C in the dark until used.

Seasonal distribution of *Chaetoceros* species in Chesapeake Bay. The abundance of diatoms belonging to the genus *Chaetoceros* was determined for stations located along the main axis of Chesapeake Bay. At each station, 125 ml of surface water was preserved using acid Lugol's fixative, 1% final concentration. Preserved samples were enumerated using 5 ml Zeiss settling chambers and an inverted microscope with phase contrast optics (Leitz Diavert or Olympus IX51; 250× and 400×, respectively). For each sample, randomly selected fields (20 when using the Leitz Diavert; 35 for the Olympus IX51) were examined, and all cells enumerated to genus or species. As a result, ~0.1 ml of each sample was counted, giving a detection level of ~10 cells ml^{-1}.

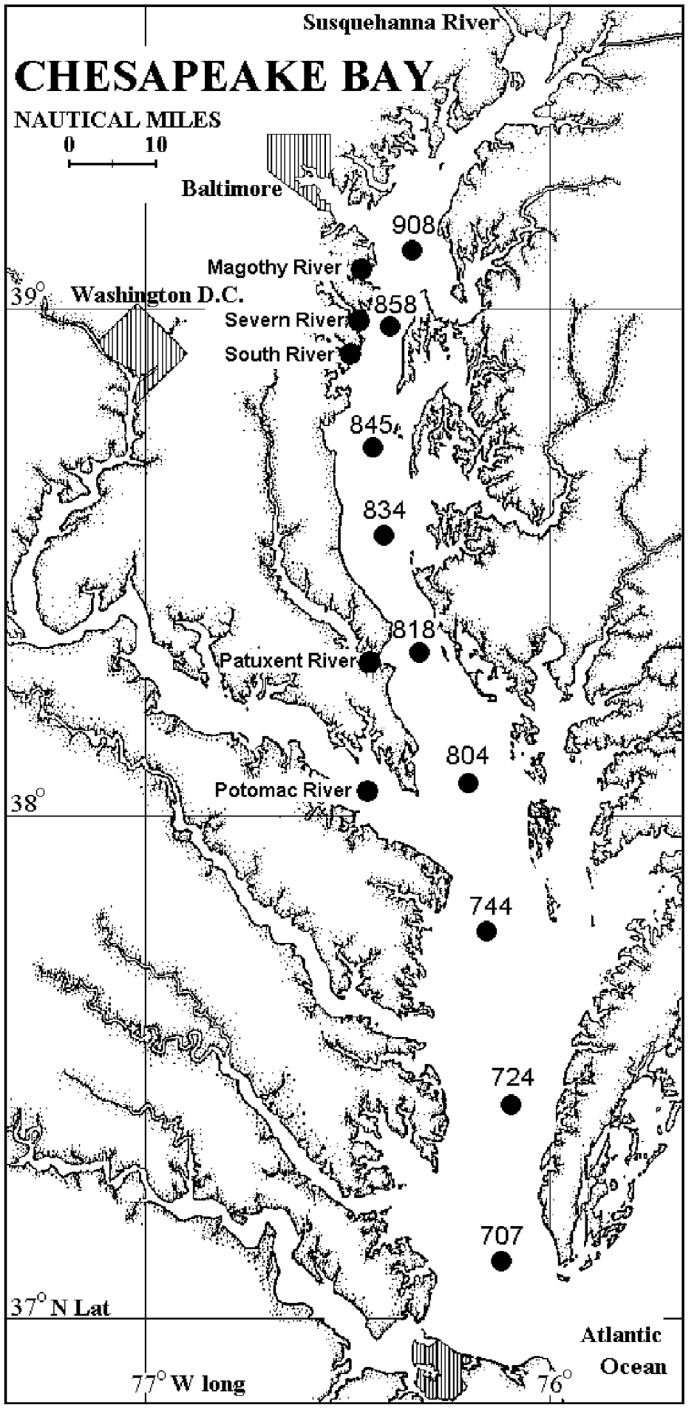

Fig. 1. Map of Chesapeake Bay showing locations of stations for cruises in 2003–2004. Station designation along the main axis of the bay are from north to south: Stn 908 (39° 08′ N, 76° 20′ W); Stn 858 (38° 58′ N, 76° 23′ W); Stn 845 (38° 45′ N, 76° 26′ W); Stn 834 (38° 34′ N, 76° 25′ W); Stn 818 (38° 18′ N, 76° 17′ W); Stn 804 (38° 04′ N, 76° 13′ W); Stn 744 (37° 44′ N, 76° 11′ W); Stn 724 (37° 24′ N, 76° 05′ W); Stn 707 (37° 07′ N, 76° 10′ W). Station designations in tributaries are from north to south: Magothy River (39° 04′ N, 76° 26′ W); Severn River (38° 59′ N, 76° 28′ W); South River (37° 55′ N, 76° 29′ W); Patuxent River (38° 18′ N, 76° 27′ W); Potomac River (38° 05′ N, 76° 30′ W)

Algal cultures. A non-axenic, clonal strain of a *Chaetoceros* sp. was isolated from the Rhode River subestuary of Chesapeake Bay in March 2003, using serial dilution technique. The morphology of *Chaetoceros* cells in our culture closely resembled *C. gracilis*, but showed variation in form likely resulting from culture conditions. We therefore tentatively identify our isolate as *C. gracilis*. Stock cultures of *Chaetoceros* cf. *gracilis* were maintained in f/2-Si medium (Guillard & Ryther 1962) at a salinity of 8‰ and temperature of 15°C, and grown under cool white fluorescent lamps supplying 50 to 100 µE m^{-2} d^{-1} on a 14:10 h light:dark cycle. The source and growth temperature of all other algal strains used in this study are listed in Table 1. These cultures were maintained in 15‰ f/2-Si medium and incubated under cool white fluorescent lamps supplying 100 to 200 µE m^{-2} d^{-1} on a 14:10 h light:dark cycle.

Susceptibility of *Chaetoceros* cf. *gracilis* and other microalgae to viral concentrates. Stock cultures of *C.* cf. *gracilis* were inoculated with VC collected during cruises and examined for viral infection, as indicated by cell lysis during a 10 d incubation period. Duplicate 10 ml volumes of exponentially growing algal stocks were inoculated with 1 ml VC to yield a ratio of 100 to 1000 viruses per algal cell. Inoculated cultures and duplicate controls (i.e. without addition of VC) were incubated under host growth conditions described above and assessed daily for *in vivo* chlorophyll *a* (chl *a*) fluorescence using a Turner Designs 10-AU fluorometer. Inoculated cultures that showed decreased *in vivo* chll *a* fluorescence >50% after 10 d, relative to controls, were considered as positive for the presence of lytic viruses. In addition, another 19 algal strains (9 diatoms, 9 dinoflagellates, 1 prasinophyte; Table 1) were tested for viral lysis as described above, using VC generated during cruises in 2003.

Isolation of viruses. A clonal pathogen isolate was obtained from the VC generated at Stn 744 in April 2003. The isolation was performed by dilution of the virus in microtiter plates containing 200 µl of exponentially growing stock culture of *C.* cf. *gracilis*. Plates were incubated for 10 d and the contents of the most diluted wells showing lysis were transferred to new microtiter plates. This procedure was repeated 3 times (Sandaa et al. 2001). The resultant lysate was filtrated through 0.2 µm pore-size polycarbonate membrane filters and designated as the clonal pathogen suspension CspNIV (*Chaetoceros* nuclear inclusion virus). Serial transfers of lysate

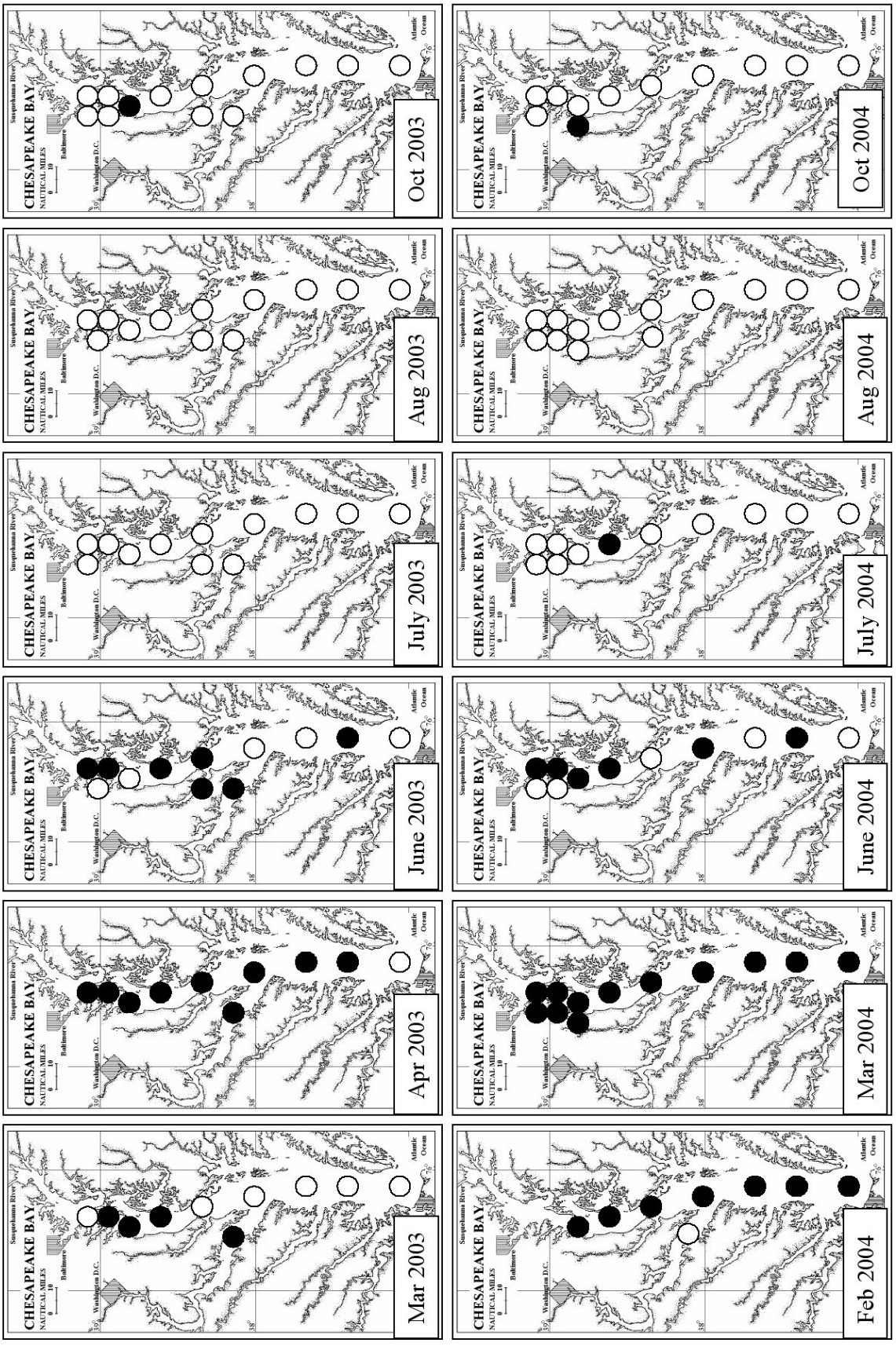

Fig. 2. Temporal and spatial patterns in the capacity of viral concentrates from Chesapeake Bay to induce lysis of *Chaetoceros* cf. *gracilis* cultures. Filled circles = lysis; open circles = lysis not detected

Table 1. Susceptibility of algal strains to viral concentrates (VC) collected in the Chesapeake Bay, USA, in 2003, and to the clonate pathogen isolated in April 2003 at Stn 744 (CspNIV). +: sensitive; –: not sensitive

		Origin of isolation	Culture temp. (°C)	Strains lysed after VC inoculation	Strains lysed by CspNIV
Dinophyceae	*Karlodinium micrum*	Chesapeake Bay	20	–	–
	Scrippsiella sp.	Chesapeake Bay	15	–	–
	Akashiwo sanguineum	Chesapeake Bay	15	–	–
	Gymnodinium instriatum	Chesapeake Bay	15	–	–
	Katodinium rotundatum	Chesapeake Bay	20	–	–
	Prorocentrum minimum	Chesapeake Bay	20	–	–
	Heterocapsa triquetra	Chesapeake Bay	20	–	–
	Alexandrium sp.	Chesapeake Bay	15	–	–
	Ceratium furca	Chesapeake Bay	15	–	–
Bacillariophyceae	*Chaetoceros* cf. *gracilis*	Chesapeake Bay	15	+	+
	Chaetoceros affinis	Narraganset Bay	15	–	–
	Chaetoceros rostratum	Narraganset Bay	15	–	–
	Thallasiosira sp.	Chesapeake Bay	20	–	–
	Pseudonitzschia seriata	Chesapeake Bay	20	–	–
	Ditylum brightwelli	Chesapeake Bay	15	–	–
	Bacteriastrum hyalinum	Narraganset Bay	15	–	–
	Asterionellopsis glacialis	Chesapeake Bay	20	–	–
	Coscinodiscus sp.	Chesapeake Bay	20	–	–
	Cylindrotheca closterium	Chesapeake Bay	20	–	–
Prasinophyceae	*Pyramimonas* sp.	Chesapeake Bay	20	–	–

were performed 5 times to amplify viruses that specifically attack *C.* cf. *gracilis.* As the purification of the pathogen could not be completed, Koch's postulates were not entirely fulfilled.

Transmission electron microscopy (TEM). CspNIV inoculated *Chaetoceros* cf. *gracilis* cultures and non-inoculated controls from a 1-step growth experiment (see below) were examined using thin-section TEM. Samples (10 ml) were fixed with glutaraldehyde (1% final concentration) and stored at 4°C. Preserved cells were pelleted in 400 µl microfuge tubes using a Beckman model 11 microfuge, rinsed with 3 changes of sterile culture medium, postfixed in 1% osmium tetroxide for 1 h at room temperature, rinsed with distilled water, dehydrated in a graded ethanol series, and transferred to Spurr's low-viscosity embedding media prior to polymerization at 60°C. Sections 900 to 1000 µm thick were cut using a diamond knife mounted on a Reichert Ultracut, transferred to uncoated copper grids, and stained with 2% uranyl acetate and 0.02% lead citrate. Sectioned cells were viewed and photographed using a Zeiss EM 10 CA at 80 kV.

Host range analysis. The host range of CspNIV was tested by adding aliquots of 10% fresh pathogen suspension to duplicate cultures (2 ml) of 20 exponentially growing clonal strains of phytoplankton belonging to classes such as Bacillariophyceae, Dinophyceae, and Prasinophyceae (Table 1). Cultures were maintained in growth media and incubated under environmental conditions appropriate for each phytoplankton species and monitored for evidence of lysis by optical microscopic observation. Inoculated cultures that had lower (<50%) cell abundance than non-inoculated controls were assumed to have succumbed to viral infection and lysis. Cultures that showed no signs of cell lysis within 15 d were considered to be unsuitable hosts for the pathogen.

One-step growth experiment. The population dynamics of *Chaetoceros* cf. *gracilis* cells, virus-like particles (VLP), and bacteria present in the cultures was followed over a 10 d 1-step growth experiment. Duplicate 1 l cultures of exponentially growing *C.* cf. *gracilis* were inoculated with 20 ml of fresh CspNIV suspension and incubated under standard growth conditions (see above). Two different controls were also run in duplicate using 1 l volumes of exponentially growing cultures of *C.* cf. *gracilis*. The first control received no inoculation, while the second received 20 ml of a 20 nm filtrate (Anodisc, Whatman) from CspNIV suspension which had been heat treated at 100°C for 5 min. Treatment and control cultures were sampled every 12 h for determination of (1) *in vivo* chlorophyll fluorescence using a Turner Designs 10-AU fluorometer; (2) abundance of VLP and bacteria in the culture media; and (3) presence of VLP inside *Chaetoceros* cells as determined by TEM and thin sectioning. For determination of abundance of free VLP and bacteria, 100 µl of the cultures was formaldehyde fixed (2% final concentration), filtered onto 0.02 µm pore-size membrane filters (Anodisc), stained with SYBR Gold, and counted using epifluorescence microscopy (Chen et al. 2001). For each sample, at least 400 VLP and 200 bacteria were counted with a minimum of 20 randomly selected fields examined.

RESULTS

Phytoplankton response to viral inoculation

Of the 20 algal species inoculated with VC obtained from Chesapeake Bay between March 2003 and February 2004, only the diatom *Chaetoceros* cf. *gracilis* repeatedly showed evidence of viral infection as indicated by cell lysis (Table 1). Typically, 6 d after inoculation with CspNIV, *C.* cf. *gracilis* cultures were cleared through cell lysis. Translucent empty frustules were commonly observed and the tint of the culture distinctly switched from brownish to greenish due to pigment degradation. Furthermore, sequential transfer of 0.2 µm-filtered lysates over multiple generations persistently resulted in lysis of *C.* cf. *gracilis*, supporting the existence, propagation, and transmission of a viral pathogen. The only other phytoplankton species to show cell lysis following inoculation with a VC from the Chesapeake Bay was the dinoflagellate *Karlodinium micrum. K. micrum* culture inoculated with a VC obtained from the Potomac River in April 2003 showed a partial crash; but the 0.2 µm-filtered lysate from that culture failed to cause cell lysis during subsequent inoculations.

Temporal and spatial patterns in viral concentrate-induced lysis

Inoculating cultures of *Chaetoceros* cf. *gracilis* with VC obtained from surface water of Chesapeake Bay during 2003 and 2004 showed a distinct seasonal pattern in cell lysis. Lytic activity was widespread in winter to late spring (December to April) of both years, decreased in early summer (May to June), virtually disappeared in mid- to late summer, and emerged again in the fall (September to November) (Fig. 2). The timing of winter-spring lysis of *C.* cf. *gracilis*, however, appeared to differ somewhat between 2003 and 2004. Lytic activity was localized in the upper Chesapeake Bay and lower Potomac River in March 2003, spreading throughout most of the bay in April. In contrast, all but 1 VC collected in February and March of 2004 induced lysis of *C.* cf. *gracilis*. The capacity of VC to induce lysis of *C.* cf. *gracilis* also exhibited a north to south gradient, with lysis appearing first in the upper bay (e.g. March and October 2003, Fig 2) and persisting in that region longer than in the lower bay (i.e. June 2003 and 2004, Fig 2.)

Seasonal distribution of *Chaetoceros* species

Diatoms of the genus *Chaetoceros* were important members of the Chesapeake Bay phytoplankton during spring, fall, and winter cruises (Fig. 3). Peak concentrations of *Chaetoceros* species (>10^3 cells ml^{-1}) occurred in the lower bay (Stns 724 and 744) during March 2003, with high densities (>10^2 cells ml^{-1}) present into April and as far north as Stn 858. *Chaetoceros* species were also widespread in the bay during fall-spring of 2003–2004, with locally high densities (>10^2 cells ml^{-1}) occurring on occasion (i.e. Stn 818 in October, Stns 707 and 724 in February and March). The winter-spring *Chaetoceros* blooms of both years largely dissipated by May, with densities in summer (June to August) usually below the detection level (~10 cells ml^{-1}). While *Chaetoceros* species were detected during summer, their densities rarely exceeded 25 cells ml^{-1} (2 of 54 summer samples).

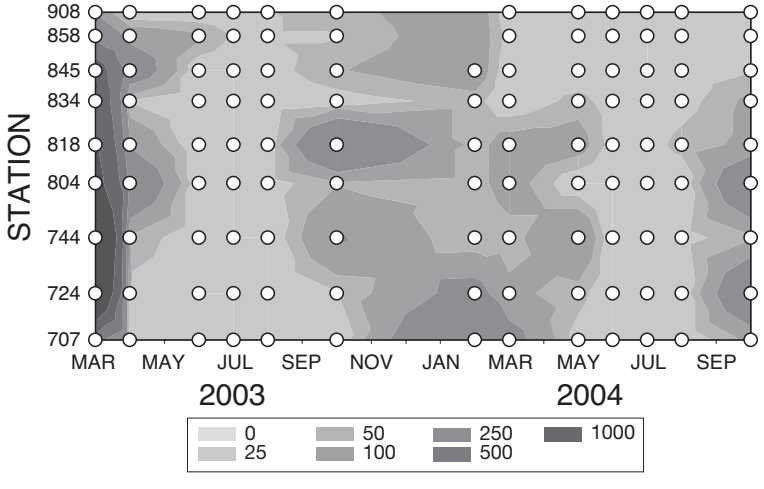

Fig. 3. Abundance of *Chaetoceros* species in the main stem of Chesapeake Bay during 2003–2004 cruises. Contours are cells ml^{-1}; open circles indicate sampling dates and locations. Plot generated using SigmaPlot 2001 (SPSS)

One-step growth experiment

Cultures of *Chaetoceros* cf. *gracilis* inoculated with CspNIV showed a marked drop in chl *a* fluorescence within 24 h, with a continued rapid decline in the fluorescence signal over the following 96 h. Minimum fluorescence values (i.e. at 108 h) were 60% less that those at the start of the incubation. Thereafter, *in vivo* fluorescence stabilized and then increased by 50% by the end of the experiment (Fig. 4A). In contrast, fluorescence in both controls (i.e. cultures with no inoculum and cultures inoculated with 0.02 µm-filtered, heat-treated lysate) increased steadily throughout the experiment.

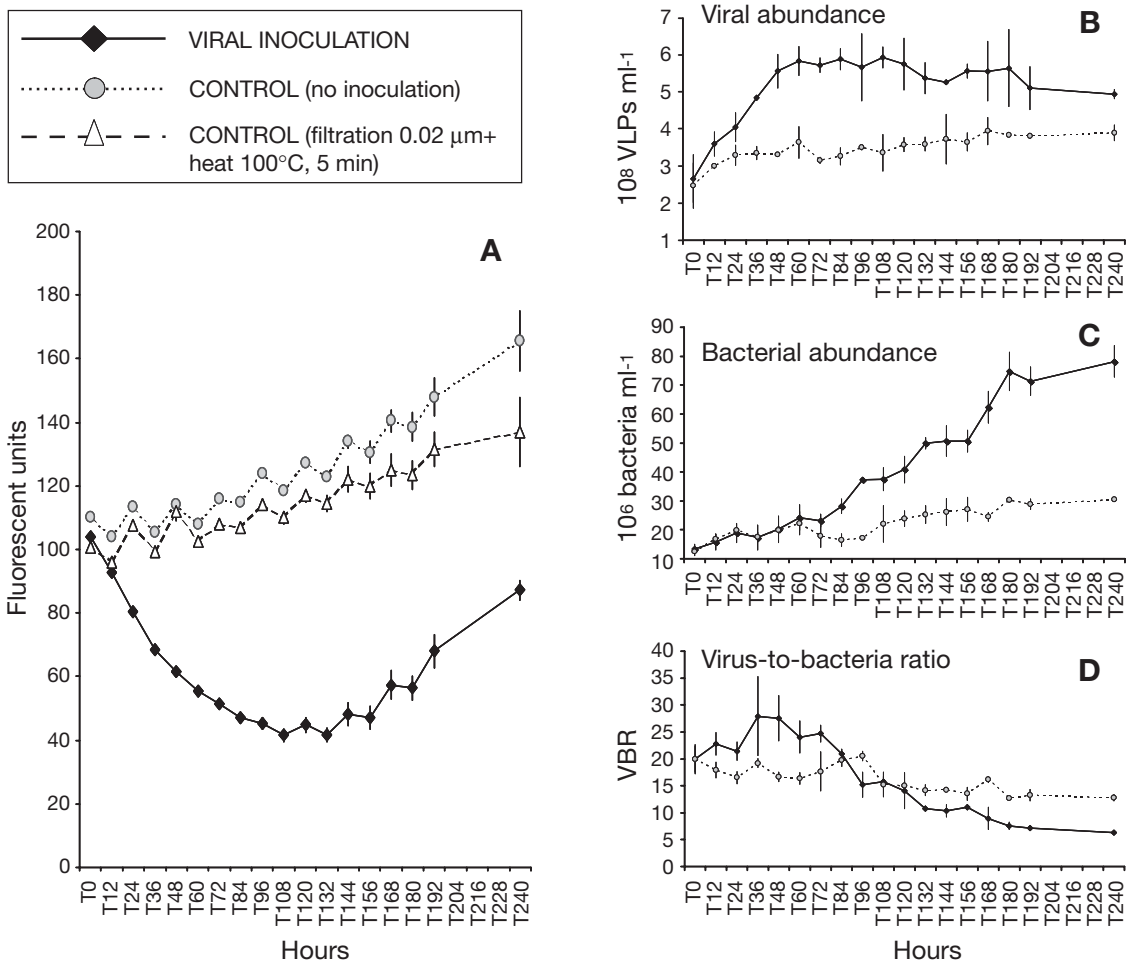

Fig. 4. (A) *In vivo* chl *a* fluorescence, (B) viral abundance, (C) bacterial abundance and (D) virus-to-bacteria ratio (VBR) for a *Chaetoceros* cf. gracilis culture inoculated with *Chaetoceros* nuclear inclusion virus (CspNIV) (diamonds) and 2 control cultures. One of the control cultures (circles) received no inoculation and the other (triangles) was inoculated with <20 nm CspNIV lysate that had been heated to 100°C for 5 min. VLPs: virus-like particles

At the start of the experiment, VLP were detected in the CspNIV inoculated cultures and the minus-inoculum controls (Fig. 4B). Viral abundance in control cultures slightly, but steadily, increased over the experiment, with values ranging from 2.5 ± 0.6 to 3.9 ± 0.2 × 10^8 VLP ml^{-1} (Fig. 4B). In contrast, viral abundance in inoculated cultures doubled from 2.7 ± 0.6 to 5.8 ± 0.4 × 10^8 VLP ml^{-1} over the first 60 h of the incubation, remained stable until 120 h, and decreased slightly thereafter.

Bacterial abundance in non-inoculated control flasks increased steadily in over the course of the experiment, showing a net growth rate of 0.09 ± 0.01 d^{-1} (Fig. 4C). Bacterial densities in inoculated cultures were comparable to the controls for the first 60 h, with values ranging from 13.4 ± 1.6 to 24.3 ± 4.5 × 10^6 cells ml^{-1}. Thereafter, bacteria grew much faster in inoculated flasks (0.16 ± 0.03) than in control cultures, with densities being 3 to 4 times higher (78.2 ± 5.5 × 10^6 and 30.5 ± 0.2 × 10^6 cells ml^{-1}, respectively) at the end of the experiment.

The virus-to-bacteria ratio (VBR) in control cultures was relatively stable over the first 108 h and then decreased slightly over the latter part of the incubation (Fig. 4D). The VBR of inoculated cultures, however, increased sharply for over 36 to 48 h, to nearly double that seen in control incubations. After 48 h, the VBR of inoculated cultures decreased steadily, eventually exhibiting values lower than the control flasks at 132 h post inoculation.

TEM observations

Chaetoceros cf. *gracilis* cells from CspNIV inoculated cultures and a non-inoculated control culture exhibited marked differences in nuclear morphology within 24 h. In thin sections, 70 to 80% of the nuclear

profiles of cells (out of a minimum of 15 inspected sections) from control flasks contained a large, centrally located nucleolus (Fig. 5A). In contrast, only 15 to 20% of nuclear sections from cells preserved 12 and 24 h after inoculation with CspNIV had a nucleolus. After 12 h, the nuclei of inoculated cells commonly showed an amorphous electron opaque matrix located peripherally (Fig. 5B) and sometimes contained an acentric paracrystalline body (Fig. 5C). By 24 h, electron opaque material was evident throughout the nucleus of some cells (Fig. 5D), and hexagonally packed VLP were present in some individuals (Fig. 5E,F). The VLP were hexagonal in cross-section, measured 20 to 30 nm in diameter, were uniformly electron dense, and lacked a visible envelope. No traces of these particles were detected within cells of the control culture.

DISCUSSION

To our knowledge this is the first report of a nuclear inclusion virus (CspNIV) which infects and lyses the diatom *Chaetoceros* cf. *gracilis* and the first observations of seasonally occurring virus-associated lysis of *Chaetoceros* cf. *gracilis* in Chesapeake Bay. This work represents only the second study showing direct evidence that diatoms are susceptible to viral attack, complementing the recent report of a virus infecting the centric diatom *Rhizosolenia setigera* (Nagasaki et al. 2004).

Preliminary characterization

The CspNIV infecting *Chaetoceros* cf. *gracilis* from Chesapeake Bay has 3 major characteristics: (1) small size, ranging between 20 and 30 nm in diameter; (2) replication within the nucleoplasm of host cells; and (3) formation of paracrystalline arrays corresponding to closely packed icosahedral viral particles. All these features tend to differentiate CspNIV from the *Phycodnaviridae*, a family of viruses characterized by a large dsDNA genome ranging from 100 to 560 kbp, replication within the host cytoplasm, and capsid diameter of 100 to 250 nm. From the very few studies on this topic, we know that phytoplankton viruses smaller than 40 nm in diameter typically contain ssRNA genomes and are related to viruses from the highly diverse picorna-like superfamily (Culley et al. 2003). This is the case for the single virus previously found in a diatom (*Rhizosolenia setigera*; Nagasaki et al. 2004), the ssRNA virus infecting the Raphidophyte *Heterosigma akashiwo* (Tai et al. 2003, Lang et al. 2004), and the ssRNA virus of the dinoflagellate *Heterocapsa circularisquama* (Tomaru et al. 2004). A dsRNA virus ranging from 65 to 80 nm in diameter was recently shown to infect the prasinophyte *Micromonas pusilla* (Brussaard et al. 2004). On the basis of virion morphology, it is possible that CspNIV is genetically related to ssRNA viruses; however, further work is needed to characterize CspNIV genome type and size.

Overall, no specific link seems obvious between viral genome type and phytoplankton host taxon. For example, *Heterosigma akashiwo* is sensitive to either dsDNA virus in coastal waters in Japan (Nagasaki & Yamaguchi 1997), or ssRNA virus in the Straight of Georgia, Canada (Tai et al. 2003). Likewise, the dinoflagellate *Heterocapsa circularisquama* (Tarutani et al. 2001, Tomaru et al. 2004) and the prasinophyte *Micromonas pusilla* (Cottrell & Suttle 1991, Brussaard et al. 2004) are both susceptible to infection by RNA and DNA viruses.

TEM observations of infected *Chaetoceros* cells revealed that virus particles appeared exclusively in the nucleoplasm. To date, only *Heterosigma akashiwo* nuclear inclusion virus (HaNIV) isolated by Lawrence et al. (2001) has been reported to proliferate within the nucleus of a planktonic microalga, while the majority of phytoplankton viruses have been shown to propagate within the host cytoplasm. Another intriguing feature shared by CspNIV and HaNIV is the formation of paracrystalline bodies. In infected *C.* cf. *gracilis* cells, the crystals appeared to be composed of an ordered array of icosahedral viral particles, with each side measuring approximately 25 nm in length. In a study of nuclear division in the dinoflagellate *Gyrodinium cohnii*, Kubai & Ris (1969) observed an unusual paracrystalline body associated with the nucleolus, but provided no further characterization of the phenomenon. More recently, nucleopolyhedroviruses infecting insects have also been shown to form occlusions in proteinaceous crystal bodies (Fuxa 2004). The temporal sequence of CspNIV development shown in Fig. 3 suggests that formation of the paracrystalline array may be connected to fragmentation of the nucleolus. Al-Lami et al. (1969) and Henry et al. (1972) showed that large masses of empty capsids of 2 parvoviruses were closely associated with, and sometimes replaced, fragmented nucleoli, suggesting the assembly and possible synthesis of viral capsids by the nucleolus. The fact that individual viral particles were not observed within infected *C.* cf. *gracilis* cells indicates that the viruses maintain their compact organisation possibly until burst of the host cell. Some yet undetermined trigger may release CspNIV from a quiescent state, causing cell lysis. Ecologically, this aggregated structure of viruses could be of importance by increasing their persistence outside the host in sediments and other environments (Lawrence et al. 2001, Fuxa 2004), although this would only be a realistic virus survival strategy in shallow coastal environments like Chesapeake Bay.

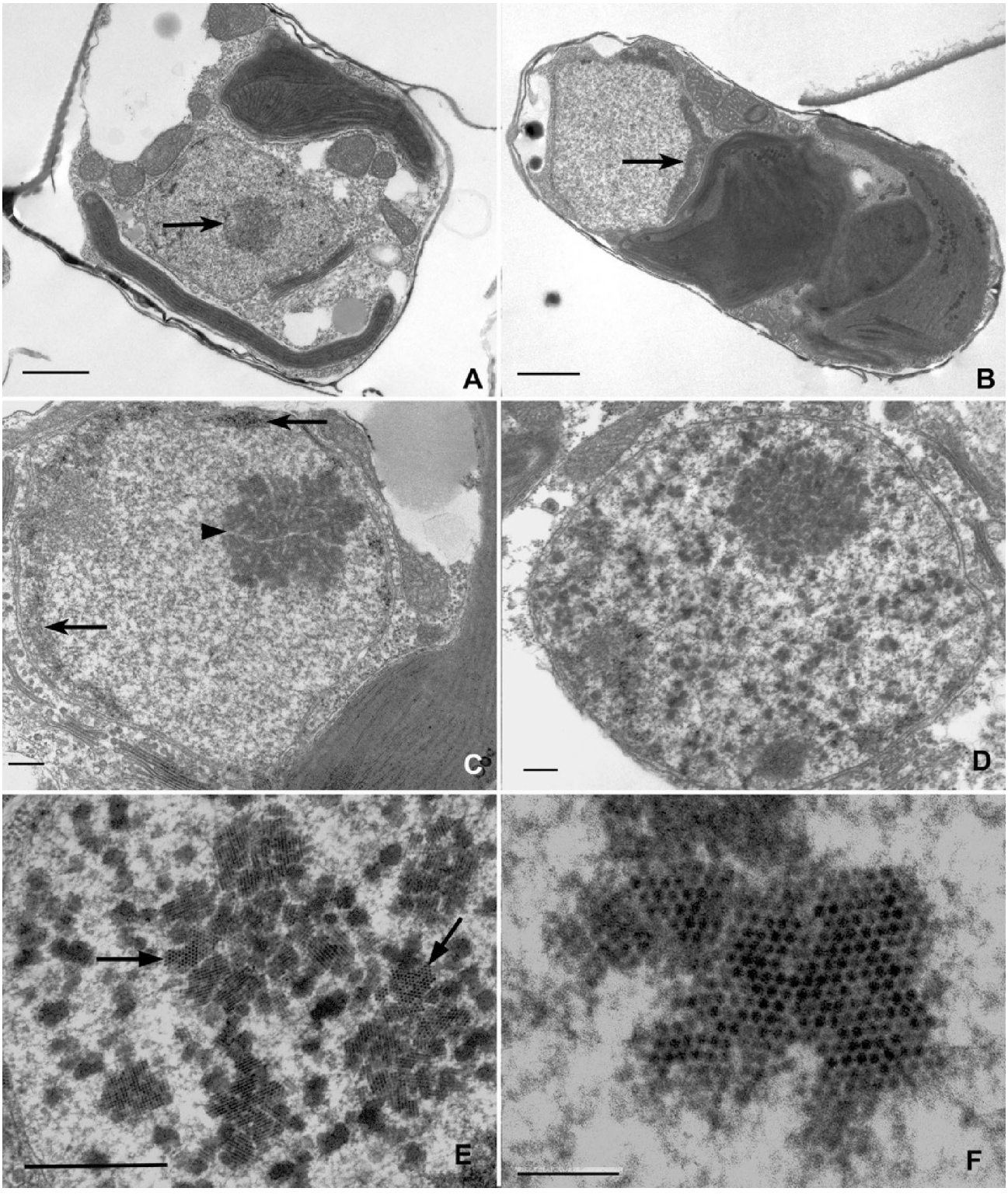

Fig. 5. Thin section electron micrographs of *Chaetoceros* cf. *gracilis* cultures. (A) Healthy cell, scale bar = 1 µm. (B–F) Cells infected by the pathogen CspNIV. (B) Post inoculation (12 h); note the accumulation of dense heterochromatin material at periphery of the nucleus, scale bar = 1 µm. (C–F) Post inoculation (24 h). Note: (C) scattered dense material at periphery of nucleus and large acentric nucleolus, scale bar = 500 nm; (D) numerous small dense masses and a large acentric mass that appears to be fragmenting, scale bar = 500 nm; (E) numerous patches of virus-like particles, scale bar = 500 nm; and (F) high magnification of virus-like particles, scale bar = 100 nm

CspNIV and HaNIV are also similar in the migration of heterochromatin to the periphery of the nucleus of infected cells. Medical studies have recently revealed that a self-destruction mechanism called apoptosis may be activated in response to biochemical alterations within the host cell, following infection by viruses (Flint et al. 2000). Apoptosis typically results in the migration of heterochromatin to the periphery of the nucleoplasm, without formation of viral progeny. This apoptosis-like phenomenon was also recently observed for the prymnesiophyte *Phaeocystis globosa* (C. P. D. Brussaard et al. unpubl. data) and linked to enhanced proteolytic activity. By preventing infected hosts from producing and releasing new viral progeny, apoptosis may therefore indirectly protect healthy individuals from viral attack (Brussaard 2004). Further investigations are needed to reveal the factors that initiate apoptosis.

Seasonal pattern in lysis of *Chaetoceros* cf. *gracilis*

Chaetoceros species were widely distributed in Chesapeake Bay during the non-summer months of 2003 and 2004, but peak densities in the 2 years differed by 1 order of magnitude. Highest concentrations of *Chaetoceros* species occurred in the middle and lower bay during late winter to early spring, coinciding with the typical pattern in the development of blooms by large centric diatoms (Harding et al. 1999). Although the abundance of *Chaetoceros* species differed considerably between 2003 and 2004, seasonal patterns in viral-associated lysis of *C.* cf. *gracilis* following inoculation with Chesapeake Bay VC were similar. Lysis of *C.* cf. *gracilis* cultures by Chesapeake Bay VC coincided with the seasonal appearance of *Chaetoceros* spp. within the same water samples from which the VC were derived. Widespread distribution of VC lytic activity followed bloom development, particularly in 2003. In addition, lytic activity of VC persisted into June of both years, approximately 1 mo after *Chaetoceros* populations had dissipated.

While seasonal patterns in abundance of *Chaetoceros* species and lytic activity of VC against *C.* cf. *gracilis* are consistent with viral control of host populations, results must be interpreted carefully. For example, we do not know how many *Chaetoceros* species present during winter-spring blooms are susceptible to viral infection, nor do we know the prevalence of viral infection in host species during the bloom cycle. Understanding the role of viruses in diatom population dynamics in Chesapeake Bay will require further research that addresses these, and related, issues.

The persistence of VC lytic activity against *Chaetoceros* cf. *gracilis* into June suggests that CspNIV remains infectious for about 1 mo after the disappearance of its host species. Factors governing loss of infectivity are uncertain, but include solar radiation, particulate matter, bacterial cells and temperature, as reported for other viral-host systems (Suttle & Chen 1992, Wommack, et al. 1996, Noble & Fuhrman 1997, Nagasaki & Yamaguchi 1998). The susceptibility of CspNIV to inactivation in the water column is not known, but bay sediments may offer a longer-term refuge for infectious particles (Lawrence et al. 2001).

Growth characteristics

With a latent period of less than 24 h, CspNIV falls within the range (3 to 48 h) reported for microalgal viruses (Brussaard 2004). In the 1-step growth experiment, the culture did not completely crash, and surviving cells were still viable as signs of fluorescence were still detectable after 108 h. A partial demise of infected host culture is not uncommon, having also been observed by Nagasaki et al. (2004) in the diatom *Rhizosolenia setigera* and the dinoflagellate *Heterocapsa circularisquama* (Nagasaki et al. 2003). In both cases, the presence of immobile, round cells resembling temporary cysts that might be more resistant to viral attack were reported. The same phenomena may have occurred in our experiments, as resting spores were relatively numerous in the inoculated treatment compared to control cultures (data not shown). It is likely that our viral inoculation was initiated towards the end of the exponential growth phase of the *Chaetoceros* culture, when a fraction of the population was no longer physiologically active and thus susceptible to viral infection. Because of this, viral propagation did not occur in all *Chaetoceros* cells. This exact scenario has been reported for a *Chlorella* virus (Van Etten et al. 1983). One may also think that the rebound in fluorescence may be due to an increase in a sub-population of resistant individuals as previously demonstrated with bacteria in chemostats (Lenski & Levin 1985). The question of whether survival of our *Chaetoceros* culture was facilitated by the physiological status of cells, or by acquired resistance needs to be elucidated.

Ecological implications

Among the major bloom forming taxa representative of Chesapeake Bay phytoplankton assemblages that were challenged with the seasonal VC, the diatom *Chaetoceros* sp. exclusively showed evidence of lytic infection. So far no algal viruses have been isolated from Chesapeake Bay, the largest estuary on the US East Coast. Given the generally poor impact of her-

bivory in regulating the appearance or persistence of microalgal blooms in the bay (Sellner & Olson 1985, Sellner et al. 1993), it is possible that virioplankton, including viruses which infect diatoms, are a prominent agent in shaping phytoplankton annual succession in the bay. In this specific case, further study is needed to determine the prevalence of CspNIV and its impact on the population dynamics of *Chaetoceros* sp. in Chesapeake Bay

Diatoms account for up to 25% of all organic carbon fixation on the planet; they also represent a major food resource for marine and freshwater microorganisms, zooplankton, and animal larvae, and are a major source of atmospheric oxygen. In addition, the sinking of diatom silica frustules to the benthos contributes to the diagenesis of 'diatomite' on the floor of the oceans (Werner 1977). Thus, diatom susceptibility to viral attack has significant biogeochemical and ecological implications. The host-virus system isolated here is expected to be an attractive tool to further elucidate the role of virioplankton in marine systems.

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LITERATURE CITED

Al-Lami F, Ledinko N, Toolan H (1969) Electron microscopic study of human NB and SMH cells infected with the parvovirus, H-1: involvement of the nucleolus. J Gen Virol 5:485–492

Bergh Ø, Børsheim KY, Bratbak G, Heldal M (1989) High abundance of viruses found in aquatic environments. Nature 340:467–468

Brussaard CPD (2004) Viral control of phytoplankton populations—a review. J Eukaryot Microbiol 51:125–138

Brussaard CPD, Thyrhaug R, Marie D, Bratbak G (1999) Flow cytometric analyses of viral infection in two marine phytoplankton species, *Micromonas pusilla* (Prasinophyceae) and *Phaeocystis pouchetii* (Prymnesiophyceae). J Phycol 35:941–948

Brussaard CPD, Short SM, Frederickson CM, Suttle CA (2004) Isolation and phylogenetic analysis of novel viruses infecting the phytoplankton *Phaecystis globosa* (Prymnesiophyceae). Appl Environ Microbiol 70:3700–3705

Chen F, Suttle CA, Short SM (1996) Genetic diversity in marine algal virus communities as revealed by sequence analysis of DNA polymerase genes. Appl Environ Microbiol 62:2869–2874

Chen F, Lu JR, Binder B, Liu YC, Hodson RE (2001) Application of digital image analysis and flow cytometry to enumerate marine viruses stained with SYBR Gold. Appl Environ Microbiol 67:539–545

Cottrell MT, Suttle CA (1991) Wide-spread occurrence and clonal variation in viruses which cause lysis of a cosmopolitan, eukaryotic marine phytoplankter, *Micromonas pusilla*. Mar Ecol Prog Ser 78:1–9

Culley AI, Lang AS, Suttle CA (2003) High diversity of unknown picorna-like viruses in the sea. Nature 424: 1054–1057

Evans CGT, Archer SD, Jacquet S, Wilson AC (2003) Direct measurements of the contribution of viral lysis and microzooplankton grazing to the decline of a *Micromonas* spp. population. Aquat Microb Ecol 30:207–219

Flint SJ, Enquist LW, Krug RM, Racaniello VR, Skalka AM (2000) Principles of virology—molecular biology, pathogenesis, and control. ASM Press, Washington, DC

Fuhrman JA (1999) Marine viruses and their biogeochemical and ecological effects. Nature 399:541–548

Fuxa JR (2004) Ecology of insect nucleopolyhedroviruses. Agric Ecosyst Environ 103:27–43

Guillard RRL, Ryther JH (1962) Studies on marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detinula confervatonula* (Cleve) Gran. Can J Microbiol 8:229–239

Harding LWJ, Degobbis D, Precali R (1999) Production and fate of phytoplankton: annual cycles and interannual variability. In: Malone TC, Malej A, Harding LWJ, Smodlaka N, Turner RE (eds) Coastal and estuarine studies: ecosystems and the land–sea margin, Vol 55. American Geophysical Union, Washington, DC, p 131–172

Henry CJ, Merkow LP, Pardo M, McCabe C (1972) Electron microscope study on the replication of AAV-1 in herpes-infected cells. Virology 49:618–621

Jacobsen A, Bratbak G, Heldal M (1996) Isolation and characterization of a virus infecting *Phaeocystis pouchetii* (Prymnesiophyceae). J Phycol 32:923–927

Kubai DF, Ris H (1969) Division in the dinoflagellate *Gyrodinium cohnii* (Schiller). A new type of nuclear reproduction. J Cell Biol 40:508–528

Lang AS, Culley AI, Suttle CA (2004) Genome sequence and characterization of a virus (HaRNAV) related to picorna-like viruses that infects the marine toxic bloom-forming alga *Heterosigma akashiwo*. Virology 320:206–217

Lawrence JE, Chan AM, Suttle CA (2001) A novel virus (HaNIV) causes lysis of the toxic bloom-forming alga *Heterosigma akashiwo* (Raphidophyceae). J Phycol 37: 216–222

Lenski RE, Levin BR (1985) Constraints on the coevolution of bacteria and virulent phage—a model, some experiments, and predictions for natural communities. Am Nat 125: 585–602

Nagasaki K, Yamaguchi M (1997) Isolation of a virus infectious to the harmful bloom causing microalga *Heterosigma akashiwo* (Raphidophyceae). Aquat Microb Ecol 13: 135–140

Nagasaki K, Yamaguchi M (1998) Effect of temperature on the algicidal activity and the stability of HaV (*Heterosigma akashiwo* virus). Aquat Microb Ecol 15:211–216

Nagasaki K, Tomaru Y, Tarutani K, Katanozaka N, Yamanaka S, Tanabe H, Yamaguchi M (2003) Growth characteristics and intraspecies host specificity of a large virus infecting the dinoflagellate *Heterocapsa circularisquama*. Appl Environ Microbiol 69:2580–2586

Nagasaki K, Tomaru Y, Katanozaka N, Shirai Y, Nishida K, Itakura S, Yamaguchi M (2004) Isolation and characterisation of a novel single-stranded RNA virus infecting the bloom-forming diatom *Rhizosolenia setigera*. Appl Environ Microbiol 70:704–711

Noble RT, Fuhrman JA (1997) Virus decay and its causes in coastal waters. Appl Environ Microbiol 63:77–83

Proctor LM, Fuhrman JA (1990) Viral mortality of marine bacteria and cyanobacteria. Nature 343:60–62

Rensel JE (1993) Severe blood hypoxia of Atlantic salmon (*Salmo salar*) exposed to the marine diatom *Chaetoceros concavicornis*. In: Smayda TJ, Shimizu Y (eds) Toxic phytoplankton blooms in the sea. Elsevier, New York, p 625–630

Rines JEB, Hargraves PE (1988) The *Chaetoceros* Ehrenberg (Bacillariophyceae) flora of Narragansett Bay, Rhode Island, USA. Bibl Phycol 79:1–196

Rines JEB, Theriot EC (2003) Systematics of Chaetocerotaceae (Bacillariophyceae). I. A phylogenetic analysis of the family. Phycol Res 51:83–98

Sandaa RA, Heldal M, Castberg T, Thyrhaug R, Bratbak G (2001) Isolation and characterization of two viruses with large genome size infecting *Chrysochromulina ericina* (Prymnesiophyceae) and *Pyramimonas orientalis* (Prasinophyceae). Virology 290:272–280

Schroeder DC, Oke J, Malin G, Wilson WH (2002) Coccolithovirus (Phycodnaviridae): characterisation of a new large ds DNA algal virus that infects *Emiliana huxleyi*. Arch Virol 147:1685–1698

Sellner KG, Olson MM (1985) Copepod grazing in red tides of Chesapeake Bay. In: White AW, Baden DG (eds) Toxic dinoflagellates. Elsevier, New York, p 245–251

Sellner KG, Brownlee DC, Bundy MH, Brownlee SG, KR Braun (1993) Zooplankton grazing in a Potomac River cyanobacteria bloom. Estuaries 16:859–872

Suttle CA (2000) Ecological, evolutionary, and geochemical consequences of viral infection of cyanobacteria and eukaryotic algae, 1st edn. In: Hurst CJ (eds) Viral ecology. Academic Press, San Diego, CA, p 247–296

Suttle CA, Chan AM (1995) Viruses infecting the marine Prymnesiophyte *Chrysochromulina* spp.: isolation, preliminary characterization and natural abundance. Mar Ecol Prog Ser 118:275–282

Suttle CA, Chen F (1992) Mechanisms and rates of decay of marine viruses in seawater. Appl Environ Microbiol 58: 3721–3729

Tai V, Lawrence JE, Lang AS, Chan AM, Culley AI, Suttle CA (2003) Characterization of HaRNAV, a single-stranded RNA virus causing lysis of *Heterosigma akashiwo* (Raphidophyceae). J Phycol 39:343–352

Tarutani K, Nagasaki K, Itakura S, Yamaguchi M (2001) Isolation of a virus infecting the novel shellfish-killing dinoflagellate *Heterocapsa circularisquama*. Aquat Microb Ecol 23:103–111

Tomaru Y, Katanozaka N, Nishida K, Shirai Y, Tarutani K, Yamaguchi M, Nagasaki K (2004) Isolation and characterization of two distinct types of HcRNAV, a single-stranded RNA virus infecting the bivalve-killing microalga *Heterocapsa circularisquama*. Aquat Microb Ecol 34:207–218

Van Etten JL, Burbank DE, Xia Y, Meints RH (1983) Growth cycle of a virus, PBCV-1, that infects *Chlorella*-like algae. Virology 126:117–125

Werner D (1977) Introduction with a note on taxonomy. In: Werner D (ed) The biology of diatoms. Botanical monographs, Vol 13. Blackwell Scientific, Victoria, p 1–23

Wilson WH, Tarran GA, Schroeder D, Cox M, Oke J, Malin G (2002) Isolation of viruses responsible for the demise of an *Emiliana huxleyi* bloom in the English Channel. J Mar Biol Assoc UK 82:369–377

Wommack KE, Colwell RR (2000) Virioplankton: viruses in aquatic ecosystems. Microbiol Mol Biol Rev 64:69–114

Wommack KE, Hill RT, Muller TA, Colwell RR (1996) Effects of sunlight on bacteriophage viability and structure. Appl Environ Microbiol 62:1336–1341

Wommack KE, Ravel J, Hill RT, Chun J, Colwell RR (1999) Population dynamics of Chesapeake Bay virioplankton: total-community analysis by pulsed-field gel. Appl Environ Microbiol 65:231–240

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