Winter 1997

Distribution of Free Marine Viruses of Lower Chesapeake Bay and Their Effects on Life-History Parameters of the Estuarine Copepod *Acartia tonsa* Dana

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DISTRIBUTION OF FREE MARINE VIRUSES OF LOWER CHESAPEAKE BAY AND THEIR EFFECTS ON LIFE-HISTORY PARAMETERS OF THE ESTUARINE COPEPOD *ACARTIA TONSA* DANA

by

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A Dissertation Submitted to the Faculty of
Old Dominion University in Partial Fulfillment of the
Requirement for the Degree of

DOCTOR OF PHILOSOPHY

OCEANOGRAPHY

OLD DOMINION UNIVERSITY
December 1997

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ABSTRACT

DISTRIBUTION OF FREE MARINE VIRUSES OF LOWER CHESAPEAKE BAY AND THEIR EFFECTS ON LIFE-HISTORY PARAMETERS OF THE ESTUARINE COPEPOD *ACARTIA TONSA* DANA.

Lisa A. Drake
Old Dominion University, 1997
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Naturally occurring viruses are very abundant in fresh, estuarine, and marine waters, with densities on the order of 10^5-10^8 viruses ml^{-1}. Research has focused on virus effects on bacteria, cyanobacteria, and phytoplankton, as well as mechanisms of virus production and decay. However, little is known about the distribution of viruses in benthic environments or virus effects on organisms in higher trophic levels.

To determine the distribution of virus-like particles (VLPs) and bacteria in the lower Chesapeake Bay, vertical profiles of VLPs and bacteria were determined through the water column and 15-25 cm into the sediment at five stations. VLPs were about ten times more abundant in sediment pore water (3.7×10^8 VLPs ml^{-1}) than in the water column (3.8×10^7 VLPs ml^{-1}). Similarly, bacteria counts were about three times higher in sediment pore water (6.4×10^6 bacteria ml^{-1}) than in the water column (2.4×10^6 bacteria ml^{-1}). In the water column, VLP and bacteria counts exhibited significant differences among stations, with highest values on the southern side of the Bay mouth. In the sediment pore water, VLP abundance varied with depth and was negatively correlated with grain size. Bacteria abundance was highest at the sediment-water-interface, decreased in the first cm

of sediment, was uniform in the deeper horizons, and showed no significant relationship with grain size. These are the first data indicating the abundance of VLPs below the surface layer of sediment in aquatic systems and demonstrate that VLPs are components of the sediment microbial community.

To evaluate virus effects on zooplankton, concentrated VLPs were added to cultures of *Acartia tonsa*, then egg production, egg hatching success, and mortality of copepods were measured. Elevated VLP concentrations were obtained by concentrating the virus-size fraction of fresh seawater or water from copepod cultures. Across six experiments, no detrimental effects of viruses on copepods were demonstrated. Similarly, adding pulverized copepods and copepod exudates to water containing healthy copepods yielded no measurable detrimental effect. Therefore, there was no support for the hypothesis that viruses infect and negatively affect the longevity and fecundity of *A. tonsa*.

ACKNOWLEDGMENTS

I am grateful for the advice and input from my dissertation committee members, Drs. Fred Dobbs, William Dunstan, Darcy Lonsdale, Lita Proctor, and Anthony Provenzano, Jr., whose range of expertise was very helpful in outlining and conducting this research. Drs. Dunstan and Provenzano graciously provided equipment and laboratory space in which to conduct experiments. My major professor, Dr. Fred Dobbs, not only provided guidance and the opportunity to do this research, but he is also a role model of the creative and thorough researcher, effective and inspiring teacher, and outstanding leader.

R.C. Kidd and Captains Robert Bray and Donnie Padgett have always been dependable and ready to help during many cruises aboard the R. V. *Linwood Holton*, and I appreciate their willingness to help me succeed. I am especially thankful for Captain Bray's encouragement and for his instruction in the art of knot tying.

Gail Bargerstock, Xianhao Cheng, Curtis Hickman, Kimani Kimbrough, Cathy McConaugha, Dr. John McLain, C. John Neely, Brian Weid, Rudy Zervigon, and Li Zhang provided assistance in the field and in the laboratory, and I am grateful to all. Michael Adam, Pamela Blades-Eckelbarger, and Dr. Keith Carson graciously shared their expertise in electron microscopy with me. In particular, I appreciate Michael Adam teaching me to prepare specimens for electron microscopy. His patience, enthusiasm, and curiosity made working with him delightful. Keun-hyung Choi and A.G. Edward Haskell helped work up data for the sediment pore water work, and Brian Parsons made the map of sampling stations.

Thank you to all three. I thank the always helpful members of the ODU Science Shop, Thurman Gardner, Bob Kizziar, and Bobby Powell. The sediment sampling device cleverly made by Mr. Gardner was an integral part of sampling for microbes in sediment pore water.

Financial assistance to complete this research was generously provided by Dr. Jacques S. Zaneveld and Sigma Xi, The Scientific Research Society. The Department of Oceanography at ODU provided support in the form of student stipends as well as ship time aboard the R. V. *Linwood Holton*. Thanks are due to Drs. David Burdige and Glen Wheless for providing financial support, as well as the opportunity to learn information and acquire skills outside the area of my dissertation research, during my tenure as a Ph.D. candidate.

I appreciate the efficient work and good humor of the ODU Oceanography Technical Support Staff, consisting of Dana Oblak, Holly Park, Andrea Haggins, and Mimi Terry (formerly at ODU), whose problem-solving proved invaluable in unraveling tuition-waiver, scholarship, and billing problems. Drs. Larry Atkinson and Greg Cutter stood up for me when I really needed it, and I am grateful for their faith in me.

Karen and Rich Richardson, Sarah Drake, Julius Drake, Anne West-Valle, Beth and Tony Foyle, Mary-Beth Moore, and Juli Homstead provided friendship and unflagging support during my pursuit of this degree. My parents, John and Cathi Drake, deserve special mention not only for their years of support and encouragement during my time at ODU, but also for encouraging me to pursue a career in oceanography. I thank them for believing in me and thank the "Drake

Foundation" for its generous contributions to this cause.

Lastly, I thank my husband and best friend, Rob Brumbaugh. Our countless discussions about this project certainly improved this research, in addition to making me a better scientist. I am most grateful for his unwavering confidence in me.

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CHAPTER I

INTRODUCTION

Naturally-occurring viruses in seawater are abundant, with densities on the order of 10^4-10^8 viruses ml^{-1} (e.g., Larsson et al., 1978; Torrella and Morita 1979; Bergh et al., 1989; Børsheim et al., 1990; Heldal and Bratbak, 1991; Weinbauer and Suttle, 1997; see review by Proctor, 1997). Viruses are found in a variety of environments, including estuarine (Wommak et al., 1992), coastal (Børsheim et al., 1990), coral reef (Paul et al., 1993), oceanic (Cochlan et al., 1993), deep water (Hara et al., 1996), and high-latitude systems (Bird et al., 1993; Steward et al., 1996). They infect bacteria (Smith and Krueger, 1954; Spencer, 1955; Frank and Moebus, 1987; Proctor and Fuhrman, 1992; Børsheim, 1993; Weinbauer and Suttle, 1996; Wilson and Mann, 1997), cyanobacteria (Proctor and Fuhrman, 1990; Suttle et al., 1990; Suttle and Chan, 1993; Waterbury and Valois, 1993; Wilson et al., 1993), eukaryotic phytoplankton (e.g., Mayer and Taylor, 1979; Seiburth et al., 1988; Suttle et al., 1990; Müller et al., 1996; see review by Van Etten et al., 1991 and reviews cited therein), including bloom-forming species (Milligan and Cosper, 1994; Nagasaki et al., 1994; Jacobsen et al., 1996), heterotrophic nanoflagellates (Nagasaki et al., 1993; Garza and Suttle, 1995), and crustaceans (Vago, 1966; Kuris et al., 1979; see review by Johnson, 1983). Although their widespread occurrence in marine organisms has only become evident within the last 20 years, it is not surprising, given the widespread occurrence of viruses in terrestrial and

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freshwater bacteria (Safferman and Morris, 1963), plants (Fry, 1996), and animals (Adams and Bonami, 1991).

The term "virus", Latin for "slimy liquid" or "slime", has been used since at least the first century A.D. In his discussion of canine rabies, Roman encyclopedist Cornelius Aulus Celsus stated, "Especially if the dog is rabid, the virus should be drawn out with a cupping glass" (Hughes, 1977). Celsus' use of the word "virus" in his writing either may indicate his understanding that rabies was transmitted via saliva, or reflect the most common connotation of virus at that time, which was "poison" or "venom" (Waterson and Wilkinson, 1978).

It was not until the late nineteenth century that scientists recognized the infectivity of particles smaller than most bacteria. The discovery of the virus is usually attributed to Dimitri Isoifovich Ivanovski and Martinus Willem Beijerinck. Both documented that tobacco mosaic virus, the agent which causes mottling of tobacco leaves, remained infectious after passage through a bacterial filter. Ivanovski demonstrated this phenomenon in 1892; Beijerinck independently discovered the same in 1898 and described the agent as "contagium vivum fluidum" (Waterson and Wilkinson, 1978). Although Ivanovski was technically the first person to discover that particles smaller than bacteria were infectious, he incorrectly postulated that the infectious quality was due to a bacterial toxin or perhaps a bacterium which had passed through a large crack in the filter (Hughes, 1977). Six years later, Beijerinck correctly hypothesized that the infectious, filterable fluid was a noncellular entity.

Shortly after the first discovery of a plant virus, animal and bacterial viruses

were discovered. The first documented animal virus, the agent of bovine foot and mouth disease, was reported in 1898 by Friedrich Johannes Löffler and Paul Frosch. Viruses of the bacteria were discovered by Frederick W. Twort in 1916 and by Felix d'Hérelle in 1917; D'Hérelle called the viruses "bacteriophage", for "bacteria eaters" (Levine, 1992). Since the coining of the word bacteriophage, it has been shortened to "phage". A virtual explosion of virus research has occurred since these landmark discoveries. Technological advances, such as the development of the electron microscope and the elucidation of the structure of DNA (Watson and Crick, 1953), have helped us determine the biochemical structures as well as life histories of viruses that infect both terrestrial and marine organisms.

Viruses in the Marine Environment

Life histories of marine viruses suggest that their abundance is either tied to the processes that govern the abundances of microbial and larger organisms, or that viral abundances are linked directly to the organisms. Free-living viruses exhibit 10-4000 fold seasonal variations in abundance (Bergh et al., 1989; Bratbak et al., 1990; Wommack et al., 1992). Virus concentrations decrease from coastal waters to offshore waters and from surface to deeper waters (Hara et al., 1991; Paul et al., 1991; Cochlan et al., 1993). These trends in abundance are also seen in organisms in the classical (Ryther, 1969) and the microbial (Hara et al., 1991) food webs. Other aspects of viral life cycles that suggest their importance in carbon flow are the rapid changes in virus concentrations that can occur, the sometimes sudden decay of viruses (Bratbak et al., 1992), and the presence of virus-like particles in sediment-trap debris (Proctor and Fuhrman, 1991; Gowing,

1993).

Historically, marine viruses were not considered important components of marine ecosystems. It was assumed until recently that the number of marine bacteriophage was low, 10^0-10^4 plaque forming units (PFU) per ml of seawater (Moebus, 1987). In retrospect, this assumption emerged because counting methods were poor. As summarized by Moebus (1987), "The failure of attempts to detect marine bacteriophages is probably due to the lack of suitable methods, as was the case during the first half of this century when no phage could be found in offshore waters". Densities of bacteria-infecting viruses were estimated by a plate method; PFUs on host bacterial lawns were counted. A plate method was also used to determine the abundance of bacteria in marine waters, but has been virtually discarded because fewer than 1% of naturally occurring marine bacteria can be cultured (Jannasch and Jones, 1959). If laboratory-grown bacteria are underestimated by two or more orders of magnitude, it is probable that the number of bacteriophage are grossly underestimated when enumerated by number of PFUs. Indeed, the PFU method of enumeration of bacteriophage has been shown to be inadequate (e.g., Bergh et al., 1989). New counting methods using transmission electron microscopy and epifluorescent microscopy have shown that free virus concentrations in seawater can range from 10^4-10^8 viruses ml^1 (e.g., Proctor and Fuhrman., 1990; Heldal and Bratbak, 1991; Hara et al., 1991; Cochlan et al., 1993; Weinbauer and Suttle, 1997). Another factor that probably contributed to the earlier underestimation of marine bacteriophages is host specificity of viruses. If a seawater sample that contained bacteriophage was plated onto a

bacteria strain for which the bacteriophage was not specific, no plaques would be produced, causing a researcher to erroneously conclude that no bacteriophage were present. Indeed, marine bacteriophage and cyanophage can exhibit host specificity (Spencer, 1955; Suttle and Chan, 1993).

Statement of the Problem

To date, most research on viruses in seawater has focused on either the ecology of viruses in the water column or the human-health risks of specific pathogens found in sediments near sewage outfalls or in shellfish tissues. Research results from the last decade suggest that the hosts for the majority of free viruses in marine and freshwater systems are bacteria. Thus, any investigation of free viruses should include a concurrent investigation of naturally-occurring bacteria. Given the widespread distribution of viruses in seawater and freshwater and their occurrence in surface sediment samples, I hypothesized that viruses are also found in subsurface sediments in marine environments and they play a role in structuring sedimentary communities, as they do in the water column. To address this hypothesis, I examined a subset of the microbial community--viruses, bacteria, and phytoplankton--and physical characteristics of the sediments and the water column across the mouth of Chesapeake Bay.

Research on the ecology of viruses in the water column has concentrated on microbial populations and primary producers, with little investigation of virus effects on organisms in higher trophic levels. Experimental evidence has shown that cell lysis by viruses occurs in bacteria and phytoplankton, and causal relationships between viruses and overall health of aquaculture crustaceans have

been demonstrated. Given the widespread occurrence of viruses and virus-like particles in bacteria, cyanobacteria, phytoplankton, crustaceans, sediment trap debris, and in the water column, it is reasonable to assume that crustacean zooplankton are also infected with viruses. Due to the prominence of copepods in the marine ecosystem, the motivation exists to investigate the interactions between copepods and viruses. I hypothesized that viruses infect copepods and negatively influence copepod populations by reducing their egg production and increasing their mortality. I performed a series of experiments to demonstrate infection of the copepod *Acartia tonsa* Dana by viruses.

CHAPTER II

VERTICAL PROFILES OF VIRUS-LIKE PARTICLES AND BACTERIA IN THE WATER COLUMN AND SEDIMENTS OF CHESAPEAKE BAY

Although the existence of aquatic viruses has been known for decades (Spencer, 1955; Valentine et al., 1966; Torrella and Morita, 1979), their prominence as functional members of aquatic microbial communities first was hypothesized when high numbers, on the order of 10^6-10^8 viruses and virus-like particles (VLPs) ml^{-1}, were counted in fresh- and saltwater environments (e.g., Berg et al., 1989; Børsheim et al., 1990; Klut and Stockner, 1990; Hara et al., 1991; Paul et al., 1993). Subsequent field and laboratory experiments demonstrated that viruses can control aquatic microbial populations at various trophic levels. At the bottom of the microbial food web, for example, bacteria can be killed by viruses at a rate equal to or greater than mortality caused by the agents thought to be their primary grazers, heterotrophic protozoans (Proctor and Fuhrman, 1990; Weinbauer and Peduzzi, 1994; Fuhrman and Noble, 1995; Weinbauer et al., 1995). Furthermore, up to 62% of the mortality of free-living marine bacteria and 52% of particle-associated marine bacteria may be attributable to viruses (Proctor et al., 1993), with an average of 20% of marine bacteria infected (Suttle, 1994).

Viruses can exert profound effects on phytoplankton populations. Viruses and VLPs have been found in marine phytoplankton and cyanobacteria (e.g., Proctor and Fuhrman, 1990; Van Etten et al., 1991; Müller and Stache, 1992;

Müller et al., 1996), and viruses specific for eukaryotic algae (Mayer and Taylor, 1979; Cottrell and Suttle, 1991; Lanka et al., 1993; Milligan and Cosper, 1994; Cottrell and Suttle, 1995; Müller et al., 1996) and cyanobacteria (Waterbury and Valois, 1993; Suttle and Chan, 1993; Wilson et al., 1993) have been isolated. The presence of viruses nearby (Bratbak et al., 1990) and within (Sieburth et al., 1988; Nagasaki et al. 1993; Bratbak et al., 1993; Brussard et al., 1996) marine phytoplankton cells during and at the end of algal blooms suggests that viruses negatively affect high-density algal populations. Laboratory experiments have demonstrated viruses indeed can have detrimental effects on phototrophs. Suttle et al. (1990) added concentrated viruses to a wide variety of primary producers and observed a 70-95% decrease in *in vivo* fluorescence. Similarly, primary productivity decreased as much as 78% following addition of concentrated viruses to natural seawater samples (Suttle, 1992). The genetic variation in marine algal viruses (Cottrell and Suttle, 1991; Chen et al., 1996) may indicate analogous complexity within the entire marine viral community.

Higher up the trophic gradient, marine microzooplankton are also infected by viruses. Nagasaki et al. (1993) estimated that 20% of an unidentified apochlorotic flagellate population associated with a dinoflagellate bloom was infected with VLPs. Garza and Suttle (1995) isolated a virus that infects and lyses 2 strains of the heterotrophic nanoflagellate genus *Bodo,* and it can reduce flagellate abundance almost 100-fold in 48 h. In another ecological niche, viruses can be ingested by flagellates (Suttle and Chen, 1992; González and Suttle, 1993). At high virus concentrations, flagellates grazing on viruses can acquire up to 9%

of the carbon, 14% of the nitrogen, and 28% of the phosphorus that they acquire by grazing on bacteria (González and Suttle, 1993).

Given the potential for viruses to control marine and freshwater microbial communities (Hennes and Simon, 1995; Hennes et al., 1995; Mathias et al., 1995; Middelboe et al., 1996) and, therefore, production of dissolved organic matter (DOM) and nutrient cycling, the effects of viruses have been incorporated into models of marine pelagic microbial food webs (Bratbak et al., 1992; Bratbak et al., 1994; Murray and Eldridge, 1994). On the other hand, much less is known about the abundance, distribution, and functional role of viruses in sediments. There are only a few reports concerning benthic viruses, all of which have demonstrated viruses to be more abundant in surface sediment and sediment pore water than in the overlying water. Viruses were 10-1,000 times more abundant in the top 1 cm of sediment in Lac Gilbert, Canada than in the water column (Maranger and Bird, 1996). Similarly, viral counts in surface sediments in Key Largo and Tampa Bay, U.S.A. were almost 100 to 1,000 times greater than counts from the water column (Paul et al., 1993; Rose and Reynolds, unpublished data). Viruses in pore water squeezed from the uppermost cm of sediment from the Chukchi Sea were about 9 times more abundant than viruses in the surface water (Steward et al., 1996).

There have been no reports of viruses below the surface horizon of the sediment. In this dissertation, I present vertical profiles of virus-like particles and bacteria at the mouth of a large estuary, profiles that extend from surface waters, through the water column, and into the sediment. I use these data to infer possible functional roles of VLPs in the sediment communities of coastal areas.

Materials and Methods

Study site. Samples were collected on 4 March 1997, during the spring phytoplankton bloom (Dobbs et al., unpublished data), at five stations along a transect across the mouth of the Chesapeake Bay (Fig. 1; station coordinates listed in Appendix A).

Water-column samples. A Seabird Model 25 CTD was used to determine vertical profiles of water temperature, salinity, and density. A submersible pump was used to collect three independent water samples from the surface, mid-depth, and one meter above the bottom at each station. The pump was flushed for 1 minute at each depth before three independent samples were taken for direct counts of virus-like particles (VLPs) and bacteria, and for determination of chlorophyll *a* concentration. For VLP counts, approximately 1 ml of seawater was poured into sterile microfuge tubes. For bacteria counts, 10 ml of seawater was transferred to glass vials containing 750 μl of filtered (0.2 μl) formalin (2.6 % final concentration of formaldehyde). Chlorophyll *a* samples were taken by filtering 100 ml of seawater onto 47 mm-diameter glass fiber filters (GF/F Whatman) at a vacuum pressure of 150 mm Hg.

Box-coring device. A spade-type box coring device with a rectangular core (10.5 cm x 17.5 cm x 35 cm) was used to collect sediment with a minimum of disturbance. The device had a hinged cutting arm that sealed the sample *in situ*.

Sediment pore-water samples. At each station, the boxcore was deployed three times, thus, triplicate sediment samples were collected. The vertical profile of sediment collected by the boxcore was exposed, and samples were removed

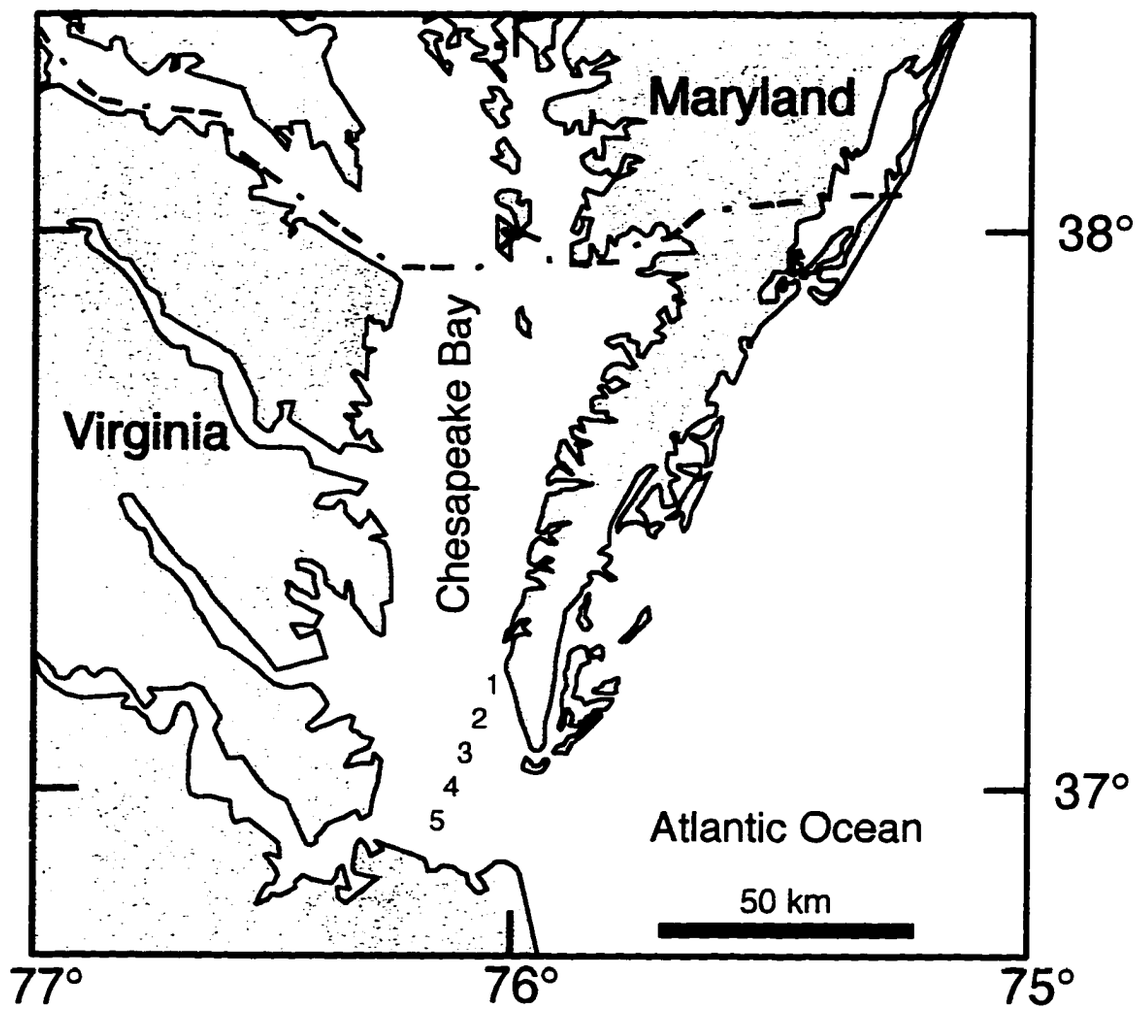


FIG. 1. Map of lower Chesapeake Bay, with stations indicated by numbers.

from six horizons: sediment-water interface, 0-1 cm, 1-2 cm, 2-3 cm, 3-4 cm, and deep (1 cm sample between 11-27 cm depending on the depth of the core) (depths are relative to the sediment-water interface). Samples of sediment for VLP and bacteria counts in sediment pore water were collected in sterile 15 ml disposable centrifuge tubes. Samples for determining sediment characteristics were collected in glass vials. Samples for VLP and bacteria counts were centrifuged in a Damon/IEC Division HN-S centrifuge for 10-50 minutes at ca. 745 x g to express pore water. Water-column and sediment pore-water samples for VLP counts were diluted 2x and 100x, respectively, with 0.02 μm-filtered distilled, deionized water.

VLP enumeration and counting accuracy. VLPs were counted using the method of Hennes and Suttle (1995). Briefly, water samples were diluted with 0.02 μm-filtered distilled, deionized water, filtered onto 0.02 μm-pore size Acrodisc™ filters, and stained with the nucleic acid stain YO-PRO™-1 iodide (491/509) (Quinolinium,4-[(3-methyl-2(3H)-benzoxazolylidene)methyl]-1-[3-(trimethylammonio)propyl]-,diiodide). Filters were stored in the dark at -85°C until they were randomly chosen (in groups of two), thawed in the dark at room temperature for ca. 5 minutes, and VLPs on them were counted using an Olympus BX50 System Microscope with a BX-FLA epifluorescence attachment. See Plates 1a and 1b for micrographs of water-column and sediment pore-water samples collected at station 5, then diluted 2 x and 100 x, respectively, with 0.02 μm-filtered distilled, deionized water, stained with YO-PRO™-1, and examined by epifluorescent microscopy.

a

b

PLATE 1. Micrograph of water-column sample (a) and sediment pore-water sample (b) stained with YO-PRO™-1 and examined by epifluorescent microscopy. VLPs are the round, green circles; bacteria are bigger, irregularly shaped, and more yellow than VLPs; detritus particles are yellow. Magnification = 1,000 x

Five control filters were prepared using 0.02 μm-filtered distilled, deionized water and the average of their VLP counts was subtracted from field samples. In order to determine the number of microscopic fields necessary to estimate accurately the number of VLPs on a filter, a running variance:running mean ratio was calculated from VLP counts of sediment pore water collected at Station 3.

Epifluorescent and transmission electron microscopy of VLPs. To practice using the YO-PRO™-1 technique to enumerate viruses, coliphage T2 (Carolina Biological Supply Company) were stained with YO-PRO™-1 and examined by epifluorescent microscopy. To confirm that YO-PRO™-1 stained viruses, qualitative comparisons of coliphage were made using epifluorescent and transmission electron microscopy (TEM). Densities of coliphage prepared for epifluorescent and electron microscopy were 1.4×10^{7} ml^{-1} (determined by direct counts) and 1.2×10^{10} ml^{-1} (determined by Carolina Biological Supply Company), respectively.

To confirm that YO-PRO™-1 stained viruses in sediment pore water, qualitative comparisons of VLPs in sediment pore water were made using epifluorescent microscopy and TEM. The sediment pore water was expressed from sediment collected at station 5, and it was not diluted.

Samples of coliphage and sediment pore water were prepared for TEM as follows: for 60 minutes, each of 3 formvar-coated copper mesh grids was floated with the formvar side down on a drop of coliphage or sediment pore water that was fixed with electron-microscopy grade glutaraldehyde (final concentration = 1%), then each grid was rinsed by submerging it in each of 3 drops of deionized water.

Grids were stained for 10 s with uranyl acetate by floating grids upside down on a drop of stain, then grids were examined at an accelerating voltage of 60 or 80 kV and a magnification of 10,000 x - 100,000 x on a JEOL 100CX II transmission electron microscope.

Efficiency of VLP extraction from sediment pore water. A known concentration of coliphage T2 in 0.85% saline was added to archived sediment from the study site, pore water was expressed by centrifugation, and VLPs were counted as described above. Extraction efficiency was determined by dividing the VLP counts in coliphage-amended sediment pore water (n = 4) by the sum of VLP counts in un-amended pore water (n = 3) plus VLP counts of coliphage only (n = 4). Again, VLP counts were determined as described above.

VLPs vs. dissolved DNA. It is conceivable that YO-PRO™-1 could stain dissolved DNA, thereby resulting in inflated estimates of VLP abundance. Hennes and Suttle (1995) demonstrated this scenario is unlikely when YO-PRO™-1 is used to enumerate VLPs in water-column samples. However, it was appropriate to test this possibility using sediment pore water given that sediments are the ultimate burial site for DNA produced in the water column and are host to high numbers of bacteria (Rublee, 1982), a potential source of dissolved DNA.

VLP counts were compared between sediment pore-water samples treated with the DNA-degrading enzyme Deoxyribonuclease I (DNase I) and control samples. In September 1997, sediment was collected from Station 5 and pore water was expressed as previously described. Two hundred-fifty Kunitz units of DNase I (type II, from bovine pancreas, Sigma Chemical Company, D-4527) in

25 μl of 0.15 M NaCl were added to a 1-ml sample of sediment pore water and incubated at room temperature for 30 minutes (Suttle, 1993; Hennes and Suttle, 1995). (0.15 M NaCl, equivalent to 8.8 ppt NaCl, is approximately the salinity of physiological saline, and it was used as a carrier solution for the DNase according to the recipe provided by Sigma Chemical Co.) This amount of DNase will digest several μg ml^{-1} of DNA (Hennes and Suttle, 1995), a concentration 100-1000 times greater than the amount of DNA dissolved in the water column. Dissolved DNA values range from 0.2 - 44 μg l^{-1} for deep offshore water and estuarine water, respectively (DeFlaun et al., 1986). To a 1-ml control sample of pore water, 25 μl of 0.15 M NaCl were added, and the sample was also incubated. Next, both the control and experimental samples were diluted 100x, four subsamples were removed from each, and VLPs were enumerated by epifluorescence microscopy. A colleague randomly labeled the subsamples before the VLPs were enumerated so that the VLPs were counted without my knowing which treatment they represented.

To confirm the DNase digested DNA in seawater, its activity was assayed. Two hundred-fifty Kunitz units of DNase were added to 35 μg ml^{-1} of DNA (Type I, "highly polymerized" from calf thymus, Sigma Chemical Company, D-1501) in seawater (salinity = 20, assumed to be representative of the salinity of the pore water collected at stations 1-5), and the temporal change in absorbance at 260 nm was monitored using a Shimadzu UV-Visible Recording Spectrophotometer UV160U.

Bacteria enumeration. Samples were stored in the dark at 4°C for less than

six weeks until they were filtered onto 0.2 μm black polycarbonate filters (Poretics) and stained with the nucleic acid stain DAPI (4' 6-diamidino-2-phenylindole) (final concentration of 1 μg ml^{-1})(Porter and Feig, 1980). Filters were stored in the dark at -85°C until the bacteria were counted using epifluorescence microscopy (see above).

Chlorophyll *a* determination. Filters were wrapped in foil and stored at -85°C until they were homogenized, and chlorophyll *a* was extracted in acetone and measured fluorometrically (Parsons et al., 1992).

Sediment characteristics. Sediment from the study site was analyzed to determine its particle size, water content, and combustible fraction. To determine particle-size distribution, samples from the 0-1 cm horizon and deep horizon (n = 3 per horizon) were wet-sieved through a series of nested sieves (1000, 500, 250, 125, and 63 μm) using distilled water. The fraction < 63 μm was collected by vacuum filtration on dried filters (Whatman GF/F). All fractions were placed into tared aluminum dishes and dried to constant mass at 60°C. Mean grain size was calculated as Folk's M_{z50} (Folk, 1980). To determine water content, whole sediment samples from all six horizons (n = 3 per horizon) were weighed, then dried to constant mass at 60°C. To determine the combustible fraction, a surrogate measure of organic content, samples from all horizons (n = 3 per horizon) were placed in tared aluminum dishes, dried overnight at 60°C, combusted for 4 h at 450°C, cooled in a desiccator, and weighed again to determine the mass difference following combustion.

Results

Hydrographic data. The water column was strongly stratified at all stations across the mouth of the bay, based on changes of temperature and salinity with depth (Fig. 2a-c). Water temperature ranged from 7.6° (Station 1, surface) to 8.9 °C (Station 5, mid-depth), and salinity ranged from 16.2 (Station 4, surface) to 28.0 (Station 1, bottom). Density profiles (sigma-t) were forced by salinity at Stations 1-3 and by both temperature and salinity at Stations 4 and 5. Average chlorophyll *a* values in water-column samples ranged from 7.7 (Station 5, bottom) to 19.5 mg m^{-3} (Station 4, bottom). A consistent pattern among stations was an increase in the average chlorophyll *a* concentrations in the water over the core (WOC) samples relative to the deepest water-column samples (Fig. 2d).

VLP controls and counting accuracy. The mean of the control filters, 4.31×10^5 VLPs ml^{-1} (S.E.=7.0×10^4), was 1.1% and 0.12% of the grand means of VLP counts in the water column and sediment pore water, respectively. A running variance:running mean ratio was calculated to determine the number of microscopic fields necessary to estimate accurately the quantity of pore water VLPs on a filter (Fig. 3). The ratio stabilized after eight 100μm x 100μm fields were counted, with an average number of 33 VLPs per field. For all samples in this study, therefore, VLPs in ten fields were counted on each filter.

Epifluorescent and transmission electron microscopy of VLPs. A qualitative examination of coliphage T2 prepared for epifluorescent and TEM demonstrated abundant viruses in both cases (Plates 2 and 3). Examination of undiluted sediment pore water showed numerous VLPs (Plate 4), and TEM examination

FIG. 2. Water-column profiles of temperature (a), salinity (b), sigma-t (c), and chlorophyll *a* (d) at stations 1-5. Data in plot (d) are mean values (n = 2-3) ± 1 SE (for data with n = 3); data points not connected to others are samples from water over the box core (WOC).

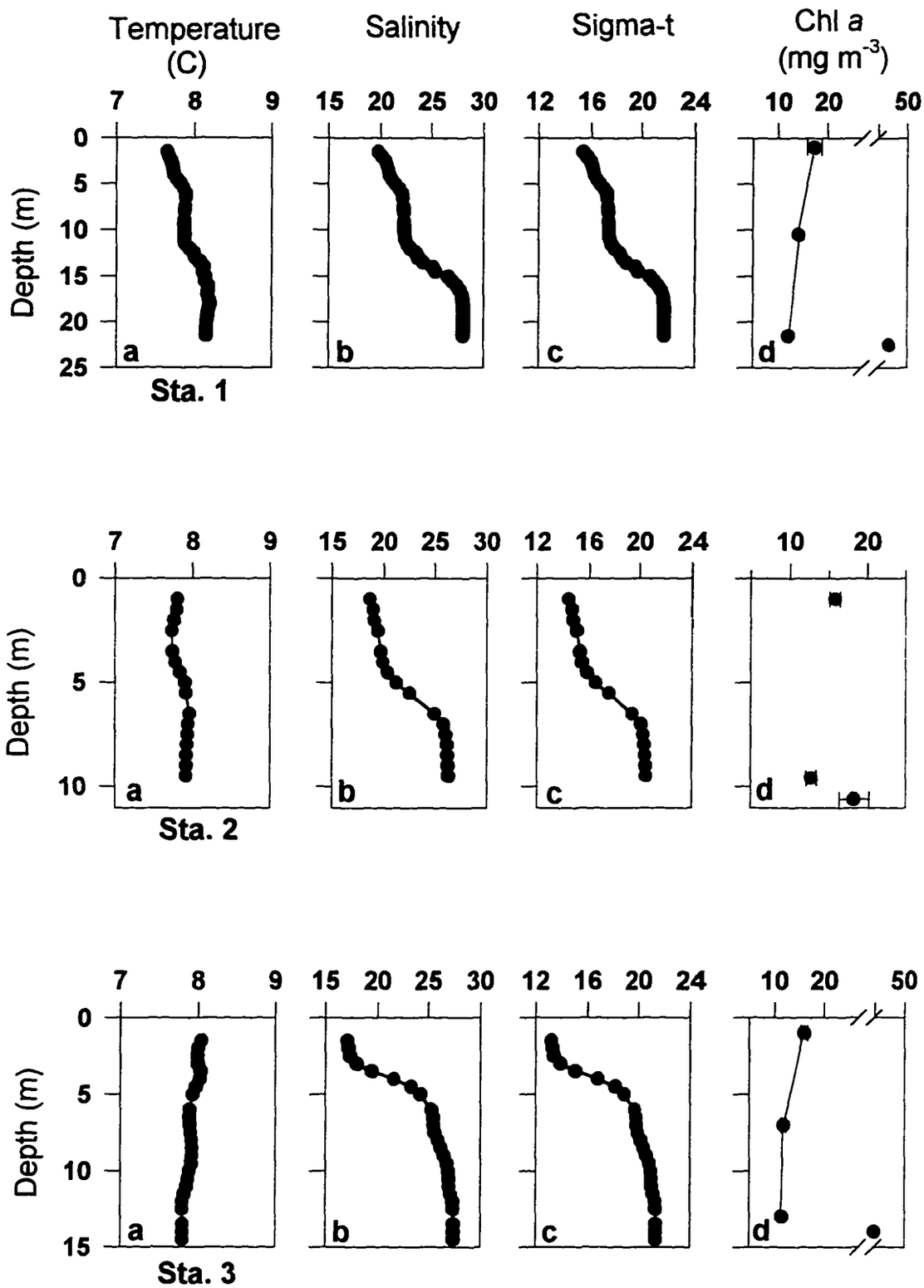
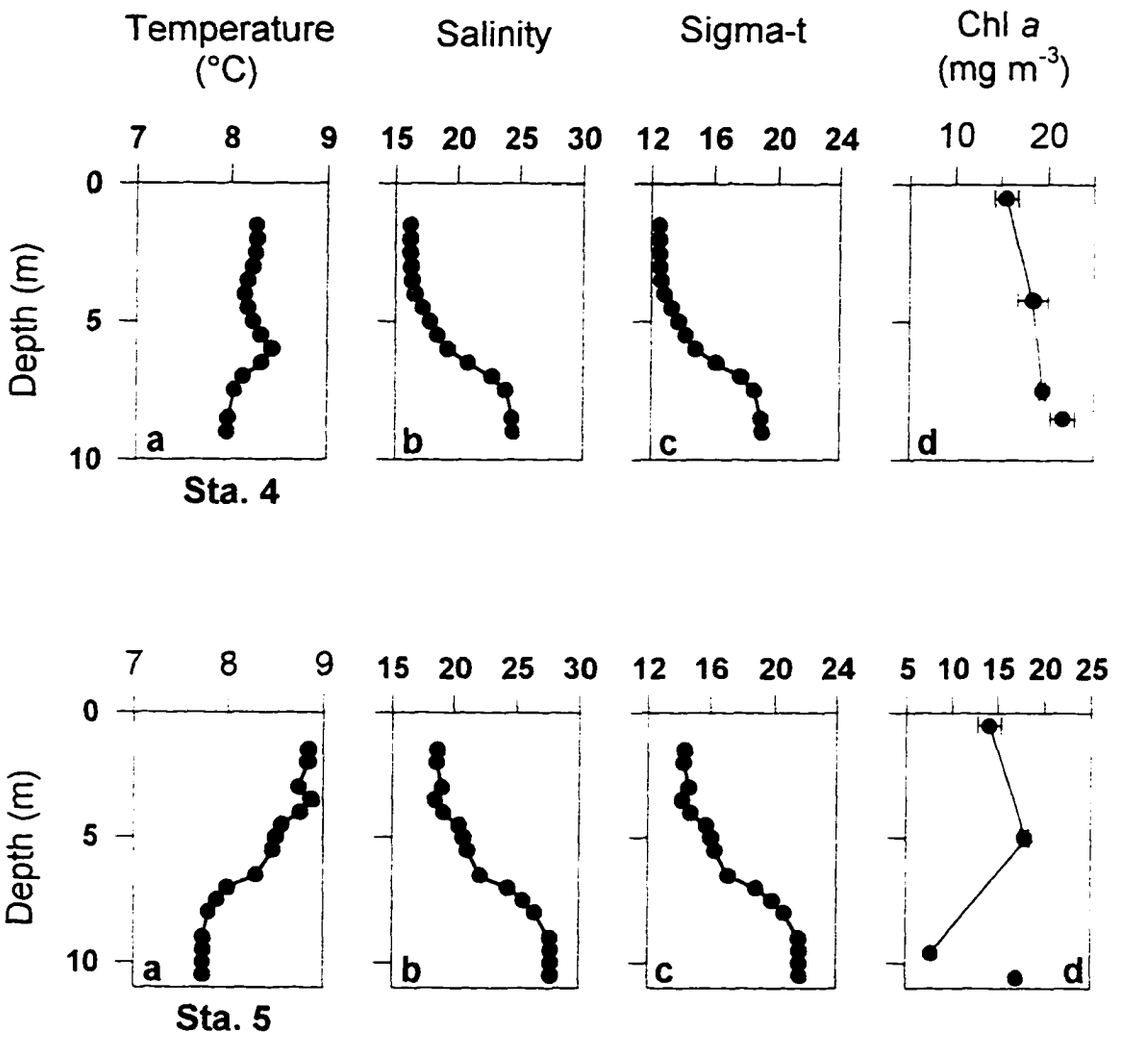


FIG. 2 Continued



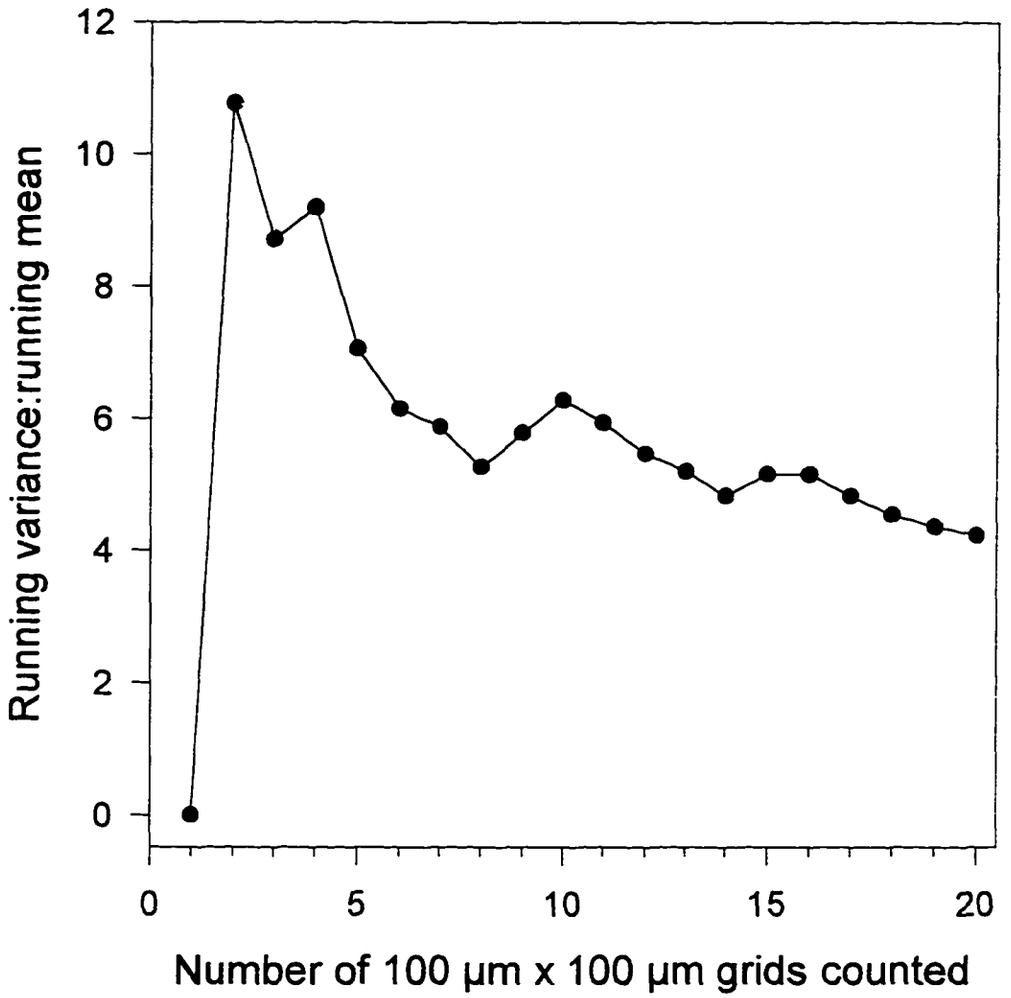


FIG. 3. Calibration of VLP counting. Sediment pore water was stained with YO-PRO-1™, counted using epifluorescence microscopy, and the running variance : mean ratio was calculated.

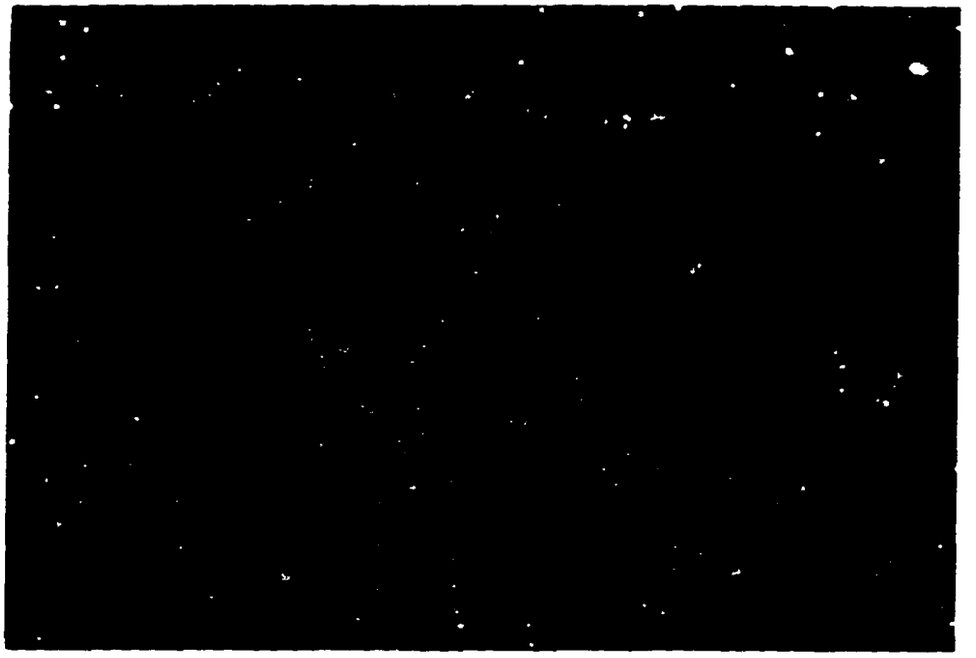


PLATE 2. Micrograph of coliphage T2 stained with YO-PRO™-1 and examined by epifluorescent microscopy. Coliphage are the round, yellow circles. Magnification = 1,000 x

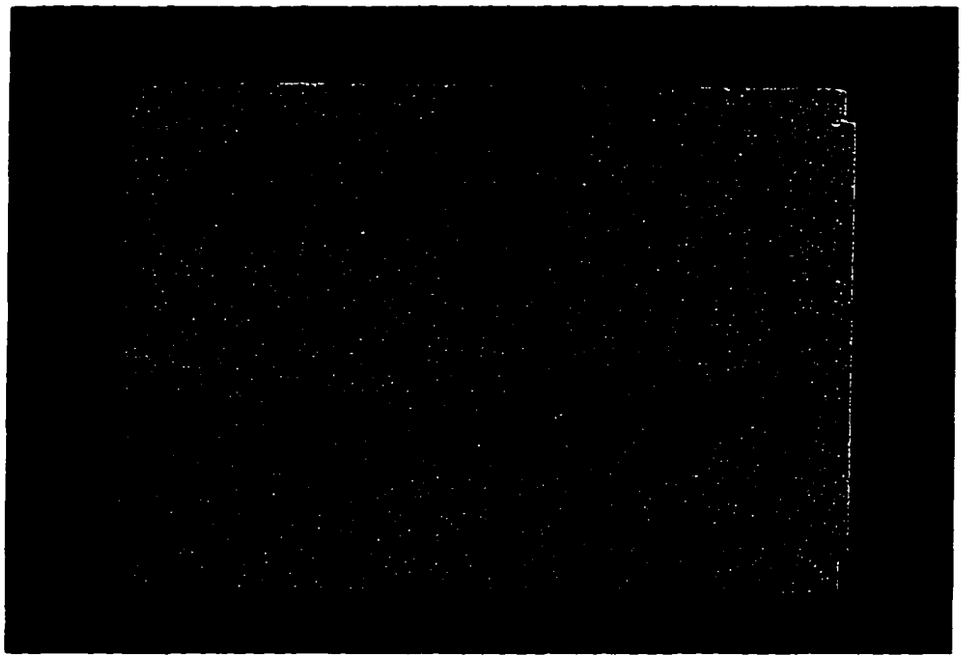


PLATE 3. Micrograph of coliphage T2 stained with uranyl acetate and examined with transmission electron microscopy. Magnification = 19,000 x.

PLATE 4. Micrograph of sediment pore water stained with YO-PRO™-1 and examined by epifluorescent microscopy. VLPs are the round, green circles; bacteria are bigger, irregularly shaped, and more yellow than VLPs; detritus particles are yellow. Magnification = 1,000 x

showed virus-like heads with diameters of 34 - 43 nm (Plate 5).

VLP extraction from pore water. Mean efficiency of VLP extraction using coliphage T2 was 65.3% (SE = 2.9) (Fig. 4). VLP counts from field samples were not corrected to reflect this efficiency however, because viruses used for the efficiency test were a monoculture of one size class (70 nm x 100 nm head diameter), and they did not reflect the size range of VLPs in the water column of natural seawater, typically 20 to 250 nm head diameter (Fuhrman and Suttle, 1993), with smaller viruses most abundant (30-60 nm, Bergh et al., 1989, Wommack et al., 1992; < 60 nm, Bratbak et al., 1990, Cochlan et al., 1993).

VLPs vs. dissolved DNA. There was no significant difference in the concentration of VLPs in DNase-treated pore-water samples and control samples ($p = 0.1564$, t-test, SAS version 6.09, Fig. 5). The DNase assay showed degradation of DNA within 10 minutes and a change in absorbance of 0.0012 min^{-1} ml^{-1} of DNA substrate ($p = 0.0001$, regression analysis, Fig.6).

VLPs in the water column and in sediment pore water. VLP concentrations were about 10 times higher in sediment pore water samples (grand mean = 3.7 x 10^8 VLPs ml^{-1}) than in the water column samples (grand mean = 3.8 x 10^7 VLPs ml^{-1}) (Fig. 7) ($p = .0001$, Wilcoxon rank sum test). Mean VLP concentrations were similar throughout the water column within each station (Table 1). Mean VLP concentrations varied significantly among stations (range = 2.6 to 5.8 x 10^7 VLPs ml^{-1}), increasing steadily from Station 1 to 5 (north to south) along the transect ($p = 0.037$, 1-way ANOVA on ranked data; Conover and Iman, 1981; Potvin and Roff, 1993) (Table 1). In contrast, mean VLP concentrations in the sediment pore water

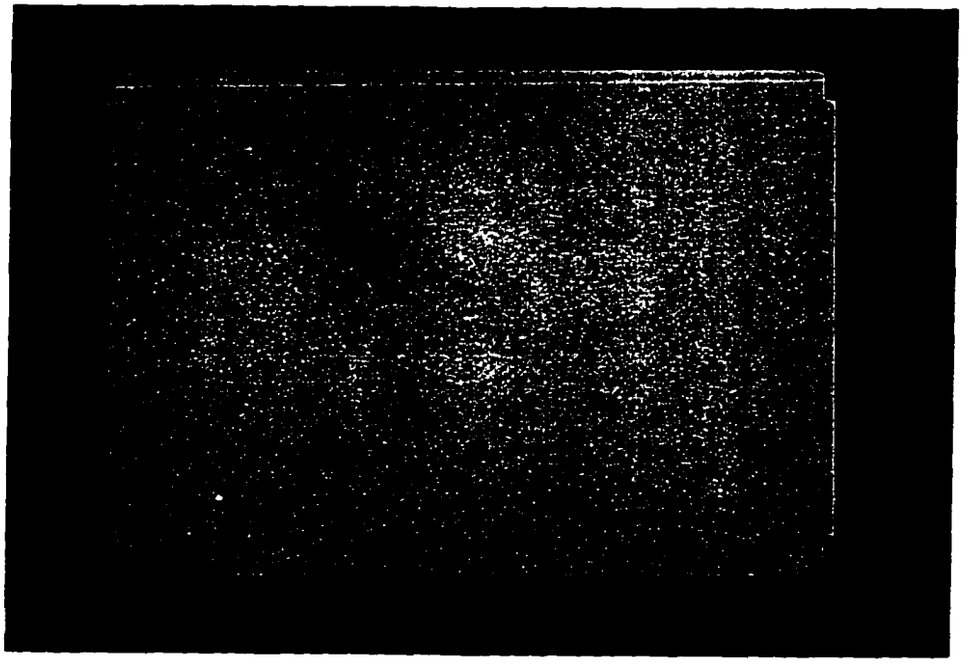


PLATE 5. Micrograph of sediment pore water examined by transmission electron microscopy. Virus-like head diameters are 34 - 43 nm. Magnification = 58,000 x.

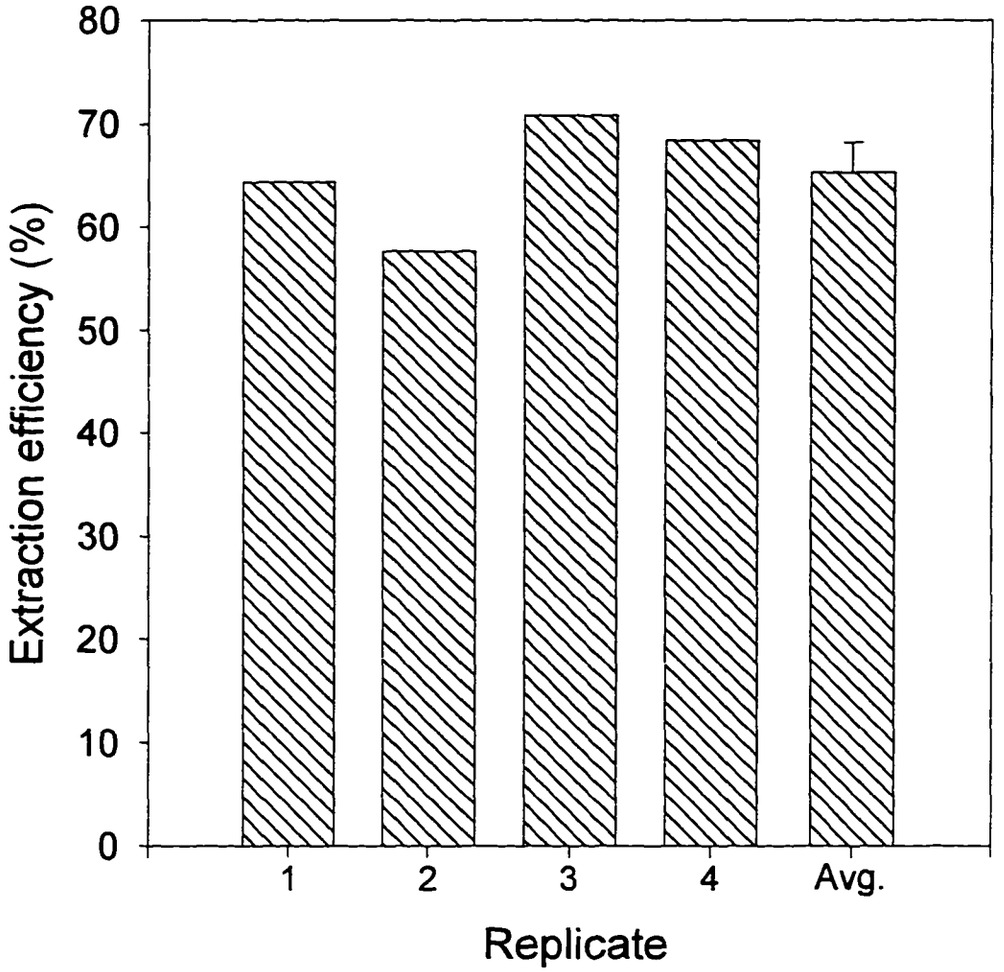


FIG. 4. Extraction efficiency of coliphage T2 from sediment. Error bar is 1 SE.

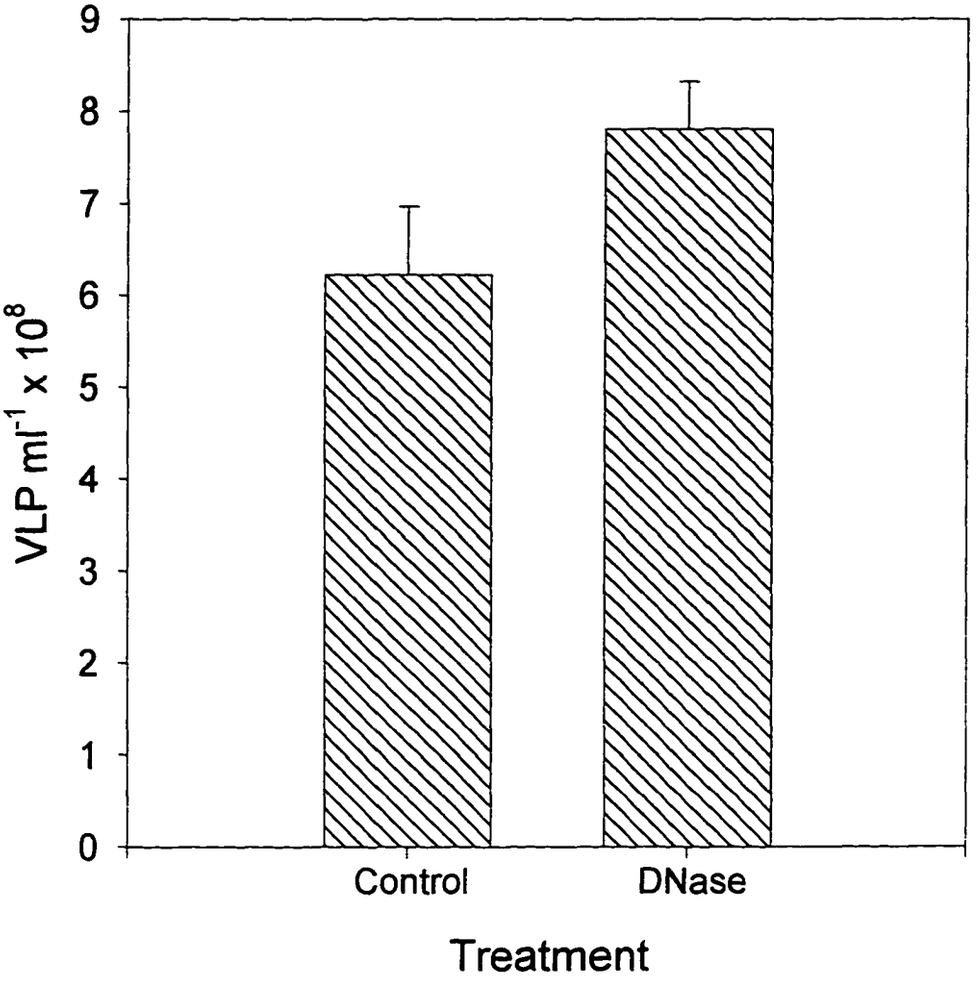


FIG. 5. VLP counts in samples of sediment pore water treated with DNase and in controls. Data are mean values (n = 4) ± 1 SE.

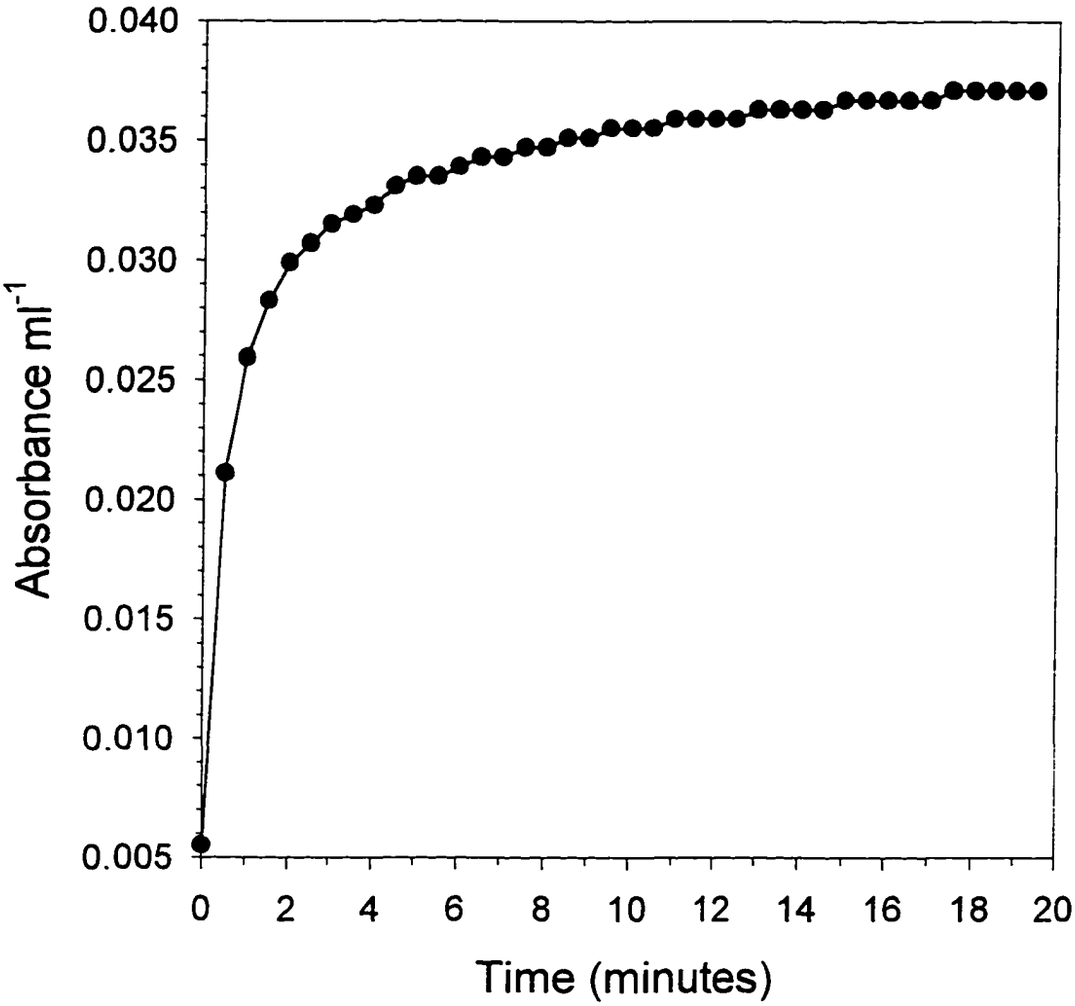


FIG. 6. Absorbance (λ = 260 nm) over time of DNA after addition of DNase.

FIG. 7. Distribution of VLPs through the water column and into the sediment at stations 1-5. Data are mean values (n = 2-3) or single observations. Error bars (± 1 SE) are used when n = 3. WOC = water over box core; dashed line indicates the sediment-water-interface.

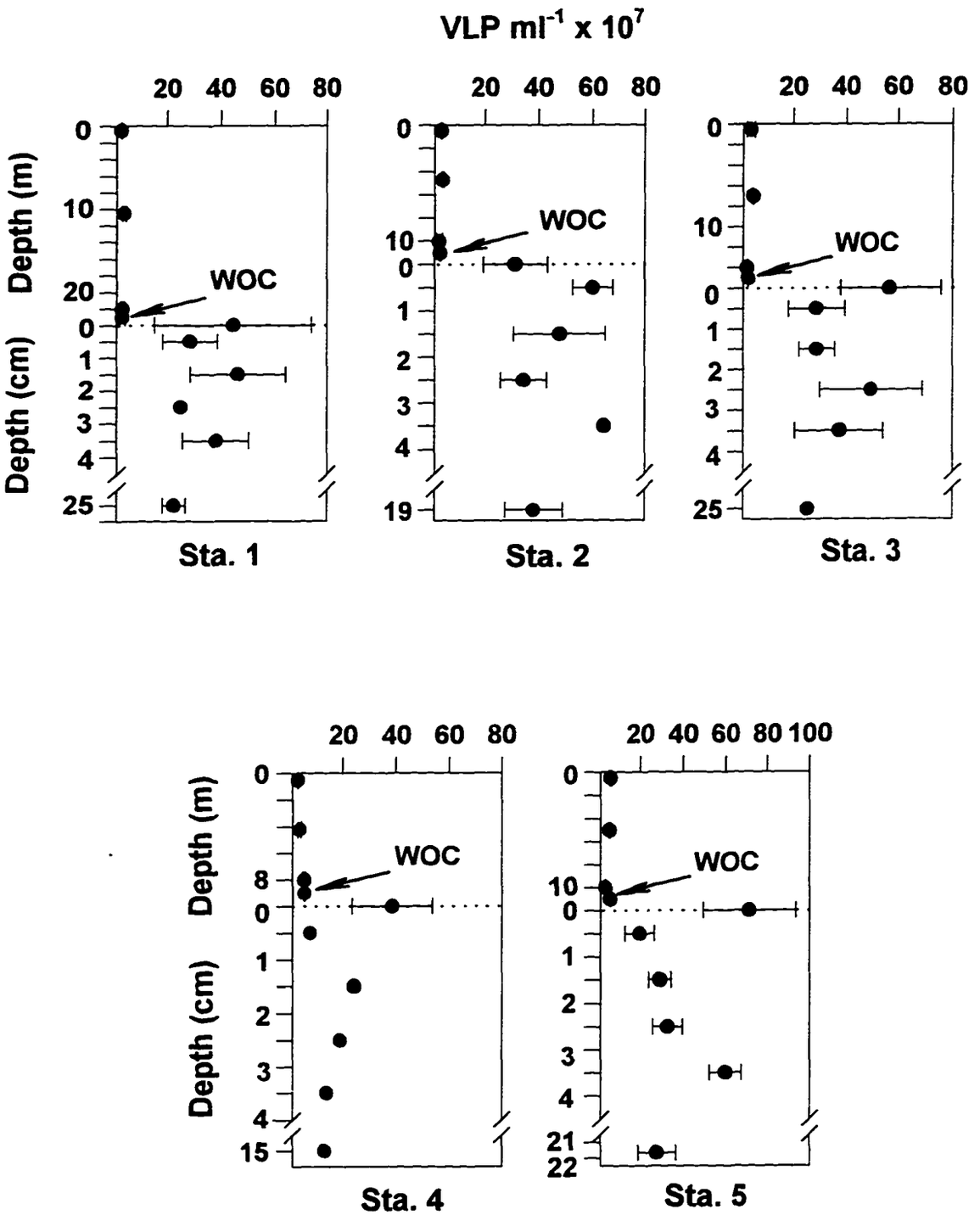


TABLE 1. Grand means[a] of VLPs and bacteria in the water column (WC) and sediment (SED) at stations 1-5[b].

Station	VLP $ml^{-1} \times 10^7$		Bacteria $ml^{-1} \times 10^6$	
	WC	SED	WC[c]	SED
1	3.03^{B}	34.16^{AB}	2.34^{AB}	6.71^{A}
2	3.25^{B}	44.43^{A}	2.13^{B}	6.16^{A}
3	3.63^{B}	38.46^{AB}	2.46^{AB}	5.93^{A}
4	4.26^{AB}	21.69^{B}	2.49^{AB}	7.34^{A}
5	5.00^{A}	39.57^{AB}	2.49^{A}	6.47^{A}

[a]Data are mean values (n= 6-18)

[b]Superscripts represent results of *a posteriori* tests. Tukey's Studentized Range (HSD) Test was used for sediment data (sample sizes were unequal), Ryan-Einot-Gabriel-Welsch Multiple F test for water-column data (sample sizes were equal). Means with different letters are significantly different within the column ($p<0.05$).

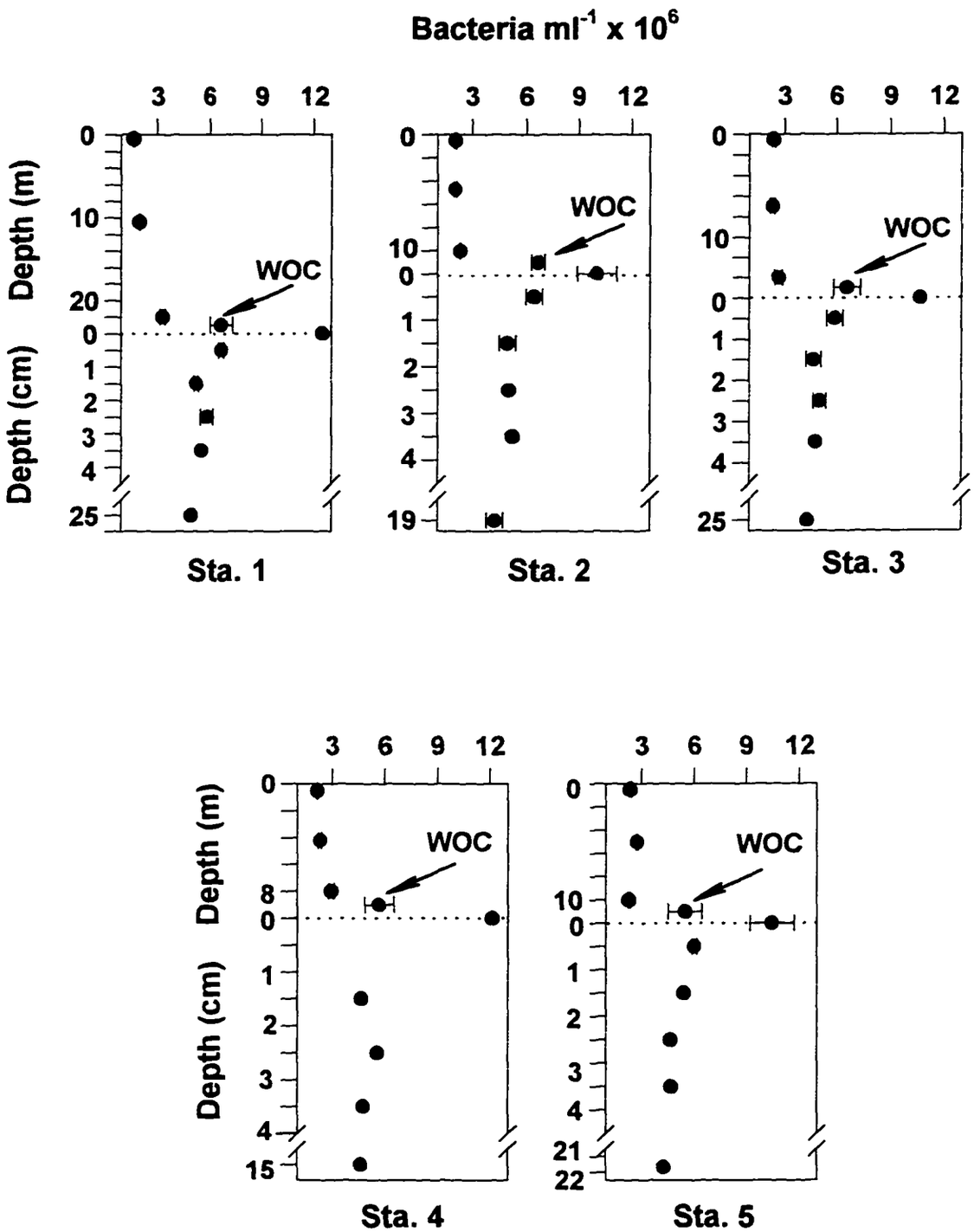
[c]Samples from water over the core (WOC) excluded from calculations.

samples were more variable (range = 1.9 to 7.1 x 10^8 VLPs ml^{-1}) at each depth horizon and with depth in the sediment. Although VLP concentrations were significantly different among stations, they did not exhibit the steady north to south increase seen in the water-column values ($p = 0.0007$, 1-way ANOVA on ranked data) (Table 1). A consistent pattern among stations was a 7.3- to 18.7-fold increase in average VLP concentrations in sediment-water-interface samples relative to WOC samples.

Bacteria in the water column and sediment pore water. Bacteria concentrations were higher in the sediment pore water samples (grand mean = 6.4 x 10^6 cells ml^1) than in the water column samples (grand mean including and excluding WOC samples = 3.3 x 10^6 cells ml^{-1} and 2.4 x 10^6 cells ml^{-1}, respectively) (*n* = 1-3) (Fig. 8), a significant difference (including and excluding WOC samples, $p = 0.0001$, Wilcoxon rank sum test). Mean bacterial densities were similar throughout the water column (range = 1.7 to 3.3 x 10^6 cells ml^{-1}), but increased about three-fold in the water overlying the sediment in the boxcore. Differences in water-column bacteria densities among stations were significant, with highest values at the southernmost station, when WOC samples were excluded from the calculations ($p = 0.038$, 1-way ANOVA on ranked data) (Table 1). Within the sediment pore water, mean bacterial concentrations were maximum at the sediment-water interface, decreased in the first cm, and ranged little (4.3 to 6.7 x 10^6 cells ml^{-1}) down to 15-25 cm depth (Table 1, Fig. 8). Bacterial densities in the sediment pore water did not vary significantly among stations (Table 1).

Virus to bacteria ratio (VBR). The average VBR in the water column was similar

FIG. 8. Distribution of bacteria through the water column and into the sediment at stations 1-5. Data are mean values (n = 2-3) or single observations. Error bars (± 1 SE) are used when n = 3. WOC = water over box core; dashed line indicates the sediment-water-interface.



among stations and ranged from 11.8 to 17.2 (Fig. 9). The lowest VBR at each station was in the WOC sample (Fig. 9), a result caused by the high bacterial counts in these samples (Fig. 8). Within sediment pore water, the average VBR was consistently higher and more variable and ranged from 29.3 to 84.7 (Fig. 9).

Sediment characteristics. The sediments from each station consisted of very fine to medium sand, with mean particle size varying from 99 to 370 μm and an average silt-clay percentage varying from 2.9 to 30.7% (Table 2). The average water content of the sediment ranged from 16.8 to 32.1% across stations (Fig. 10). The average combustible fraction of sediment from all stations ranged 0.3 to 2.1% (Fig. 10). Percent water covaried with percent combustibles, otherwise, there were no trends among stations.

Property-property plots. A series of scatter plots were constructed to infer possible relationships between the sediment parameters and the biological parameters. A significant correlation between VLPs and bacteria occurred in the water column samples when WOC data were excluded from the calculations (Spearman's coefficient, r_s, = 0.31; p = 0.04) (Fig. 11a). Note that VLP concentrations varied much less in the water column than in the sediment pore water. When only sediment samples were examined, there was a significant negative correlation between VLP counts and mean particle size (Spearman's coefficient, r_s, = -0.41, p = 0.043) (Fig. 11d). There were no significant correlations between VLP abundance and percent water content or percent combustibles (Figs. 11b,c).

FIG. 9. Virus to bacteria ratio (VBR) through the water column and into the sediment at stations 1-5. Data are mean values (n = 2-3) or single observations. Error bars (± 1 SE) are used when n = 3. WOC = water over box core; dashed line indicates the sediment-water-interface.

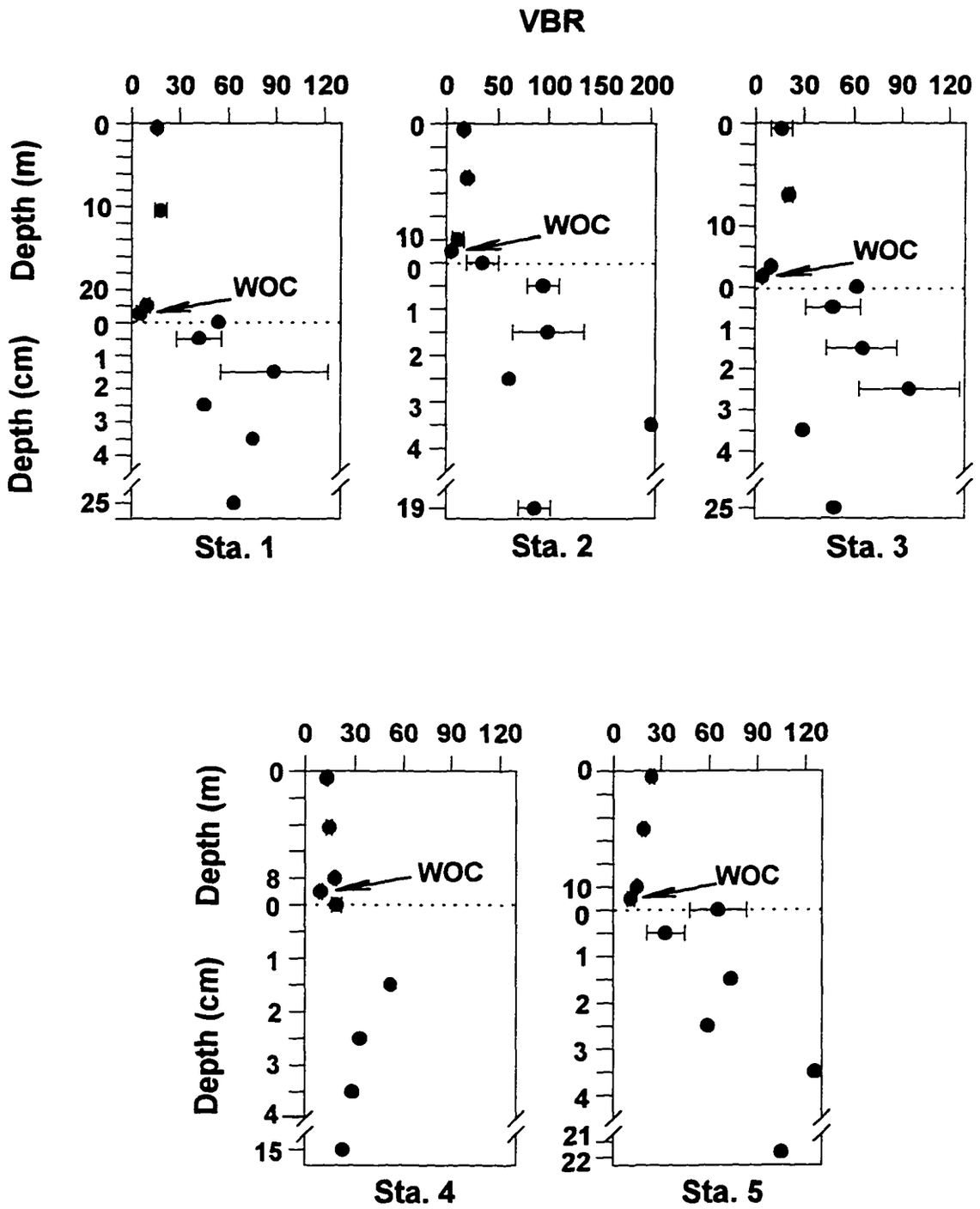
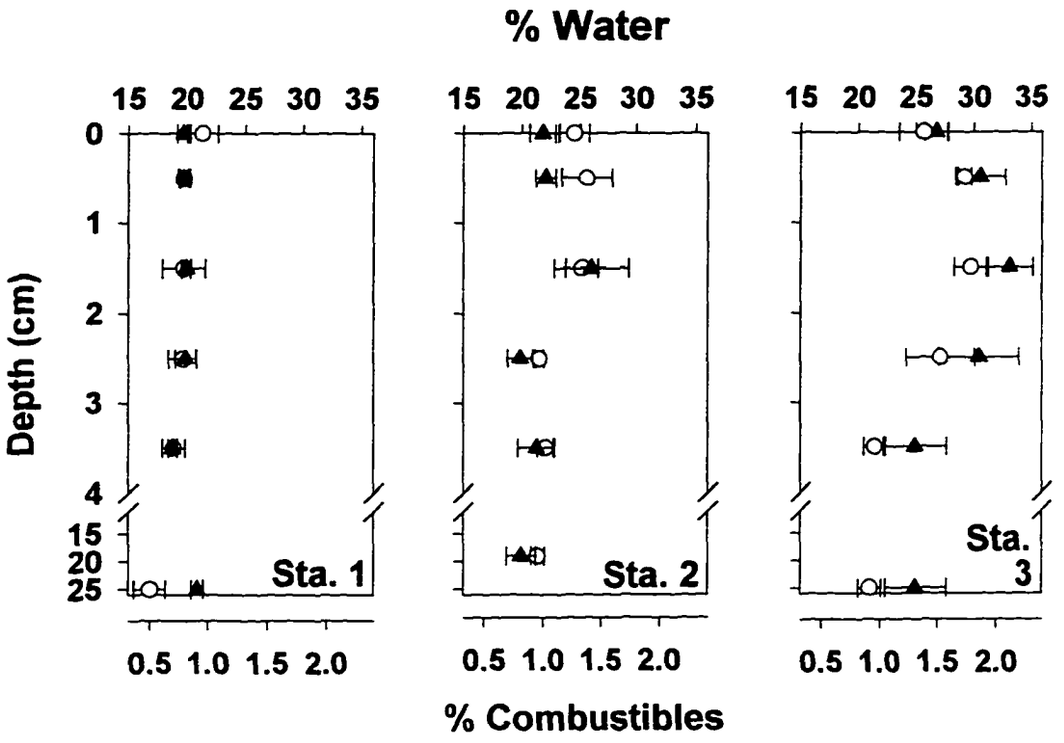


TABLE 2. Particle size and percent silt-clay in the uppermost and deepest sediment horizons at stations 1-5. Data are mean values (n = 3) with SE in parentheses.

Station	Depth (cm)	Particle Size (μm)	% Silt-Clay
1	0.5	302 (74)	6.4 (2.6)
	25.0	213 (30)	16.1 (5.2)
2	0.5	145 (2)	6.8 (0.2)
	19.0	169 (14)	10.6 (0.9)
3	0.5	160 (27)	12.5 (4.6)
	25.0	171 (22)	8.2 (1.7)
4	0.5	370 (36)	2.9 (0.2)
	15.0	299 (4)	3.2 (0.6)
5	0.5	205 (35)	5.2 (2.5)
	21.3	99 (10)	30.7 (4.5)

FIG. 10. Percent water and percent combustibles in the sediment at stations 1-5. Percent water = ○; Percent combustibles = ▲. Data are mean values (n = 3) ± 1 SE.



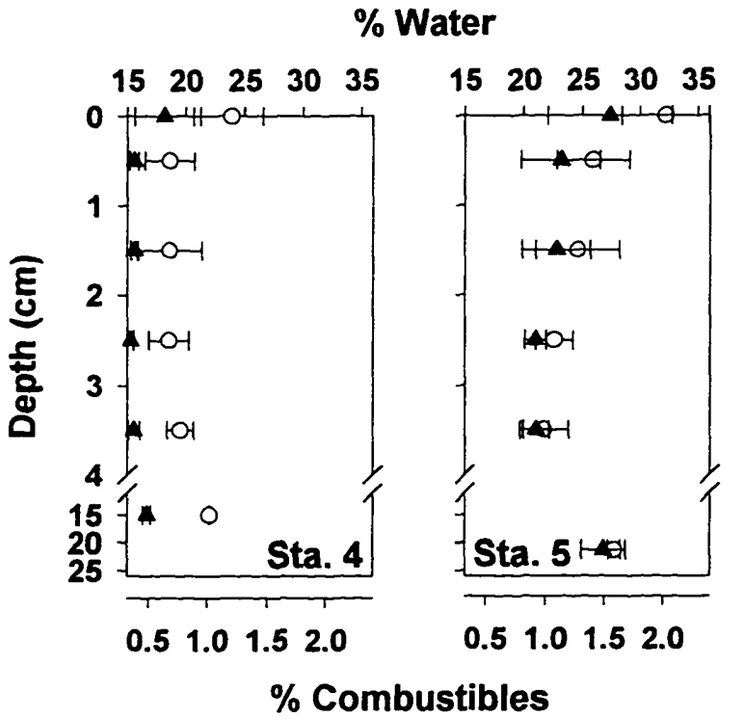
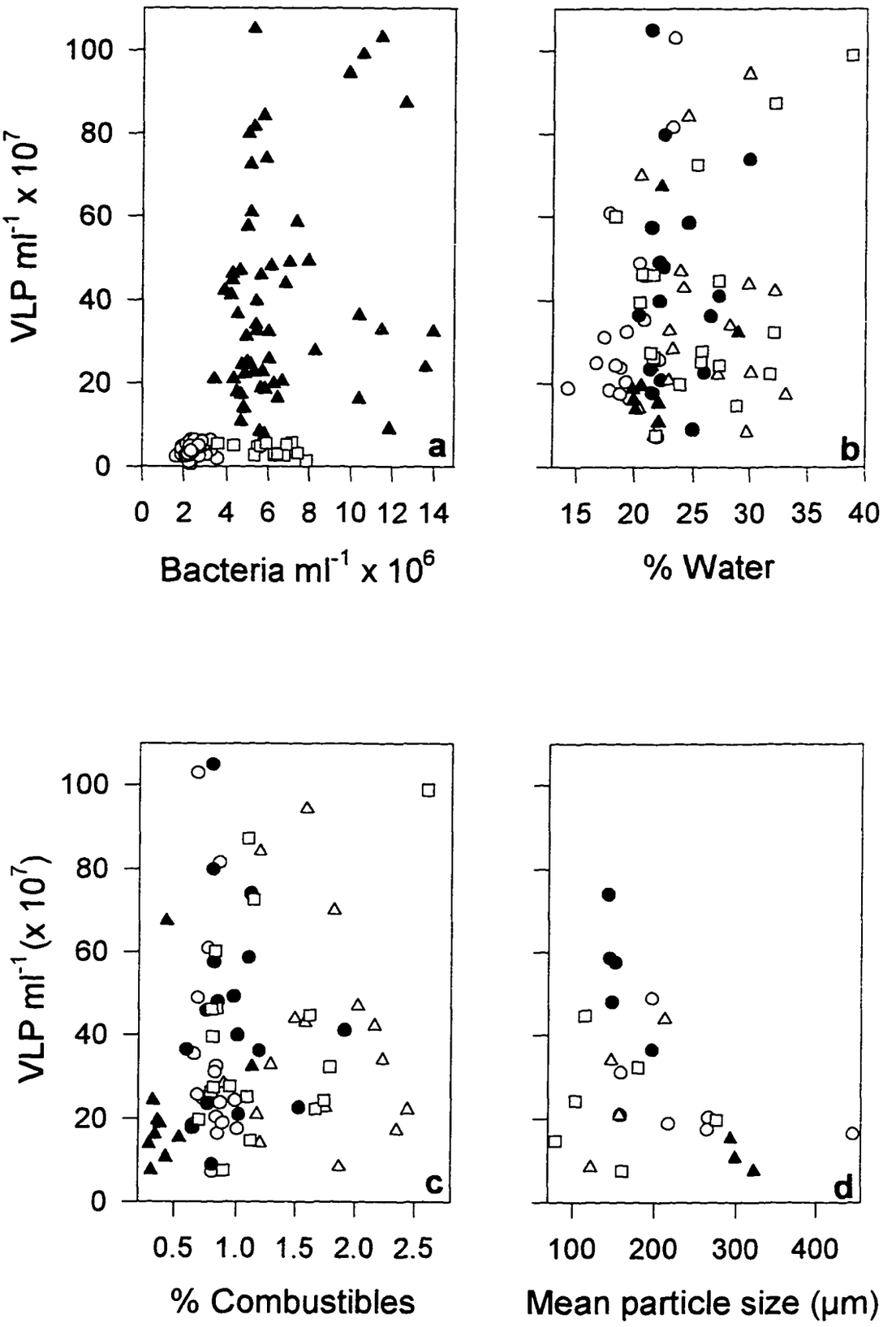


FIG. 11. Property-property plots. VLPs and bacteria in the water column (water over box core (WOC) values excluded) (○), WOC (□), and sediment pore water (▲)(a); VLPs in the sediment pore water vs. percent water (b), percent combustibles (c), and mean particle size (d). In graphs b-d, data from stations 1-5 are represented by ○ ● △ ▲ □, respectively. Graphs a-c represent data from all six sediment horizons, while graph d represents data from only the uppermost and deepest horizons.



Discussion

Bacteria and VLPs at the mouth of the Chesapeake Bay were much more abundant in sediment pore water than in the overlying water column, a result anticipated from synthesis of the ecological (Paul et al., 1993; Rose and Reynolds, unpublished data; Maranger and Bird, 1996; Steward et al., 1996;) and public-health literature (Gerba et al., 1977; Smith et al., 1978; LaBelle et al., 1980). The present study confirms previous research, therefore, and extends our knowledge of VLP distributions into deeper sediment horizons than studied previously. In this discussion, I will compare our results with those of earlier studies, and consider how virus-bacteria interactions in the sediments may contrast with those in the water column.

VLPs and bacteria in the water column. Previous water-column studies have shown that increases in virus abundances are associated with the thermocline (Weinbauer et al., 1995), increases in bacterial abundance (Bird et al., 1993; Cochlan et al., 1993; Weinbauer and Suttle, 1997), the chlorophyll *a* maximum (Boehme et al., 1993) or proximity to surface (Hara et al., 1996) and coastal waters (Cochlan et al., 1993). In contrast, Wommack et al. (1992) found no difference in viral abundance between surface and bottom-water samples in the mesohaline portion of Chesapeake Bay, even when the water column was stratified and bacteria were more abundant above the pycnocline than below it. In addition, they found no association with virus counts and sampling location. In the present study, VLP counts in the water column were not correlated with chlorophyll *a* values; however there was a positive and significant correlation with bacteria abundance,

and a significant difference with location across the bay mouth.

Water-column concentrations of VLPs and bacteria were highest at the southernmost stations of the transect. Chesapeake Bay is a partially mixed estuary (Pritchard, 1952), with the low-salinity bay plume exiting the southern side of the estuary mouth, and my hydrographic data reflect that general pattern of circulation. Other researchers have documented the increase of virus concentrations from oceanic to coastal waters (i.e., high-salinity to lower-salinity waters)(Cochlan et al., 1993). Here I see such an increase on a smaller scale, inside the bay, consistent with classical models of two-layer estuarine circulation.

VLPs and bacteria in the sediment. This first report of sub-surface distributions of VLPs demonstrates they are numerically significant components of the microbial community not only at the sediment-water-interface, but also to at least 25 cm depth. Estimates of VLPs in marine and freshwater sediments vary over three orders of magnitude, from 10^7-10^{10} ml^{-1}, with the lowest estimates from sediment pore water and the highest estimates from counts of viruses extracted from bulk sediments (Appendix B). While part of the large range in values likely arises from different methodologies (see discussion below), arguably the biggest difference among benthic VLP estimates stems from whether bulk sediment or sediment pore water was analyzed. The inventory of VLPs extracted from bulk sediment includes particle-adsorbed VLPs as well as VLPs in sediment pore water. Therefore, it is not surprising that counts of VLPs extracted from bulk sediment can be orders of magnitude higher than counts from sediment pore water alone. The potential roles of particle-adsorbed VLPs in microbial dynamics and the infectivity of adsorbed

VLPs are discussed below.

The VBR in Chesapeake Bay pore water was greater than in the water column (ratios ranged from 29-85 and 12-17, respectively). In the Chukchi Sea, the VBR in pore water (surface 1 cm) also was higher than in surface water (ratios were 12.9 and approximately 4.0, respectively) (Steward et al., 1996). The saltwater trend of a higher VBR in pore water contrasts with results from freshwater Lac Gilbert, Canada, where with the exception of one sample, the VBR in surface sediments was lower than that in the water column (ratios were approximately 1.4 and 13.3, respectively) (Maranger and Bird, 1996). In part, the comparatively high VBRs determined in the present study may arise from methodological differences. I counted viruses using epifluorescence microscopy, a technique that is 1.6 to 7 times more efficient than enumeration using transmission electron microscopy (Hennes and Suttle, 1995; Weinbauer and Suttle, 1997), the method used in the other studies (Maranger and Bird, 1996; Steward et al., 1996).

Sediment grain size can potentially be used to predict of the rates of virus-host encounter, virus infection, and subsequent cell lysis. The significant, negative correlation between grain size and VLPs determined in this study is a phenomenon documented for bacteria (e.g., Yamamoto and Lopez, 1985). Decreasing grain size is proportionately accompanied by an increase in the surface area:volume ratio of particles, resulting in a relative increase in the surface area available for molecular adsorption and bacterial colonization (Yamamoto and Lopez, 1985). Because bacteria are thought to be the hosts for most marine viruses (Proctor and Fuhrman, 1990, Wommack et al., 1992), it follows that more potential hosts for

viruses will occur in sediment with smaller grain size than in sediment with larger grain size. Furthermore, the hosts will be located closer to one another in fine-gravel sediments. Densely distributed hosts increase the likelihood of virus encounter, since encounter rates are subject to the laws of diffusion (Murray and Jackson, 1992). If the time between viral lysis and subsequent infection of new hosts is shortened, then virus cycling and release of DOM (from lysed hosts) will be greater in sediments having smaller grain size.

Temporal and spatial changes of VLP and bacteria distributions in the water column and sediments. There are seasonal changes in the abundances of viruses and bacteria in the water column of the mesohaline portion of the Chesapeake Bay (Wommack et al., 1992), and it is reasonable to assume that seasonal changes also occur at the mouth of the bay. However, the data presented here represent a single sampling effort and illustrate only the differences in VLP and bacteria abundances over mesoscale distances. As a result, one can cannot use them to describe seasonal or daily changes in microbial abundance.

The sediments are less sensitive to temperature and nutrient fluctuations that occur in the water column, so conditions vary less there than in the water column. Given the relative constancy of the sediments and that bacterial densities in marsh sediments remained high year round (Rublee, 1982), I hypothesize that at any given season or tidal state, the abundance of VLPs and bacteria in sediment pore water in the lower Chesapeake Bay is greater than the corresponding abundances in the water column.

Adsorbed vs. unadsorbed VLPs. The ratio of virus (or VLP) abundance in sediments (including pore waters) to abundance in the water column varies across three orders of magnitude (9 - ~10^3) (Table 3). The values and range reported for pore waters are lower than those reported for bulk sediments. I suggest that the discrepancy between virus counts in pore water and bulk sediment is related to adsorption. Adsorption to particles is common among viruses (Kapuscinski and Mitchell, 1980) and quantitatively removes viruses from surface waters (Suttle and Chen, 1992). In the particle-rich environment of sediments, therefore, I hypothesize that most viruses will be adsorbed and only a small percentage of them will be found in the pore water.

However, unadsorbed viruses in sediment pore waters were numerous, approximately 10 times more abundant than viruses in the water column. In contrast, bacteria in the sediment pore water were only about two times more abundant. Therefore, the VBR in the sediment pore water increased relative to the VBR in the water column. Given the higher VBR in sediment pore waters, if unadsorbed viruses are also infective, then the viruses potentially could exert greater control on bacterial mortality in the sediments than their counterparts in the water column.

Infectivity of VLPs in the sediment. Infectivity is a key issue. Consider the hypothetical scenario in which 1), all viruses ultimately bound for the benthos were inactivated in the overlying water column by sunlight (Bitton, 1980; Suttle and Chen, 1992; Murray and Jackson, 1993; Wommack et al., 1996; Weinbauer et al., 1997), bacteria (Bitton, 1980), or attachment to particles (Suttle and Chen, 1992)

and 2), no water-column bacteria were infected by viruses prior to their deposition in the sediments. In this case, then benthic bacteria could not be infected by viruses. One study (Steward et al., 1996) showed despite the presence of free virus-like particles, there were no infected bacteria in the sediment pore water. The generality of this scenario, however, seems unlikely. First, it seems impossible that all infective viruses in the water column could be inactivated. Second, some water-column bacteria undoubtedly are infected with viruses prior to their arrival at the sediment-water interface (e.g., Proctor and Fuhrman, 1991). There is no *a priori* reason to consider that the process of viral infection operates differently in pore waters than in the water column; therefore, *in situ* production must contribute in some part to the inventory of pore-water viruses. Because current methods to enumerate viruses cannot distinguish infective ones from non-infective ones, virus-production experiments are necessary to understand fully the effects of viruses in sedimentary bacteriological communities.

However, one can roughly estimate the *in situ* production rate of VLPs in the sediment pore water at my study sites across the Chesapeake Bay mouth. Assuming the deposition of water-column bacteria and VLPs to benthic communities is negligible, no bioturbation occurs, benthic VLPs are removed after 1 day, all hosts for benthic VLPs are bacteria, 30% of benthic bacteria are infected by VLPs, benthic bacteria divide once per day, 50 VLPs are released upon lysis of each infected benthic bacteria cell, the density of VLPs in the sediment pore water is 3.7×10^8 ml^{-1}, and the density of bacteria in the sediment pore water is 6.4×10^6 ml^{-1}, then 9.6×10^7 VLPs ml^{-1} day^{-1} are produced *in situ*. This estimate accounts

for only 26% of the VLP standing stock. However, the assumptions used in this calculation are from water-column studies of VLP-bacteria interactions, which may not be applicable to studies of microbial interactions in benthic environments.

Whether viruses *attached* to sediment are infective or not is a more complicated question. Viruses adsorb readily to organic and inorganic particles and are protected from inactivation when clay particles (Bitton and Mitchell, 1974), non-host bacteria (Bitton and Mitchell, 1974) and sediment (LaBelle and Gerba, 1980; LaBelle and Gerba, 1982) are added to seawater. However, different inorganic surfaces convey differing degrees of protection from virus inactivation (Murray and Laband, 1979). While it is interesting that viruses may survive longer while attached to sediment than in the overlying seawater, if they are irreversibly bound to sediments, their ecological role is diminished relative to their role in the water column. While some viruses easily desorb from organic and inorganic substrates (Bitton and Mitchell, 1974), LaBelle and Gerba (1979) recovered less than 14% of sediment-adsorbed virus in 3 of 4 strains. Maranger and Bird (1996) suggested that the low VBR in freshwater sediments may be the result of decreased viral production in sediments, owing to the tendency of viruses and bacteria to bind to particles. Similarly, Suttle and Chen (1992) could not recover appreciable numbers of infectious viruses from microscopic aggregates in seawater using several elution methods. They suggested that viruses permanently bind to aggregates in seawater and loose their infectivity (Suttle and Chen, 1992). Perhaps viruses in sediment pore water are the only infective viruses in the sediments.

CHAPTER III

THE EFFECT OF NATURAL VIRUSES ON FECUNDITY, LARVAL SURVIVAL, AND ADULT MORTALITY OF AN ESTUARINE COPEPOD

Naturally-occurring viruses are extremely abundant in aquatic systems, on the order of 10^5-10^8 viruses ml^{-1} (Bergh et al., 1989; Børsheim et al., 1990; Suttle et al., 1990; Heldal and Bratbak, 1991; Hara et al., 1991; Cochlan et al., 1993), and they infect bacteria (Proctor and Fuhrman, 1992), cyanobacteria (Proctor and Fuhrman, 1990), prokaryotic and eukaryotic phytoplankton (Suttle et al., 1990), nanoflagellates (Nagasaki et al., 1993), and crustaceans (Kuris et al., 1979). Distributions of marine viruses suggest that their abundance is either tied to the processes that govern the abundances of microbial and larger organisms, or that viral abundances are linked directly to the presence of the host organisms. Free viruses exhibit 10-4000 fold seasonal variations in abundance (Bergh et al., 1989; Bratbak et al., 1990; Wommack et al., 1992). Virus concentrations decrease from surface to deeper waters (Hara et al., 1996) and from coastal waters to offshore waters (Hara et al., 1991; Cochlan et al., 1993; Weinbauer and Suttle, 1997). These trends in abundance are also seen in organisms in the classical (Ryther, 1969) and the microbial (Hara et al., 1991) food webs .

It has been more than forty years since the first marine bacteriophage was found (Spencer, 1955), but marine viruses were little studied until the mid 1980s. Since then, there has been a proliferation of research on viruses that infect

bacteria. Up to 31% of free-living bacteria and 26% of particle-associated bacteria are infected with viruses at any time (Proctor et al., 1993), with an average of 20% of heterotrophic marine bacteria infected (Suttle, 1994). When the >0.05 μm to <0.22 μm size fraction of seawater was concentrated (high molecular weight concentrate) and added to seawater, bacterial abundances decreased significantly compared to controls (Proctor and Fuhrman, 1992). Although the decrease could have been due to high molecular weight compounds, viruses were assumed to have caused the majority of the bacteria mortality (Proctor and Fuhrman, 1992).

Viruses infect a wide variety of prokaryotic and eukaryotic marine phytoplankters (Mayer and Taylor, 1979; Suttle et al., 1990; Müller, 1991; Suttle and Chan, 1995; Cottrell and Suttle, 1995; Brussaard et al., 1996), and viruses may change phytoplankton abundance and species composition. Addition of concentrated viruses to phytoplankton cultures resulted in significant decreases in *in vivo* fluorescence in five phytoplankton isolates compared to control phytoplankton cultures (Suttle et al., 1990). This result suggests that elevated virus concentrations can control phytoplankton populations. During the course of a spring diatom bloom, a causal relationship between viruses and the termination of the phytoplankton bloom was suggested by a 30-fold change in virus concentrations (Bratbak et al., 1990). Nagasaki et al. (1994) found virus-like particles within red-tide algae at the middle and end of a red-tide bloom. They postulated viruses were responsible for the bloom's termination, based on the inverse relationship between numbers of red tide algae cells and virus-like particles. Thus, manipulative laboratory experiments and field studies suggest that

viruses may negatively affect phytoplankton populations.

Little is known about marine viruses and higher trophic levels, however, there are some well-studied host-virus interactions. Since the first discovery of a crustacean-infecting virus (Vago, 1966), research has focused mainly on economically important species, chiefly shrimps and crabs. Virus infection is documented in crustacean fishery species (Couch, 1978; Johnson, 1983, Bonami and Lightner, 1991; Overstreet et al., 1997), as well as aquaculture organisms (e.g., Lightner et al., 1983; Lightner and Redman, 1985, Adams and Bonami, 1991; Bonami et al., 1997; Edgerton and Owens, 1997).

Transmission of viruses to uninfected crustaceans in the laboratory has been accomplished using a variety of methods, including immersing larvae in water containing viruses (Johnson, 1983), inoculating uninfected animals with purified virus (Overstreet et al., 1997), inoculating uninfected animals with homogenized virus-infected tissue (Mari and Bonami, 1988), inoculating uninfected animals with virus-infected hemolymph (Johnson, 1983), feeding virus-containing material to uninfected animals (Couch, 1978), and feeding virus-infected tissue to brine shrimp and in turn feeding the brine shrimp to uninfected animals (Overstreet et al.,1988). These experiments are used to study virus infections that occur in the artificial environments of aquaculture facilities. Indeed, environmental stresses associated with rearing shrimp under crowded conditions is related to greater incidence of virus infection (Couch, 1977; Fegan et al., 1991).

The effects of viruses on decapods in the wild and in the laboratory range from no apparent effect to weakness, gill infection, disoriented swimming, or

paralysis that lead to an eventual death (Johnson, 1983). The variance in the time from initial virus infection to mortality of laboratory crustaceans is also large, from several days to two months (Johnson, 1983).

The effects of virus infection on the population dynamics of feral crustaceans are not studied, but viruses have been identified in field-collected crabs (e.g., Pappalardo et al., 1986) and shrimp (e.g., Couch, 1977), and the prevalence of virus infection in natural populations can be as high as 50 - 80% (Johnson, 1983). Similarly, the mechanisms of virus transmission among natural populations of marine crustaceans are unknown; Couch (1978) suggested that transmission of *Baculovirus penaei* occurs by cannibalism of infected shrimp by healthy shrimp.

Less evidence exists for virus infection of zooplankton, although viral lesions have been demonstrated in cultured rotifers (Comps et al., 1991), barnacles (Leibovitz and Koulish, 1989), and daphnids (Federici and Hazard, 1975; Bergoin et al., 1984). Marine zooplankton may also be vulnerable to infection by viruses.

The marine meso-zooplankton is dominated by copepods in terms of biomass and number of organisms (Conover, 1956; Heinle, 1966; Baird and Ulanowicz, 1989; White and Roman, 1992). Copepods can occupy different trophic levels in marine food webs in that they may feed on primary producers (White and Roman, 1992) as well as on protozooplankton (Kleppel, 1993). In turn, copepod eggs, nauplii, and adults are a major food source for carnivores such as larval fish and invertebrates, including predatory copepods. If virus-zooplankton relationships are of interest, then it seems well-considered to study copepods as

host organisms, given their prominent and multi-tiered position in marine food webs.

There is no reason to presume copepods are immune to viral infection, given that other crustaceans are infected by viruses. That is, I assume that the dearth of information on copepod-virus interactions is the result of a lack of investigation, rather than a lack of infection of copepods by viruses. Also, the susceptibility of copepods to infection by fungi (Redfield and Vincent, 1979), ciliates (Turner et al., 1979; Weissman et al., 1993), bacteria (Carman and Dobbs, 1997), and diatoms (Ho and Perkins, 1985; Carman and Dobbs, 1997) suggests that they are not immune to infection by microorganisms. Therefore, although I am unaware of any work on the ecology of viruses infecting natural populations of zooplankton, I hypothesize that marine copepods are vulnerable to infection by viruses.

To quantify the effects of viruses on the copepods, I measured two life-history parameters of copepods exposed to elevated concentrations of naturally occurring viruses. These parameters were mortality and egg production, the latter a characteristic of copepods well-studied in population dynamics. Mortality of copepods is attributed to predation (e.g., Lonsdale et al., 1979), temperature intolerance, interactive effects of temperature and predation (Fulton, 1983), salinity and temperature intolerances (Jeffries, 1962), fungal infection (Redfield and Vincent, 1979), and exposure to compounds found in diatoms (Poulet et al., 1994), among others. The following factors influence copepod egg production: temperature (e.g., Ambler, 1985; Kleppel, 1992), food quantity (Marshall and Orr,

1972; Dagg, 1977; Kiorboe et al., 1985), food quality (Marshall and Orr, 1972; Parrish and Wilson, 1978; Durbin et al., 1983; Støttrup and Jensen, 1990), previous feeding history (Tester and Turner, 1990), and epibiotic ciliate load (Weissman et al., 1993). Given that copepod mortality and egg production are affected by a suite of parameters, I considered that they are also influenced by viral infection.

I attempted to induce viral infection in laboratory-reared cultures of a copepod, *Acartia tonsa* Dana, by exposing them to elevated concentrations of natural viruses. My intent was to infect copepods, isolate a copepod virus, and proceed with transmission experiments and other manipulative experiments.

Materials and Methods

Laboratory cultures of copepods. *Acartia tonsa* Dana were collected from the mouth of Chesapeake Bay in July 1995 and kept continuously in culture. Copepods were reared in gently aerated 1- and 2-l polymethylpentene beakers filled with autoclaved seawater. Beakers were kept in an incubator set at 20 ± 0.5°C and illuminated on a 12L:12D cycle by cool white fluorescent light bulbs. Copepods were fed the phytoplankton *Isochrysis galbana* and *Rhodomonas salina*, clones ISO and 1319, respectively, from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton. Phytoplankton concentrations in the cultures were not strictly maintained, but copepods were fed 2-3 times weekly approximately 4×10^4 cells ml^{-1} and 2×10^4 cells ml^{-1} of *R. salina* and *I. galbana*, respectively. Phytoplankton were grown in F/2 media (minus silica) (Guillard and Ryther, 1962) in seawater (salinity 20-25) and maintained in the incubator.

Timing of *A. tonsa* bloom. I hypothesized that virus infection of *A. tonsa* would be highest in natural seawater when *A. tonsa* dominated the zooplankton. To determine when *A. tonsa* were dominant, qualitative plankton tows were taken with a 202 μm-mesh plankton net. Most of the following experiments were conducted in the spring and summer of 1996 when the zooplankton was dominated by *A. tonsa* adults and copepodites (see Table 4).

Concentration of natural virus assemblage. Seawater was collected in plastic containers that were rinsed with seawater. Twenty liters of seawater were transferred to an acid-washed carboy, then prefiltered serially through 1) a glass fiber filter (Whatman GF/F or Gelman A/E) and 2) a cellulose acetate or polyvinylidene difluoride filter into an acid-washed carboy on ice. Vacuum pressure during prefiltration was 60 mm Hg. For egg-production, egg-hatching, and nauplii-survival experiments (experiments 1-5), as well as for preparing virus-like particle (VLP) concentrate for examination by electron microscopy, cellulose acetate filters with a pore size of 0.22 μm were used (Micron Separations, Inc.); for the serial-inoculation experiment, polyvinylidene difluoride filters with a pore size of 0.45 μm were used (Millipore Corporation) . Prefiltered seawater was reduced to a volume of 100-200 ml using a MEMBREX Benchmark® Gx Vortex Flow Filtration System with a 100 kD filter. A 30 kD filter was used for the first iteration of the serial-inoculation experiment. The fraction of seawater that passed through the 0.22 μm- or 0.45 μm-pore size filter and was retained by the 100 or 30 kD filter was considered the VLP concentrate. Filters with cut-offs of 100kD and 30 kD will retain particles with spherical diameters of approximately 0.01 μm and 0.003 μm,

TABLE 3. Details of virus experiments with *A. tonsa*.

Experiment number or name	Parameters measured	Location of water collection, date	Water temperature (°C), salinity	VLP density in water before VFF[a] (x 10^7 ml^{-1})	VLP density in exp. trt. (x 10^7 ml^{-1})
1	egg production	Lafayette River 25 April 1996	18.0, 16.0	n/a	64.1
2	egg production	Lafayette River 26 April 1996	18.0, 14.0	n/a	69.5
3	egg hatching	CBM[b] 7 May 1996	15.0, 22.0	10.2	657
4	egg hatching nauplii survival	CBM 8 May 1996	15.0, 19.0	14.2	712
5	egg production egg hatching nauplii survival	CBM 31 May 1996	17.8, 17.3	5.0	12.9 359 359
serial inoculation	egg production survival egg hatching	Ocean View Beach (Chesapeake Bay) 27 August 1997	22.0, 25.0	19.1	25.4 25.4 autoclaved seawater

TABLE 3. Continued

Experiment number or name	Parameters measured	Location of water collection, date	Water temperature (°C), salinity	VLP density in water before VFF[a] (x 10^7 ml^{-1})	VLP density in exp. trt. (x 10^7 ml^{-1})
pulverized copepods	survival	CBM[c] 22 October 1995	19.6, 25.0	n/a	0.07
copepod exudates	survival	CBM[c] 22 October 1995	20.0, 28.0	n/a	0.03

[a]Vortex flow filtration, used to concentrate the virus-size fraction of seawater.

[b]Chesapeake Bay mouth.

[c]Location of copepod collection.

respectively (B. Herman, MEMBREX, Inc., pers. com.). VLP concentrate was stored in the dark at room temperature until it was added to experimental beakers or microfuge tubes, which was within one hour of concentration.

Virus enumeration. Virus-like particles were counted using the method of Hennes and Suttle (1995). Briefly, water samples were filtered onto 0.02 μm-pore size Acrodisc™ filters and the filters stained with the epifluorescent nucleic acid stain YO-PRO™-1 iodide (491/509, Molecular Probes, Inc.) (Quinolinium,4-[(3-methyl-2(3H)-benzoxazolylidene)methyl]-1-[3-(trimethylammonio)propyl]-,diiodide). Filters were stored in the dark at -85°C until the virus-like particles (VLPs) were thawed in the dark at room temperature for ca. 5 minutes, then counted at 1000 x magnification using an Olympus BX50 System Microscope with a BX-FLA epifluorescence attachment.

Examination of VLP concentrate by transmission electron microscopy.

To verify that the VLP concentrate contained viruses, I examined VLP concentrate prepared on 14 December 1995 as described above (seawater was collected from the North Channel at the Chesapeake Bay mouth; coordinates are listed in the description of Experiment 11 in Appendix C). Each of 3 formvar-coated copper mesh grids was floated with the formvar side down on a drop of glutaraldehyde-fixed viral concentrate for 60 minutes, then rinsed 3 times with deionized water. Grids were stained for 10 s with uranyl acetate, then examined at an accelerating voltage of 80,000 kV and a magnification of 10,000 - 140,000 x on a JEOL 100CX II transmission electron microscope.

Egg-production experiments. A total of three experiments was performed in

1996 to determine the effects, if any, of virus concentration on egg production (Table 4; see descriptions and results of preliminary experiments in Appendix C). In each experiment, twenty copepod eggs or first-stage nauplii were removed from cultures and placed in each of 6 acid-washed one-liter beakers filled with either autoclaved or microwaved seawater (control treatments; n = 2 or 3) or with autoclaved or microwaved seawater plus VLP concentrate (virus treatments; n = 2 or 3; volume of concentrate per beaker = 36 - 57 ml to reach a VLP concentration of 1.3 - 7.0 x 10^8 ml^{-1}). Seawater was microwaved in one-liter volumes for a total of ten minutes (3, 2, 3, 2-minute intervals with a 2-minute break between each interval (Sanborn et al., 1982; Keller et al., 1988) in a Sharp Household Microwave Oven Model R-3A36 (60 Hz). The salinity in the virus beakers was adjusted to 20 with autoclaved, deionized, distilled water. Phytoplankton cells were counted with an Improved Neubauer Hemacytometer and added to each beaker: 19,000 cells ml^{-1} of *R. salina* and 10,000 cells ml^{-1} of *I. galbana*. Beakers were arranged randomly along a shelf in the incubator. Water was changed twice before copepods reached adulthood, each time replacing the water in all beakers with autoclaved seawater and new algae. Copepods in experimental beakers were exposed to viruses from the initiation of the experiment to the time of the first water change, which was 4-7 days.

To measure egg production, 1 - 3 adult females were removed from each beaker and placed in an acrylic sleeve having a 202 μm-mesh bottom (D. Lonsdale, pers. com.). The sleeve was immersed in a 250 ml polymethylpentene beaker filled with autoclaved seawater and 19,000 cells ml^{-1} of *R. salina* (2 or 3

beakers per control and virus treatments). Egg production was monitored over 12-hour periods. Females that were not recovered after the egg laying period were assumed to have been present during the entire egg laying period. Females found dead at the end of the egg laying period in experiment 1 (1 copepod in the control and 2 in the virus treatment) were assumed to have died half way through the egg laying period.

Egg-hatching and nauplii survival experiments. Experiments were performed to quantify the hatching and survival of eggs (three experiments) and nauplii (two experiments) in the presence of VLP concentrate (Table 4). In each experiment, ten copepod eggs or 5 first-stage nauplii were put in a sterile microcentrifuge tube with 50 μl of autoclaved seawater. To the control tubes (n = 3 or 4), 950 μl of autoclaved seawater or 950 μl of microwaved seawater was added. To experimental tubes (n = 3 or 4), 950 μl of VLP concentrate was added to reach a VLP concentration of 3.6 - 7.1 x 10^9 ml^{-1}. The salinity in the experimental tubes was not adjusted because the salinity of the VLP concentrate, 17.8 - 22.0, was similar (± 11%) to the salinity in the control tubes. Tubes were incubated for 36 h in the dark at 20°C, then eggs or nauplii were checked for hatching or survival. Eggs that did not hatch during that time were monitored for an additional 48 h; eggs that did not hatch after a total of 84 h were assumed to be non-viable. Missing eggs and nauplii were excluded from calculations of hatching success and nauplii survival; 6 eggs and 3 nauplii were unaccounted for out of totals of 280 and 100, respectively, used in all egg-hatching and nauplii-survival experiments.

Serial-inoculation experiment. In an attempt to build the titer of one or more *A.*

tonsa-infecting viruses, the virus-size fraction of seawater from laboratory copepod cultures was repeatedly concentrated and added to 400-600 copepod eggs, which ranged in age from 1 - 26.5 h old (Table 4). Initially, twenty liters of seawater were collected from Chesapeake Bay, and the virus-size fraction was concentrated (as described above). To each of three one-liter beakers, the following were added: 953 ml of autoclaved seawater, copepod eggs, phytoplankton (1.9×10^4 cells ml^{-1} of *R. salina* and 4×10^4 cells ml^{-1} of *I. galbana*), and forty-seven ml of VLP concentrate to reach a VLP concentration of 2.5×10^8 ml^{-1}. After seven days, copepods were removed, stained with Rose Bengal (1:160,040 dilution), and counted to determine their percent survival. The virus-size fraction of the three liters of seawater was then concentrated and 54 ml of VLP concentrate was added to each of three one-liter beakers containing fresh copepod eggs, autoclaved seawater, and phytoplankton. The initial VLP concentrations in the beakers was 8.3×10^7 ml^{-1}. This cycle of concentrating copepod culture water and subsequently adding VLP concentrate (48 - 55 ml) to fresh copepod eggs from laboratory cultures was repeated five times, for a total of six iterations, with six or seven days between iterations. Initial VLP concentrations in the experimental beakers ranged from 4.6×10^7 ml^{-1} to 1.4×10^8 ml^{-1}. For the sixth iteration, a control treatment was added. To each of three one-liter beakers in the virus treatment, fresh copepod eggs, autoclaved seawater, phytoplankton, and 52 ml of VLP concentrate to reach a VLP concentration of 3.7×10^7 ml^{-1} were added. To each of three beakers in the control treatment, 52 ml of iced, autoclaved seawater (salinity = 20) were added in lieu of VLP concentrate. Copepods in both treatments were

reared for 14 days. On day 7, water was changed in all beakers, and fresh autoclaved seawater and phytoplankton (3.8 x 10^4 cells ml^{-1} of *R. salina* and 4 x 10^4 cells ml^{-1} of *I. galbana*) were added. On day 11, copepods were fed again (1.9 x 10^4 cells ml^{-1} of *R. salina*). On day 14, ten adult female copepods were removed from each beaker and egg production was monitored over 12 h (see above). Also on day 14, the remaining copepods were stained with Rose Bengal and counted.

Other attempts to concentrate viruses that infect *A. tonsa*: pulverized copepods and copepod exudates. To test the hypothesis that pulverized copepods release viruses that have detrimental effects on copepod survival, about 1000 field-collected *A. tonsa*, primarily adults, were rinsed in autoclaved seawater, pulverized with a mortar and pestle, and mixed with 500 ml of autoclaved seawater (Table 4). The mixture was kept in the dark at 20°C for 4 hours, then centrifuged for 16 minutes at ca. 745 x g. The supernatant, which had a VLP concentration of 7.0 x 10^5 ml^{-1}, was used immediately after centrifugation as the virus inoculum, Three hundred nauplii (0 - 33.3 h old) were removed from laboratory cultures and evenly divided among 6 acid-washed 250-ml beakers containing either 250 ml of autoclaved seawater (control treatments; n = 3; 50 nauplii per beaker) or 210 ml of autoclaved seawater plus 40 ml of the virus inoculum (virus treatments; n = 3; 50 nauplii per beaker). Ten thousand cells ml^{-1} of *R. salina* and 10,000 cells ml^{-1} of *I. galbana* were added to each beaker. Beakers were arranged randomly along two shelves in the incubator. Water was changed once; water in all beakers was replaced with autoclaved seawater and fresh algae. On day 15 of the experiment, the number of live adults and copepodites in each beaker was counted.

To determine if copepod exudates include viruses that negatively affect copepod survival, field-collected *A. tonsa* were rinsed with autoclaved seawater, and placed in an acid-washed beaker with 30,000 cells ml^{-1} of *R. salina* and autoclaved seawater (Table 4). The density of copepods was about 400 copepods l^{-1}. The beaker was kept on a laboratory bench (temperature range = 20.5 - 24.0°C). Twenty-four hours later, water in the beaker passing through a 53 μm-mesh screen was used as the virus inoculum, which had a VLP concentration of 3.0×10^5 ml^{-1}; this mesh size excluded copepods but included fecal pellets. A total of twenty male and female adults and copepodites were removed from laboratory cultures and placed in each of 6 acid-washed one-liter beakers filled with either autoclaved seawater (control treatments; $n = 3$) or with autoclaved seawater plus 250 ml of the virus inoculum (virus treatments; $n = 3$). Phytoplankton were added to each beaker: 10,000 cells ml^{-1} of *R. salina* and 7,650 cells ml^{-1} of *I. galbana*. The beakers were arranged randomly along a laboratory bench. On day 4 of the experiment, 10,000 cells ml^{-1} of *R. salina* were added to each beaker. On day 8 of the experiment, the number of live adults and copepodites in each beaker was counted.

Results

Examination of VLP concentrate by transmission electron microscopy. Many of the viruses visible in the VLP concentrate had angular heads with attached tails; examples are shown in Plate 6. Head diameters of the viruses in the micrographs range from 43-64 nm (Plates 6a, 6b).

Egg-production experiments. Preliminary experiments of virus effects on egg

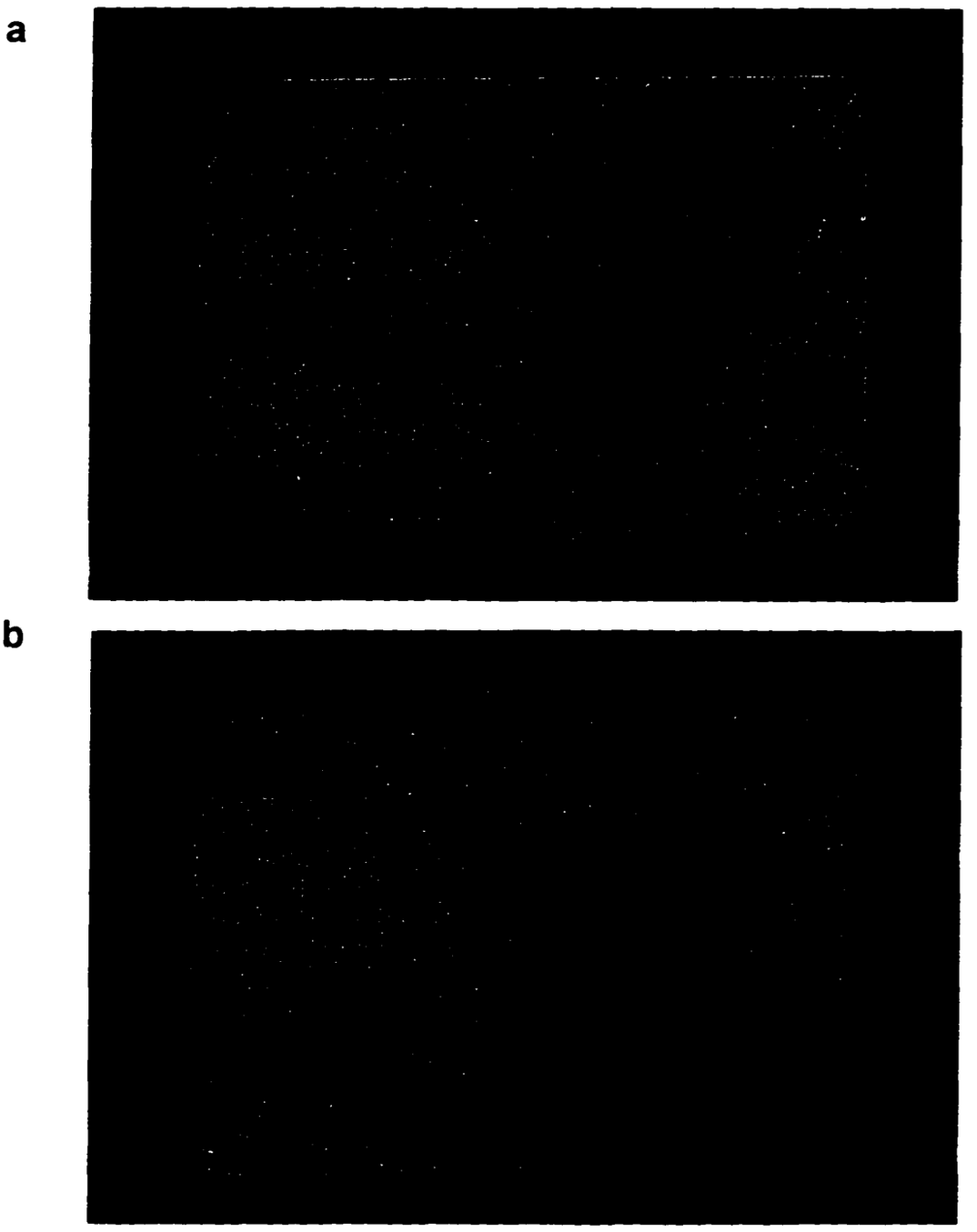


PLATE 6. Micrographs of viruses concentrated from a natural water sample and examined by transmission electron microscopy. Virus head diameters are 43 and 64 nm (a) and 50 nm (b). Magnification = 140,000 x for both plates.

production performed in late summer and fall 1995 showed no significant difference in egg production between control and virus treatments (data in Appendix C). I repeated the experiments during the 1996 spring *A. tonsa* bloom, presumably when viruses infecting *A. tonsa* would be most abundant. There was no significant difference in egg production between control and virus treatments in any of the three experiments ($p = 1.000$, Wilcoxon 2-sample test, Fig. 12a; $p = 0.6579$, Wilcoxon 2-sample test, Fig. 12b; $p = 1.0$, 1-way ANOVA on ranked data, Fig. 12c),

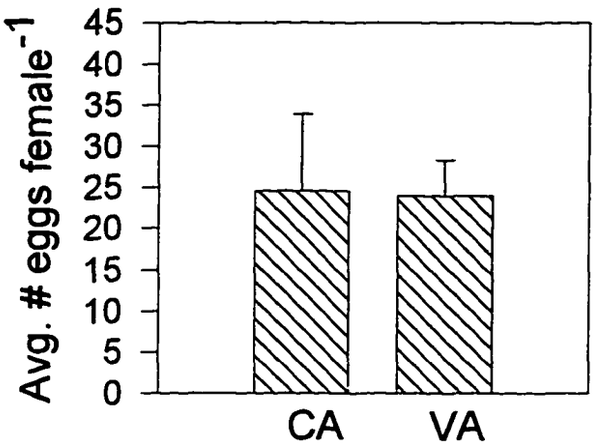
Egg-hatching and nauplii-survival experiments. In two of the egg-hatching experiments, 100% of the eggs in both control and virus treatments hatched (Fig. 13a, b). In the third experiment, all eggs hatched except for a few in two replicates of the control treatment with autoclaved seawater (Fig. 13c). These results indicated no significant difference among any treatment groups in all three experiments ($p = 0.055$, 1-way ANOVA on ranked data).

In the first nauplii-survival experiment, nauplii survival was 100% in both the control and virus treatments (Fig. 14a). In the second experiment, survival was lower in the virus treatment with autoclaved seawater than in its corresponding control treatment (Fig. 14b). Nauplii survival was 100% in both treatments using microwaved seawater (Fig. 14b). Again, there was no statistically significant difference among treatments in both experiments ($p = 0.108$, 1-way ANOVA on ranked data).

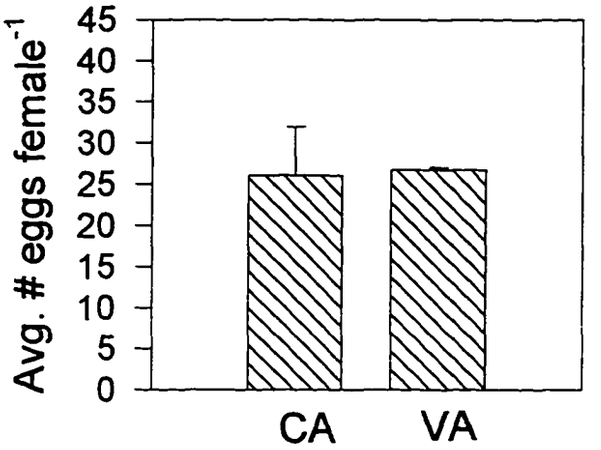
Serial-inoculation experiment. There was no significant difference in survival of each cycle of copepods during the first five iterations of the experiment ($p = 1.0$; 1-way ANOVA on ranked data, Fig. 15). VLP and bacteria abundances

FIG. 12. Measurements of egg production in experiments 1, 2, and 5 (spring, 1996), labeled as (a), (b), and (c), respectively. Egg-production was monitored over one nighttime 12-hour period with 1-3 females in each replicate. Data are mean values (n = 3) ± 1 SE in (a) and (b); n = 2 for (c). C = control treatment of autoclaved seawater; V = virus concentrate added to autoclaved seawater; CM = control treatment of microwaved seawater; VM = virus concentrate added to microwaved seawater.

a



b



c

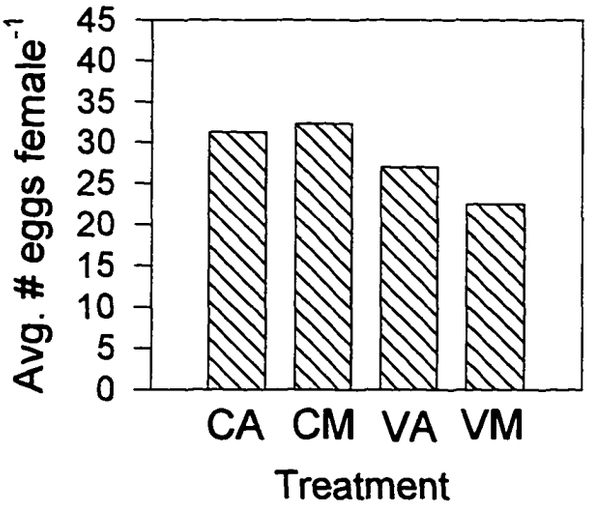
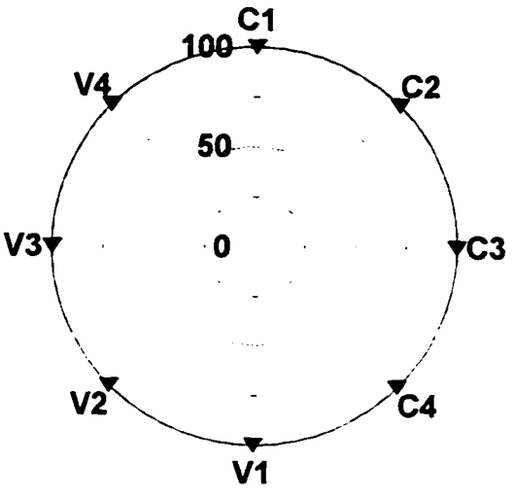


FIG. 13. Measurements of egg hatching in experiments 3, 4, and 5 (spring, 1996) labeled as (a), (b), and (c), respectively. Concentric circles represent the scale; triangles represent the percentage of ten eggs that hatched. Thus, the outer circle represents 100% hatching. C = control treatment of autoclaved seawater; V = virus concentrate added to autoclaved seawater; CM = control treatment of microwaved seawater; VM = virus concentrate added to microwaved seawater. Arabic numerals following the letters indicate replicate number.

a



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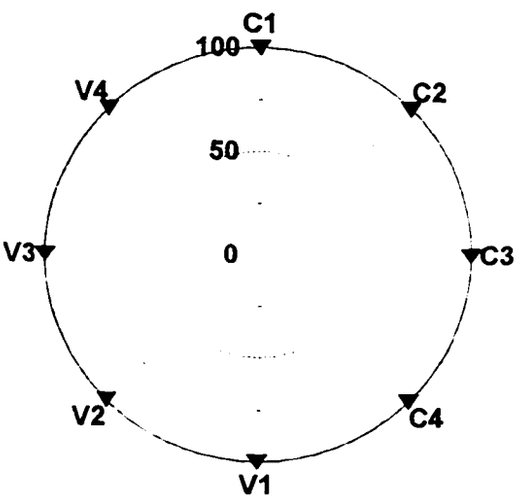


c

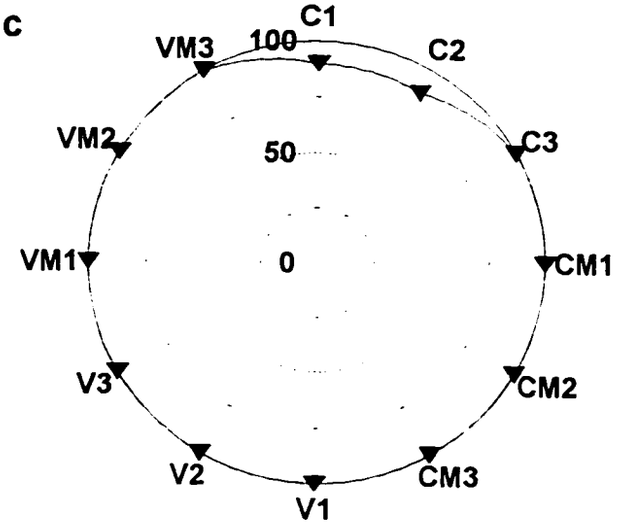
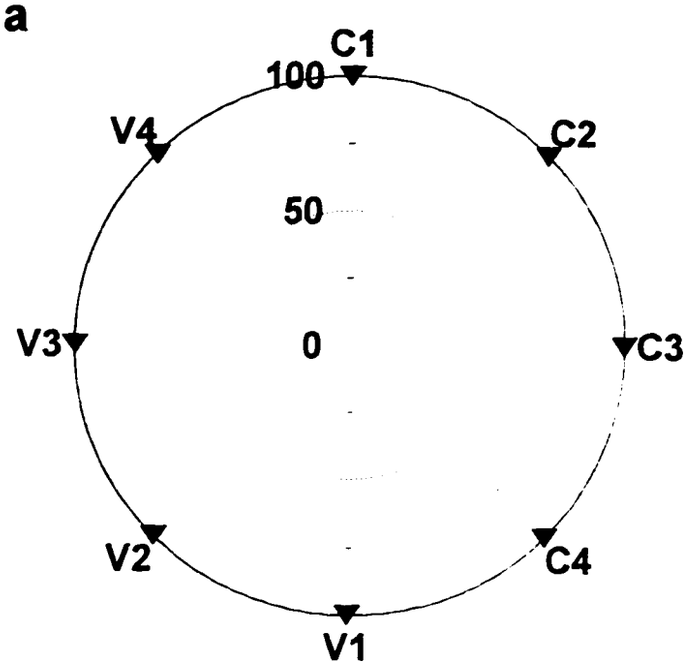
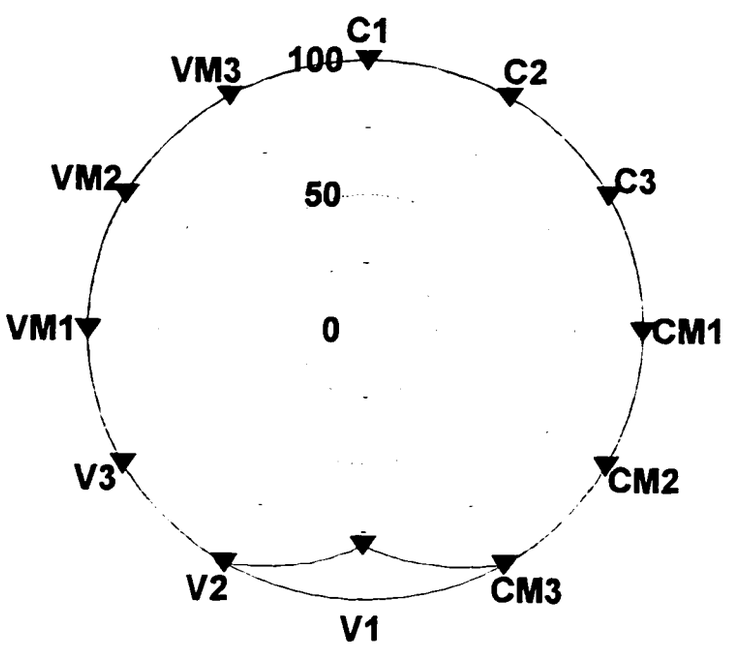


FIG. 14. Measurements of nauplii survival experiments 4 and 5 (spring, 1996), labeled as (a) and (b), respectively. Concentric circles represent the scale; triangles represent the percentage of ten eggs that hatched. Thus, the outer circle represents 100% hatching. C = control treatment of autoclaved seawater; V = virus concentrate added to autoclaved seawater; CM = control treatment of microwaved seawater; VM = virus concentrate added to microwaved seawater. Arabic numerals following the letters indicate replicate number.

a



b



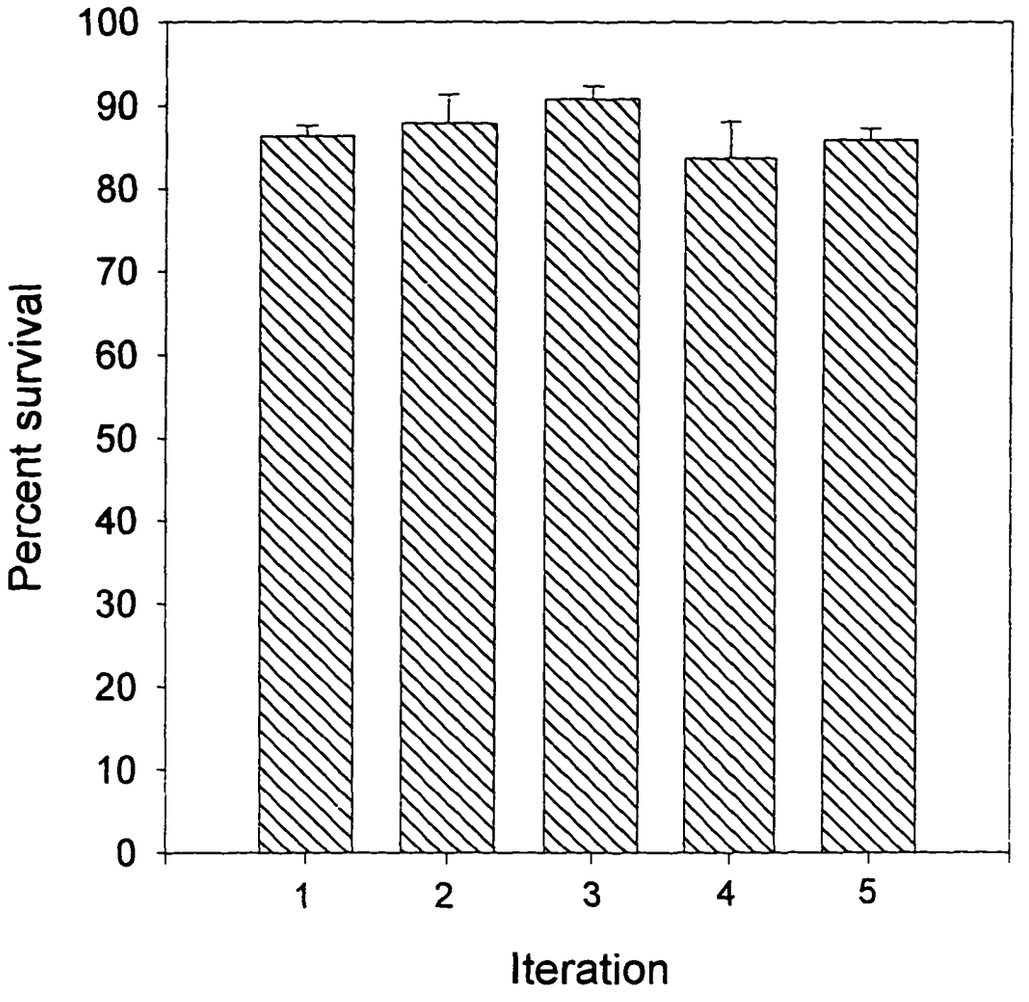


FIG. 15. Percent survival of copepods in the serial-inoculation experiment (experiment 6, summer 1997). Percent survival was measured 6 or 7 days after the beginning of the cycle.

in the copepod water varied inversely over time (Fig. 16). Lastly, there was no difference between the control and virus treatment groups of the experiment's sixth iteration with respect to survival ($p = 0.3827$, Wilcoxon 2-sample test), egg production ($p = 1.0$, Wilcoxon 2-sample test), or egg hatching ($p = 0.3865$, Wilcoxon 2-sample test) (Fig. 17).

Other attempts to concentrate viruses that infect *A. tonsa*. There was no difference in mean survival between control and virus treatments when pulverized copepods were used as virus inoculum ($p = 0.6625$, Wilcoxon 2-sample test) (Fig. 18). Similarly, there was no difference in survival between control and virus treatments in the experiment in which exudates of copepods were used as virus inoculum ($p = 1.0$, Wilcoxon 2-sample test) (Fig. 19). However, the variance between replicates is higher in the virus treatment versus the control treatment in both experiments.

Discussion

These experiments did not demonstrate any detrimental effects of exposure to viruses on copepods. There are at least two possible ways to interpret these results. The first line of reasoning involves the modes of virus infection and the second concerns the probability of encounter of copepods by viruses.

Infection. Animal viruses can effect host cells in four ways--via lytic infection, latent infection, persistent infection, and transformation of cells to tumor cells (Brock et al., 1994). A virus that causes a lytic infection is adsorbed by the host, initiates host cell production of virus copies, and lyses the host cell, thereby releasing virus progeny. On the other hand, an infection is latent when the viruses

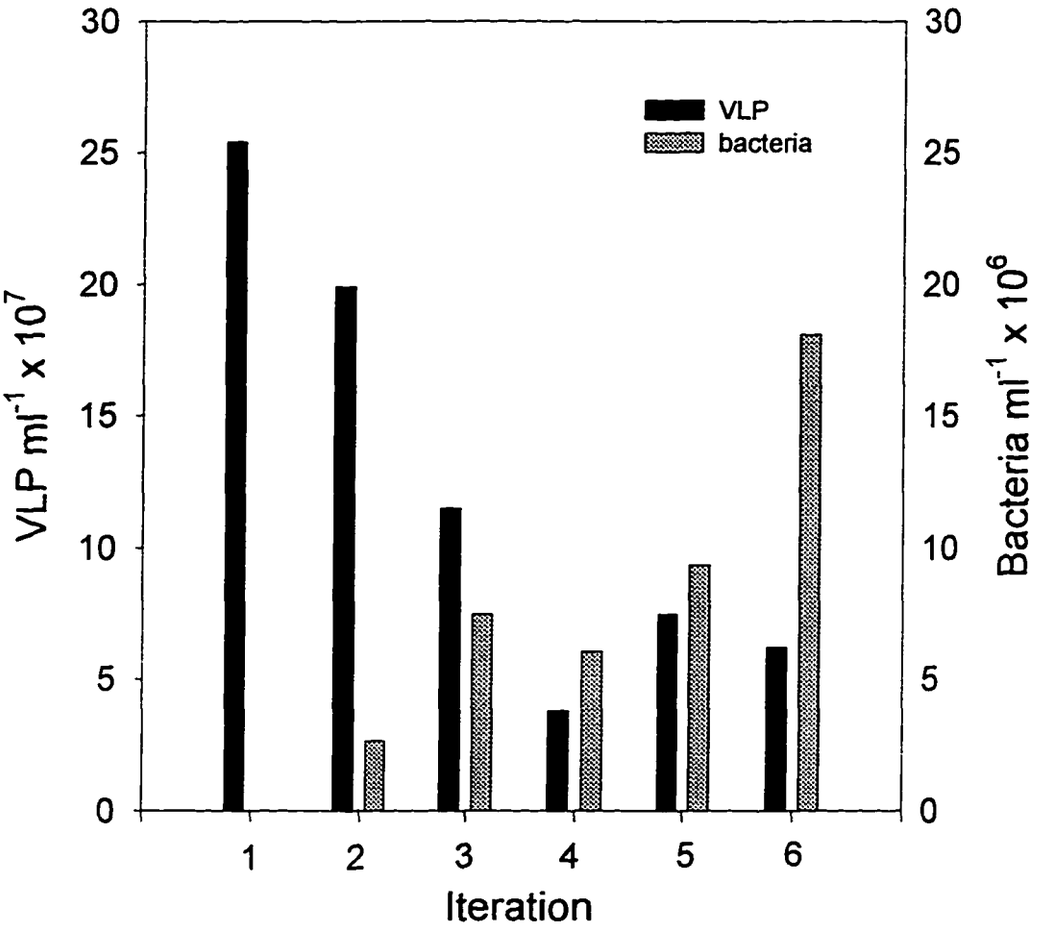


FIG. 16. Quantity of VLPs and bacteria in the six iterations of the serial-inoculation experiment (summer 1997). For iteration 1, the first day of the experiment, the VLP value represents the final concentration of VLPs in autoclaved seawater after addition of viral concentrate. No data for bacteria are shown for iteration 1 because bacteria were removed by filtration prior to concentrating the virus-size fraction. Data for iterations 2-6 are VLP and bacteria abundances in the copepod culture water 6 or 7 days after the addition of virus concentrate.

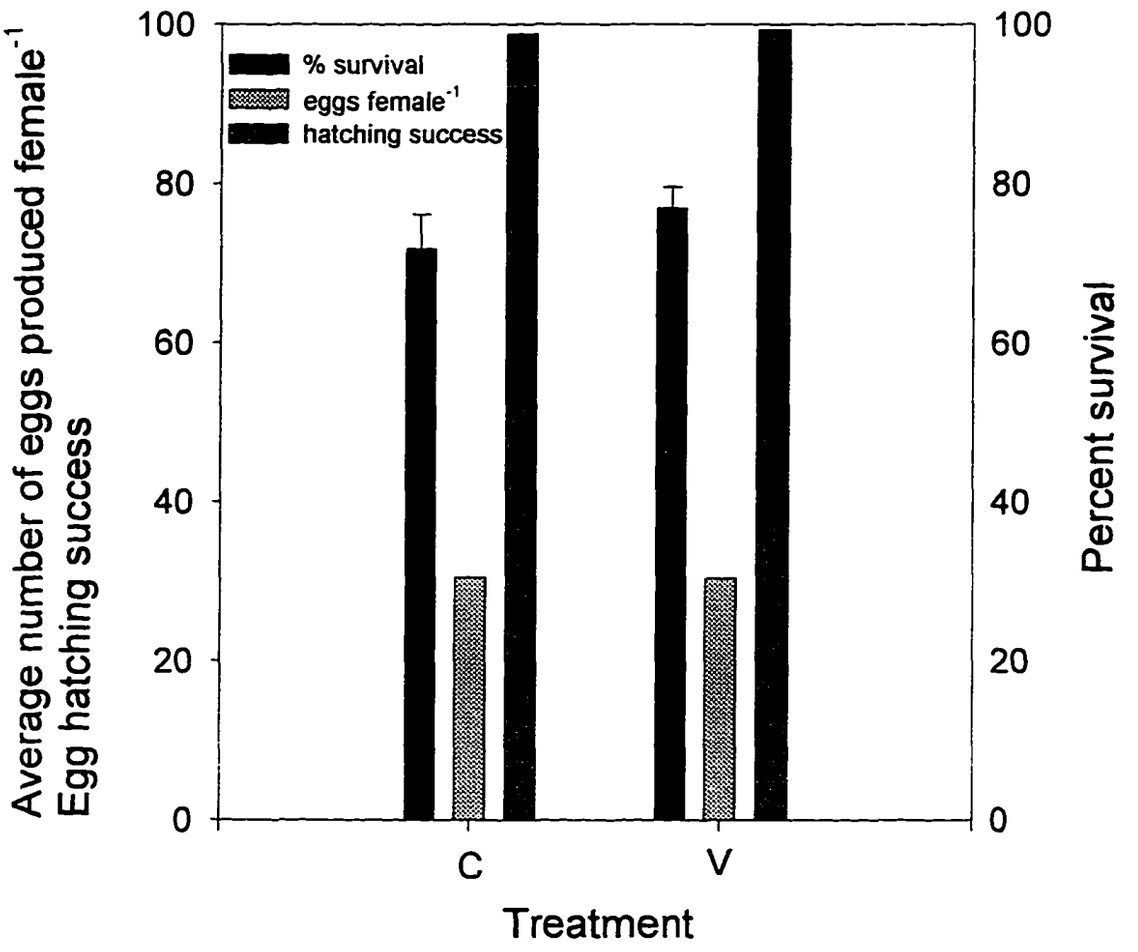


FIG. 17. Percent survival, egg production, and egg hatching success in iteration 6 of the serial-inoculation experiment (summer, 1997). Percent survival of all copepods was measured 14 days after the beginning of the cycle. Data are mean values (n = 3) ± 1 SE. Egg production per female (ten females per replicate) was measured over one nighttime 12-hour period. Twenty-five hours later, hatching success of the eggs was measured and represented as a percentage. Data are mean values (n = 2 for control treatment; n = 3 for virus treatment). C = control; V = virus.

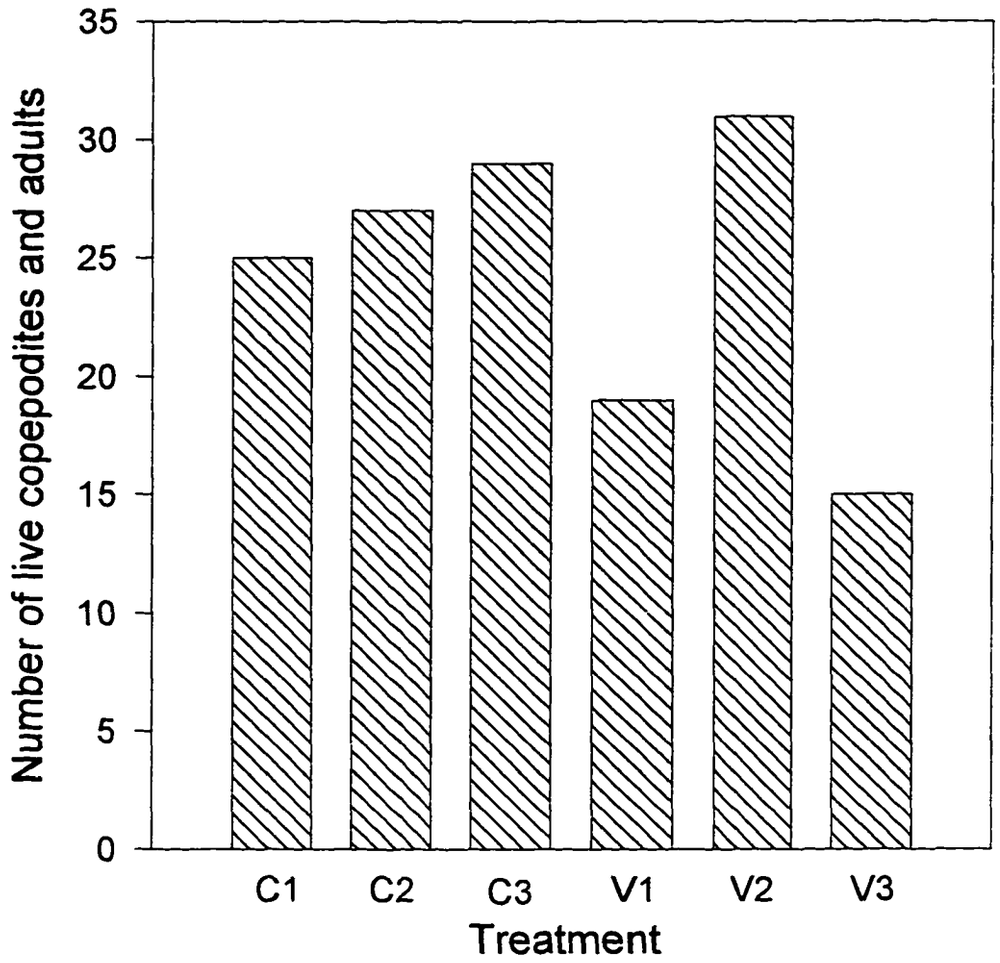


FIG. 18. Survival of copepods in the presence of pulverized copepods used as virus inoculum (fall, 1995). Each bar represents a beaker containing 50 nauplii at the start of the experiment. C = control; V = virus. Arabic numerals following the letters indicate replicate number.

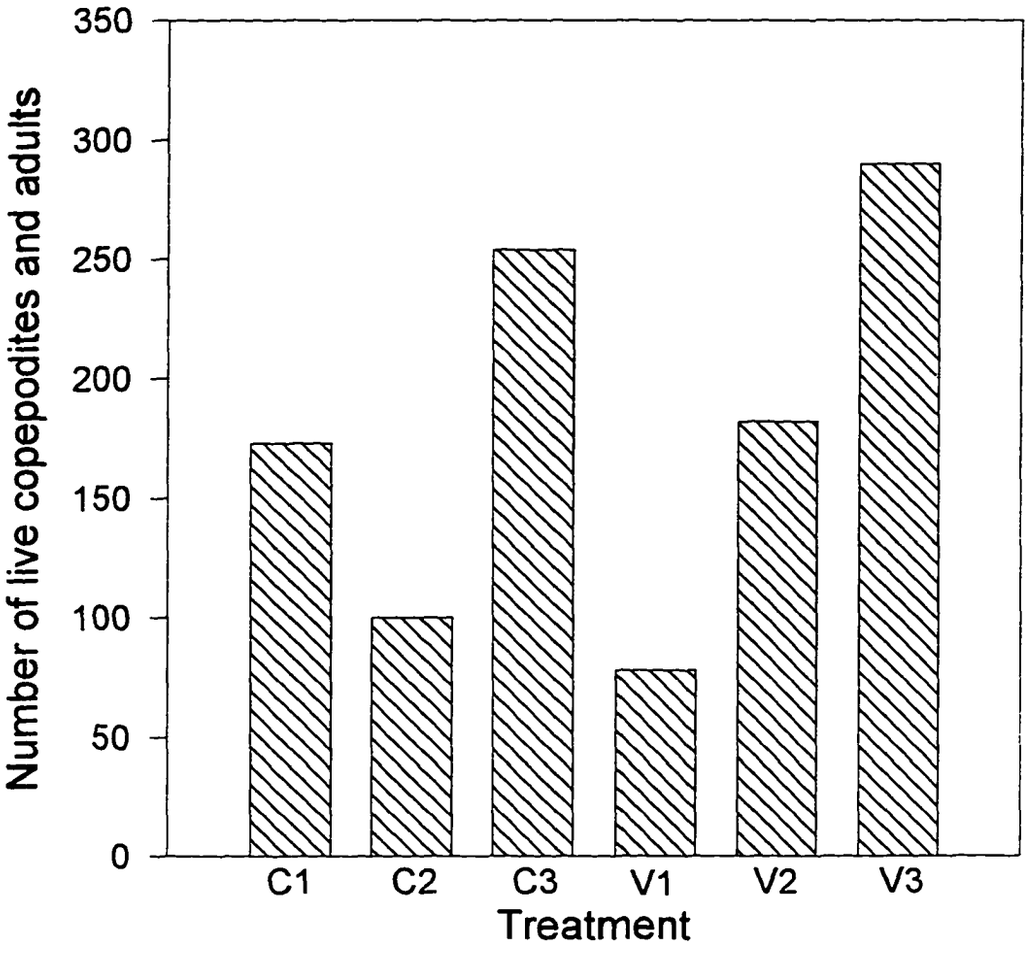


FIG. 19. Survival of copepods in the presence of copepod exudates used as virus inoculum (fall, 1995). Each bar represents a beaker containing a total of 20 adults and copepodites at the start of the experiment. C = control; V = virus. Arabic numerals following the letters indicate replicate number.

are present in cells but do not effect on host metabolism. Subsequently, lytic infection can occur. Persistent infections are manifested in a continual release of viruses with out killing the host cell. Viruses that cause tumors cause uncontrolled growth. It is possible that the copepods in these experiments were infected with either a lytic, persistent, or tumorous infection or all three, but if so, evidence of infection was not manifested in mortality or diminished fecundity.

Similarly, I may have infected the copepods with a latent virus or viruses. That is, a copepod-infecting virus may have been incorporated into copepod cells but did not affect the normal functioning of the copepod. Some factor could potentially induce the latent infection to become lytic and kill the copepod. Since I did not manipulate environmental conditions to induce such an effect, I do not know if the copepods had a latent infection.

Encounter rates. Another explanation for the negative results, not mutually exclusive to the first, is that low encounter rates between viruses and copepod eggs or nauplii resulted in no infection. It is hypothesized that most viruses in seawater infect the most numerable organisms, the bacterioplankton (Wommack et al., 1992). This hypothesis is supported by data on the size distribution and morphology of free viruses in seawater (Berg et al., 1989; Proctor and Fuhrman, 1990; Cochlan et al., 1993; Wommack et al., 1992). These data are consistent with the expected shape (typical phage shape with angular head and attached tail) of bacteria-infecting viruses, as well as calculations based on the probability of viruses preferentially encountering bacteria cells rather than larger particles (Murray and Jackson, 1992). If the viruses I concentrated from natural seawater

contained mostly bacteria-infecting viruses with few *A. tonsa*-infecting viruses, the *A. tonsa*-infecting viruses may have been inactivated by adhering to particles, such as plankton, bacteria, or microaggregates (Mitchell and Jannasch, 1969; Bitton and Mitchell, 1974; Noble and Fuhrman, 1997) before encountering a copepod egg or nauplius. However, It is notable that under the artificial conditions of aquaculture facilities viruses can be transmitted via water (e.g., Johnson, 1983; Overstreet et al., 1997).

Virus effects on *A. tonsa* population dynamics.

Regardless of the negative results of these experiments, copepods may have been infected by a virus or viruses. In fact, despite virus-host encounter arguments against viral infection of large "particles", marine organisms 10-1000 times larger than copepods are infected by viruses, including fish (Fryer, 1996) and dolphins (Van Bressem et al., 1994). Perhaps viruses that infect organisms larger than prokaryotes are transmitted only vertically to increase the likelihood of host encounter and minimize the chance of becoming adsorbed to a particle and subsequently buried in the sediments.

However, these experiments demonstrated no support for the hypothesis that viruses infect and detrimentally affect the longevity and fecundity of *A. tonsa*. If these results are applicable to other life-history parameters and to other dominant species of copepods, then it may be that viruses exert no significant control on copepod population dynamics.

There are decreasing effects of viruses on marine organisms as trophic level increases (Suttle, 1994). That is, an estimated 10-20% of marine heterotrophic

bacteria are lysed daily by viruses, while at the next trophic level, it is likely that 2-3% of primary production is removed by viral infection (Suttle, 1994). The decrease between trophic levels is roughly six-fold. An extrapolation of that decrease to the next trophic level would yield 0.4% of copepod production lost to viral lysis. Perhaps the effects of viruses do decrease from primary to secondary producers, and virus effects, on secondary producers are nonexistent, episodic, or below the level of detection.

CHAPTER IV

CONCLUSIONS

Despite advances made in the last decade, the study of marine viruses remains fraught with questions. Much has been learned from investigations of water-column distributions of viruses and virus effects on bacterioplankton and eukaryotic phytoplankton. However, our knowledge of viral ecology in benthic communities and in higher trophic levels is less extensive. Information on viruses in benthic communities is limited to a few reports of viral abundance in surface sediments, with no data on ecological parameters such as production and decay rates of viruses. The subject of viruses affecting higher trophic levels has some areas of concentrated research, notably on viruses that infect economically valuable crustaceans (e.g., Overstreet et al., 1997), but little work has been done on the viral ecology of zooplankton. In this chapter, I will review the highlights of my investigations and discuss avenues of future research.

Results

Bacteria and virus-like particle (VLP) profiles in the water column and sediments of Chesapeake Bay. As hypothesized, VLPs were found in subsurface sediments of Chespeake Bay, on the order of 10^8 VLPs ml^{-1} of porewater, and they were about ten times more abundant in sediments than in the water column. VLP distributions in the water column showed a maximum at the southern side of the Bay, consistent with the pattern of higher VLP abundances in coastal versus oceanic water (Hara et al., 1991; Cochlan et al., 1993). Abundance of VLPs in the sediments showed a significant, negative correlation with grain size, a trend also

seen in bacteria distributions (e.g., Yamamoto and Lopez, 1985). Given the high abundance of VLPs in the subsurface sediment, I hypothesize that VLPs play an equally important role in structuring microbial communities in the benthos, as they do in the water column.

Experiments on virus-copepod interactions. The experiments presented here demonstrated no negative effect on egg production or survival of *A. tonsa* when the copepods were exposed to natural viruses using four different methods--concentrating natural seawater, concentrating copepod culture water, pulverizing copepods, and concentrating copepod exudates. As stated in Chapter III, the possibility exists that copepods were infected, but manifestations of the infection were not obvious (see Kinne, 1984). Despite these results, *A. tonsa* nonetheless may be a host to viruses, since many terrestrial and marine metazoans have shown to be vulnerable to viral attack. However, I conclude that if virus infection is important in structuring the population dynamics of *A. tonsa*, it is episodic at best and is dwarfed by other factors such as food availability, temperature suitability, and predation.

Future Work

VLPs in benthic environments. As discussed in Chapter II, the discovery of high numbers of VLPs in the sediment invites many questions concerning their role. For instance, we do not know what portion, if any, of the VLPs in the sediment pore water or adsorbed to sediments are infective. That issue will be difficult to resolve, given the tools now used to study marine viruses. When using transmission electron microscopy or epifluorescence microscopy to enumerate

viruses, we cannot determine whether or not they are infective. A current method used to determine virus infectivity, plating viruses on bacteria lawns and counting the number of plaques that form, would be difficult to use in this situation, since most marine bacteria are unculturable (Jannach and Jones, 1959). Additionally, we do not know which bacteria, if any, are dominant in a given sediment community. Thus, obtaining multiple host-phage isolates that are representative of benthic microbial communities would be difficult.

Examining thin sections of benthic bacteria with transmission electron microscopy would show whether or not benthic bacteria are infected by viruses, but one would not know if the bacteria and viruses that infect them were produced *in situ* or if they were deposited from the water column. Given that the doubling times of bacteria are short, on the order of days, it would seem that bacteria that survive the trip from the water column to the benthos eventually become benthic bacteria, which complicates questions regarding dynamics of "water column" vs. "benthic" bacteria. Additionally, the absence of viruses from thin sections does not necessarily preclude infection. Mature, assembled viruses are present and visible in marine bacteria by transmission electron microscopy for only a small portion of the lytic cycle, on the order of 20% of the cycle (Proctor et al., 1993).

The biological differences in sedimentary and water-column environments may mean that the viral ecology of the two areas are vastly different. That is, given the density of potential hosts in the sediment pore water relative to the water column, one has to wonder whether rates of host infection, lysis, and subsequent DOM production are greater in the sediment than in the water column. Also, since

benthic bacteria generally have greater biovolume than water column bacteria, perhaps the burst size, i.e., the number of viruses released during cell lysis, is greater for benthic bacteria than the burst size of water-column bacteria. This question could be answered by with transmission electron microscopy, using as a model the latent period work done using samples from the water column (Proctor et al., 1993).

Another intriguing question regarding benthic viruses is borrowed from terrestrial microbiology–what role do surface layers of bacteria ("S-layers") play in virus recognition of potential hosts? S-layers are outer layers of protein or glycoprotein found in more than 300 Bacteria and Archaea strains (Beveridge, 1994). Research with *Bacillus sphaericus* isolated from soil suggests that changes occur in its S-layer when oxygen concentration is decreased, rendering them unrecognizable to a bacteriophage (Rodriguez and Lewis, 1996). Is this interaction paralleled in marine bacteria? If bacteria produced in the water column are buried in sediment, perhaps their S-layers change under reduced oxygen conditions, and viruses associated with them can no longer attach and infect them.

Isolating a copepod-infecting virus. Even though I concluded that viruses are not important to copepod population dynamics, at least to *A. tonsa*, it is worthwhile to try to isolate a copepod-infecting virus. If a host-phage system could be maintained, some of the interesting questions to be addressed would be: How is the virus transmitted? What is its prevalence? Is it responsible for gene flow between different copepod populations? The simplest way to determine virus presence would be to look for plaques on tissue lawns. Unfortunately, no cell lines

exist for copepods. The effort necessary to establish and maintain the line might be enormous with little reward. One would have to choose which type(s) of tissue to keep in culture, although it is possible that no viruses exist in nature that infect it.

Another way to infect copepods with a virus may be to feed them virus-infected food. This method is used by researchers who study shrimp viruses (e.g., Overstreet et al., 1988). *A. tonsa* eat their nauplii (Lonsdale et al., 1979); therefore, feeding infected nauplii to healthy adults may cause infection in the adults. One would need to find visibly virus-infected nauplii in nature, which might be distinguished from healthy nauplii by malformity, weak swimming, or colonization by microbes. Although these signs could be due to infection by other microbes such as bacteria or fungi, rather than viral infection, this method might be useful in isolating a copepod-infecting virus.

Combining stress and elevated virus concentrations may be an effective way to induce infection. Possible methods to stress copepods are as follows: 1) rear copepods under sub-optimum temperature or food conditions; 2) rear copepods in very crowded conditions; 3) use copepods that may be naturally stressed, old copepods, as the test organisms; 4) exposing copepods to chemicals; and 6) expose a healthy population of copepods to concentrated viruses from another geographic area. The prevalence of *Baculovirus penaei* virus is increased in host penaeid shrimp when the shrimp are stressed by being kept in aquaria or are exposed to polychlorinated biphenyls (Johnson, 1983). Similarly, penaeid shrimp showed decreased numbers of inclusion bodies of infectious hypodermal and

hematopoietic necrosis virus as water exchange rates increased from 50 to 100% in experimental tanks (Browdy et al., 1993). Therefore, stressing copepods with one of these methods then exposing the copepods to elevated virus concentrations may lead to the isolation of a copepod-infecting virus.

Infection of copepods by benthic viruses.

One link between the two areas of research discussed in this dissertation is the reproductive life cycle of copepods. Many species of copepods, including *Acartia tonsa*, produce two types of eggs, subitaneous and resting eggs (Uye, 1985; Dahms, 1995). During seasons of active growth, copepods produce subitaneous eggs, which hatch immediately, within hours or days. At the onset of unfavorable conditions, copepods may produce resting eggs (either true diapause eggs or quiescent eggs), which sink and hatch as long as weeks, months, or years later. Because resting eggs are deposited in the sediment before they hatch, they are subject to infection by microbes in the sediment, including viruses. Subitaneous eggs produced in shallow, coastal areas may also be deposited in the sediments prior to hatching and could be infected by viruses during their brief stay in the benthos. In fact, calculations show that *A. tonsa* eggs sink to the sediment in Chesapeake Bay before they hatch (Appendix D). The potential effects of viruses on copepod eggs range from sub-lethal, such as the incorporation of a virus that later causes reduced egg production, to lethal, such as a virus that prevents egg hatching. Therefore, virus distributions and abundances in sediments may affect the life-history parameters of pelagic copepods.

A relatively quick way to check for VLP attachment on copepod eggs

deposited in the sediment is to collect copepod eggs from the sediment, stain them with the nucleic acid stain YO-PRO™-1, and examine the eggs by epifluorescent microscopy. Staining *A. tonsa* eggs collected from laboratory cultures showed that eggs were stained, but there was no obvious attachment of VLPs on the egg surface (pers. obs.). An alternative way to examine copepod eggs for virus attachment is by transmission or scanning microscopy. However, both methods require labor-intensive specimen preparation and are expensive to use.

One way to test the hypothesis that benthic viruses infect *A. tonsa* is to expose copepod eggs to elevated virus concentrations, using methods similar to those described in this dissertation:

collect surface sediment
↓
express pore water via centrifugation
↓
pre-filter through GF/F + 0.45 μm filters
↓
further concentrate viruses in pore water via vortex flow filtration
↓
add concentrated viruses + *A. tonsa* eggs to microfuge tubes
↓
monitor eggs for hatching success + compare to hatching success in control treatments
↓
rear eggs that hatch to adulthood in beakers
↓
monitor survival of all copepods + egg production of females + compare to survival + egg production in control treatment

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APPENDIX A

Coordinates of stations from water-column and sediment sampling

Station	Coordinates
1	37° 13.48' N 76° 03.27' W
2	37° 09.88' N 76° 04.92' W
3	37° 06.22' N 76° 06.70' W
4	37° 02.68' N 76° 08.50' W
5	36° 59.13' N 76° 10.12' W

APPENDIX B

Concentration of benthic viruses (or VLPs) and ratio of benthic viruses (or VLPs) to water-column viruses (or VLPs)

All sampling locations represent saltwater environments except for Lac Gilbert, which is freshwater.

Location	Source of benthic viruses	Viruses (or VLPs)	Ratio (benthic: water column)	Reference
Chesapeake Bay	Pore water	1.9 - 7.1 x 10^8 ml^{-1}	3-27	This study
Chukchi Sea	Pore water	0.27 x 10^8 ml^{-1}	~9	Stewart et al., 1996
Florida Keys	Sediment	1.4 - 5.3 x 10^8 cm^{-3}	~10^2	Paul et al., 1993
Tampa Bay, Florida	Sediment	~100 x 10^8 cm^{-3}	~10^3	Rose and Reynolds, unpubl. data
Lac Gilbert, Canada	Sediment	6.5 - 183 x 10^8 ml^{-1}	10^1-10^3	Maranger and Bird, 1996

APPENDIX C

Notes on preliminary experiments to demonstrate negative effects of viruses on the survival and egg production of *A. tonsa*

Experiment number = 1
Start date = 27 May 1995
(Note: All of my experiments were numbered consecutively, however only the experiments designed to test hypotheses about copepod-virus interactions are included in this appendix.)

-prefilter 20 L of 1-month old Chesapeake Bay water through glass fiber filters + 0.22μm cellulose acetate filters
-concentrate VLPs using a MEMBREX Benchmark® Gx Vortex Flow Filtration System (VFF) set up in recirculation mode with a 100 kD filter.
-add 60 copepod eggs + 135 ml VLPC (virus-like particle, VLP, concentrate) + 865 ml seawater (salinity = 25) to 1-L beaker (n = 1) (virus treatment)
-add 60 copepod eggs + 100 ml seawater (salinity = 25) to beaker (n = 1) (control treatment)
-add *I. galbana* to both beakers
-18 days later, measure egg production for 12 h; feed females *I. galbana* and *R. salina*
-measure egg production in 250-ml beakers (n = 2 beakers for control; n = 3 beakers for virus) with a sleeve having a 202 μm mesh bottom submerged inside beaker ; put 2-3 females in each sleeve:

Beaker i.d. (# of females per beaker)	**# eggs + nauplii per female**	**Total # eggs**	**Total # live nauplii**	**Total # dead nauplii**
C1 (3)	9	18	8	1
C2 (3)	10	27	3	0
V1 (3)	18.3	36	19	0
V2 (3)	8	12	12	0
V3(2)	44	58	30	0

-all beakers had green ciliates in them
→no difference in egg production in control vs. virus treatments over a 12-hour egg- laying period
→16 days later, only 1 copepodite in each treatment was alive.

Experiment number = 2
Start date = 19 June 1995

-prefilter 22 L of 1-day old Chesapeake Bay mouth water through glass fiber filters + 0.22μm cellulose acetate filters

-concentrate VLPs using VFF set up in recirculation mode with a 100 kD filter.
-add a few copepod eggs + 140 ml VLPC (salinity = 27) + 110 ml seawater (salinity = 27) to a 250-ml polymethylpentene (PMP) beaker (n = 1) (virus treatment)
-add a few copepod eggs + 250 ml seawater to a PMP beaker (n = 1) (control treatment)
-(few eggs were available from lab cultures for experiments)
-add 50 ml of a mix of *I. galbana* + *R. salina* to both beakers
➜All copepods died.

Experiment number = 4
Start date = 4 July 1995

-collect 24 L of seawater in an acid-rinsed carboy with a 5-L Niskin bottle from a depth of 2-3 m 1.5 mi E of the Chesapeake Bay Bridge Tunnel trestle bridge
-collect *A. tonsa* from the same location
-water temperature = 25°C and salinity = 25
-transport to laboratory in darkness and on ice
overnight, prefilter seawater through glass fiber filters + 0.22μm cellulose acetate filters into acid-rinsed carboy on ice
-the next day, concentrate VLPs using VFF set up in recirculation mode with a 100 kD filter.
-put 15 copepod eggs + 48 ml VLPC + 200 ml autoclaved GF/F-filtered seawater (seawater = 27) in 250 ml PMP beakers (n = 3) (virus treatment)
-put 15 copepod eggs + 250 ml autoclaved GF/F-filtered seawater (seawater = 27) in 250 ml PMP beakers (n = 3) (control treatment)
-add a 10 ml mix of *I. galbana* + *R. salina* to all beakers
-arrange beakers randomly on shelf in incubator; temperature = 20°C; light = 12L:12D (approximately 60 μmoles m^{-2} s^{-1})
➜After 12 days, the only live copepod was one copepodite in a virus beaker.

Experiment number = 8
Start date = 21 August 1995

-collect 30 L of seawater in an acid-rinsed carboy with a submersible pump from a depth of 6 m at the North Channel in Chesapeake Bay
-water temperature = 25.6°C and salinity = 28.9
-transport to laboratory in darkness and on ice
-immediately prefilter seawater through glass fiber filters + 0.22μm cellulose acetate filters into an acid-rinsed carboy on ice with a vacuum pressure $\leq$ 60 mm Hg
-immediately concentrate VLPs using VFF set up in recirculation mode with a 100 kD filter; VLPC checked by light microscopy, and it looked clear
-put 15 copepod eggs + 33 ml VLPC + 217 ml autoclaved GF/F-filtered seawater in 250 ml PMP beakers (n = 3) (virus treatment)
-put 15 copepod eggs + 250 ml autoclaved GF/F-filtered seawater in 250 ml PMP

beakers (n = 3) (control treatment)
-add *I. galbana* to all beakers
-put beakers in incubator; temperature = 20°C; light = 12L:12D (approximately 60 μmoles m^{-2} s^{-1})
-6 days later (day 6 of experiment), change the water in all beakers; replace with autoclaved seawater + *I. galbana* and *R. salina*
-11 days later (day 17 of experiment), measure egg production over 24 h
-measure egg production in 250-ml beakers filled with autoclaved GF/F-filtered seawater with *R. salina* (n = 2 beakers for control; n = 3 beakers for virus) with a sleeve having a 202 μm mesh bottom submerged inside beaker; put 1-3 females in each beaker:

Beaker i.d. (# of females per beaker)	**# eggs + nauplii per female**	**Total # eggs**	**Total #live nauplii**	**Total # dead nauplii**	**Total # live + dead nauplii + eggs 24 h after eggs were counted**
C1 (1)	32	22	10	0	3 live 17 dead 3 mia 0 eggs
C2 (1)	33	16	17	0	1 live 15 dead 0 eggs
V1 (1)	40	23	17	0	1 live 20 dead 2 mia 0 eggs
V2 (2)	26	29	21	2	21 live 6 dead 2 eggs
V3(2)	23	51	17	0	9 live 30 dead 9 eggs

➜There was no difference in egg production in control vs. virus treatments over a 24-hour egg-laying period.

Experiment number = 9

Start date = 1 September 1995

-collect 19 L of seawater in an acid-rinsed carboy with a submersible pump from a depth of 5 m at Sand Shoal Inlet, Virginia, on the eastern side of the Delmarva peninsula.
-water temperature = 25°C and salinity = 30
-transport to laboratory in darkness and on ice
-immediately prefilter seawater through glass fiber filters + 0.22μm cellulose acetate filters into an acid-rinsed carboy on ice with vacuum pressure < 60 mm Hg
-immediately concentrate VLPs using VFF set up in recirculation mode with a 100 kD filter and keep seawater on ice during concentration; VLPC checked with light microscopy, and it looked clear
-put 20 copepod eggs + 80 ml VLPC + 920 ml autoclaved GF/F-filtered seawater (salinity = 27) in 1-L plastic beakers (n = 3; V1-V3) (virus treatment)
-put 20 copepod eggs + 1000 ml autoclaved GF/F-filtered seawater (salinity = 27 in 1-L plastic beakers (n = 3; C1-C3) (control treatment)
-add 1.25 x 10^5 cells ml^{-1} of *I. galbana* to all beakers
-arrange beakers randomly on shelf in incubator; temperature = 20°C; light = 12L:12D (approximately 60 μmoles m^{-2} s^{-1})
-7 days later (day 7 of experiment), change water in all beakers; replace with autoclaved GF/F-filtered seawater and 19,000 cells ml^{-1} of *R. salina*
-8 days later (day 15 of experiment), measure egg production over 12 h
-measure egg production in 125-ml Erlenmeyer flasks with autoclaved GF/F-filtered seawater and 19,000 cells ml^{-1} of *R. salina*; experiment designed in a nested fashion: 2-4 groups of 1-2 female copepods were removed from each experimental beaker and placed in a flask (n = 10 flasks for control; n = 6 flasks for virus). Arrange flasks randomly on incubator shelf. Egg production was not measured for any copepods in beaker V2 because on day 15, there was a lot of green lettuce-like algae in the beaker with only 2 copepods. Beaker V2 was at the end of the shelf in the incubator--too little light?

Beaker i.d. (# of females per beaker)	**# eggs + nauplii per female**	**Total # eggs**	**Total #live nauplii**	**Total # dead nauplii**	**Total # nauplii + eggs 36 h after eggs were counted**
C1a (2)	16	32	0	0	30 nauplii 2 eggs

Beaker i.d. (# of females per beaker)	# eggs + nauplii per female	Total # eggs	Total #live nauplii	Total # dead nauplii	Total # nauplii + eggs 36 h after eggs were counted
C1b (1)	10	10	0	0	9 nauplii 1 mia
C1c (2)	10.5	21	0	0	19 nauplii 2 eggs
C1d (2)	30.5	61	0	0	59 nauplii 2 eggs
C2a (1)	14	14	0	0	13 nauplii (1 egg accidently smashed with pipet)
C2b (1)	16	16	0	0	9 nauplii 7 eggs
C3a (2)	12	24	0	0	23 nauplii 1 egg
C3b (2)	19.5	39	0	0	39 nauplii 0 eggs
C3c (1)	30	30	0	0	29 nauplii 1 egg
C3d (1)	24	24	0	0	23 nauplii 1 egg
V1a (1)	17	17	0	0	0 nauplii 17 eggs unfertilized female?
V1b (1)	19	19	0	0	2 nauplii 17 eggs
V3a (1)	17	17	0	0	17 nauplii 0 eggs

Beaker i.d. (# of females per beaker)	# eggs + nauplii per female	Total # eggs	Total #live nauplii	Total # dead nauplii	Total # nauplii + eggs 36 h after eggs were counted
V3b (1)	6	6	0	0	6 nauplii 0 eggs
V3c (1)	18	18	0	0	0 nauplii 18 eggs unfertilized female?
V3d (1)	15	15	0	0	13 nauplii 2 eggs

Grand means of control beakers = 16.75 (C1), 15 (C2), 21.4 (C3)
Grand means of virus beakers = 18 (V1), 14 (V3)

→Egg production in control vs. virus treatments was similar over a 12-hour egg-laying period (note that low egg production in V3 was due to female in flask V3b, which produced only 6 eggs).

Experiment number = 10
Start date = 11 September 1995

-collect 30 L of seawater in a seawater-rinsed carboy with a submersible pump from a depth of 7 m at the North Channel at Chesapeake Bay mouth; location approximately 37° 10.90 ' N, 76° 01.12' W
-water temperature = 24.5°C and salinity = 27.6
-transport to laboratory in darkness and on ice
-immediately prefilter seawater through glass fiber filters + 0.22μm cellulose acetate filters at vacuum pressure = 50 -60 mm Hg
-immediately concentrate VLPs using VFF set up in recirculation mode with a 100 kD filter and keep seawater on ice during concentration. Light microscopy of VLPC showed stick-like debris in concentrate
-put 40 copepod eggs + 44 ml VLPC + 948 ml autoclaved GF/F-filtered seawater in 1-L plastic beakers (n = 3) (virus treatment)
-put 20 copepod eggs + 1000 ml autoclaved GF/F-filtered seawater in 1-L plastic beakers (n = 3) (control treatment)
-add 2.0 x 10^4 cells ml^{-1} of *I. galbana* to all beakers
-arrange beakers randomly on shelf in incubator; temperature = 20°C; light = 12L:12D (approximately 60 μmoles m^{-2} s^{-1})

-5 days (day 5 of experiment) later, change the water in all beakers; replace with autoclaved GF/F-filtered seawater (salinity = 27) and 1.9 x 10^4 cells ml^{-1} of *R. salina*
-4 days later (day 9 of experiment), change the water in all beakers; replace with autoclaved GF/F-filtered seawater (salinity = 27) and 1.9 x 10^4 cells ml^{-1} of *R. salina*
-another 4 days later (day 13 of experiment), change the water in all beakers; replace with autoclaved GF/F-filtered seawater (salinity = 27) and 1.9 x 10^4 cells ml^{-1} of *R. salina*
→1 day later (day 14 of experiment), the experiment was terminated because of diatom bloom in all 3 virus beakers. There was only 1 live copepod in the V1 and V2 beakers; V3 had some live adults

Experiment number = 11
Start date = 11 September 1995

-collect 30 L of seawater in an acid-rinsed carboy with a submersible pump from a depth of 7 m at the North Channel at Chesapeake Bay mouth; location approximately 37° 10.90 ' N, 76° 01.12' W
-water temperature = 21.8°C and salinity = 29
-transport to laboratory in darkness and on ice
-immediately prefilter seawater through glass fiber filters + 0.22μm cellulose acetate filters at a vacuum pressure of < 60 mm Hg into an acid-washed carboy on ice
-immediately concentrate VLPs using VFF set up in recirculation mode with a 100 kD filter and keep seawater on ice during concentration
-put 3 adult female copepods and 1 adult male copepod + 30 ml VLPC + 220 ml autoclaved GF/F-filtered seawater (salinity = 27) in 250-ml PMP beakers (*n* = 3; V1, V2, V3) (virus treatment)
-put 80 first-stage nauplii + 30 ml VLPC + 220 ml autoclaved GF/F-filtered seawater (salinity = 27) in a 250-ml PMP beaker (*n* =1; V1a) (virus treatment)
-put 3 adult female copepods and 1 adult male copepod + 250 ml autoclaved GF/F-filtered seawater (salinity = 27) in 250-ml PMP beaker (*n* = 3; C1, C2, C3) (control treatment)
-put 80 first-stage nauplii + 220 ml autoclaved GF/F-filtered seawater (salinity = 27) in a 250-ml glass beaker (*n* =1; C1a) (control treatment)
-add 1.9 x 10^4 cells ml^{-1} of *R. salina* to all beakers
-arrange beakers randomly on shelf in incubator; temperature = 20°C; light = 12L:12D (approximately 60 μmoles m^{-2} s^{-1})
-2 days later (day 2 of experiment), add enough *R. salina* to all beakers to bring the concentration of *R. salina* to 1.9 x 10^4 cells ml^{-1}
-3 days later (day 5 of experiment), add enough *R. salina* to all beakers to bring the concentration of *R. salina* to 2.8 x 10^4 cells ml^{-1}
→1 day later (day 6 of experiment), the copepods in beakers C1 and C2 were dead; the experiment was terminated.

Experiment number = 22
Start date = 14 January 1996

-The purpose of this experiment was to immerse live, laboratory-reared copepods in virus concentrate, then fix copepods and examine them for the presence of viruses with transmission electron microscopy (TEM)
-collect 10 L of surface seawater in a seawater-rinsed bucket from Harrison's Fishing Pier, Chesapeake Bay
-water temperature = 2.5°C and salinity = 20
-immediately prefilter seawater through glass fiber filters + 0.22μm cellulose acetate filters at a vacuum pressure of < 60 mm Hg into an acid-washed carboy
-immediately concentrate VLPs using VFF set up in recirculation mode with a 100 kD filter
-put adult female copepods + 175 ml VLPC + 1400 ml autoclaved GF/F-filtered seawater (salinity = 25) in 250-ml PMP beakers (n = 1) (virus treatment)
-put adult female copepods + 175 ml autoclaved GF/F-filtered seawater (salinity = 25) in 250-ml PMP beaker (n = 3) (control treatment)
-add 1.5 x 10^4 cells ml^{-1} of *I.* galbana and 3.5 x 10^3 cells ml^{-1} of *R. salina* to both beakers
-put beakers on shelf in incubator; temperature = 20°C; light = 12L:12D (approximately 60 μmoles m^{-2} s^{-1})
-3 days later (day 3 of experiment), fix copepods for TEM in 1% electron-microscopy grade glutaraldehyde using the protocol of Blades-Eckelbarger (1991)
→Examination of thin sections of copepods in virus and control treatments showed no presence of viruses or virus-like particles in copepod tissues.

Experiment number = 33
Start date = 31 May 1997

-The purpose of this experiment was to collect eggs produced by females that were potentially infected by viruses, prepare the eggs for TEM using the protocol of Blades-Eckelbarger and Marcus (1992), and examine the eggs for the presence of viruses by TEM.
-eggs used were produced by females used in egg-production experiment 5 (described in Chapter III)
→examination of thin sections of eggs in virus and control treatments showed no presence of viruses or virus-like particles inside the eggs
→However, there were virus-size structures on the surface of eggs in both control and virus treatments that, at first glance, appeared to be viruses. The structures had "heads" that were composed of electron-dense tops and bottoms separated by regions that were not electron dense. The heasds were attached to the egg surface by a tail-like structure. The structures were not viruses, but they have also been seen on eggs of *A. tonsa* collected in Florida by P. Blades-Eckelbarger (pers. com.).

APPENDIX D

Calculation of the sinking time of subitaneous *A. tonsa* eggs in Chesapeake Bay

Assume average depth of Chesapeake Bay, including tributaries, = 689 cm
R. Brumbaugh, pers. com.

Assume fall velocity of subitaneous *A. tonsa* eggs = 0.0373 cm s^{-1}
from measurement of *A. clausi* egg settling velocity at 20°C, salinity = 32
(Uye, 1980, as cited in Marcus and Fuller, 1986; egg type unknown--assume it was subitaneous)*

Assume average time to 100% hatch of subitaneous *A. tonsa* eggs at 20°C in East Lagoon, Galveston, Texas = 19 h
(Ambler, 1985)

distance = rate x time
689 cm = 0.0373 cm s^{-1} x time
time = 18472 s
time = 5.1 hours

sinking time = 5.1 hours < average time to 100% hatch = 19 hours

Therefore, subitaneous eggs of *A. tonsa* should be deposited in sediments prior to hatching (unless they are eaten, adsorbed to a buoyant particle, or kept in suspension by mixing before they sink to the benthos)

*The fall velocity of diapause eggs of the calanoid copepod *Labidocera aestiva* is about 25% greater than the fall velocity for subitaneous eggs of *L. aestiva* (Marcus and Fuller, 1986). If Uye's (1980) calculations were for diapause eggs, applying the Marcus and Fuller (1986) correction yields a fall velocity of 0.028 cm s^{-1}, which translates to a sinking time of 6.8 hours. Eggs of *A. tonsa* would still sink to the benthos before hatching.

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