Distribution of Viruses in the Chesapeake Bay†

K. ERIC WOMMACK,[1] RUSSELL T. HILL,[1] MARTIN KESSEL,[1] ESTELLE RUSSEK-COHEN,[2]
AND RITA R. COLWELL[1]*

*Center of Marine Biotechnology, University of Maryland, 600 East Lombard Street, Baltimore, Maryland
21202,[1] and Department of Animal Sciences, University of Maryland, College Park, Maryland 20742[2]*

High virus counts were found in water samples collected from the Chesapeake Bay. Viruses were enumerated by ultracentrifugation of water samples onto grids which were visualized by transmission electron microscopy. Virus counts in September 1990, April 1991, June 1991, August 1991, and October 1991 ranged between 2.6 × 10⁶ and 1.4 × 10⁸ viruses ml⁻¹ with a mean of 2.5 × 10⁷ viruses ml⁻¹. Virus counts were usually at least three times higher than direct bacterial counts in corresponding samples. Virus counts in August and October were significantly higher than at the other sampling times, whereas bacterial counts were significantly lower at that time, yielding mean virus-to-bacterium ratios of 12.6 and 25.6, respectively. From analysis of morphology of the virus particles, it is concluded that a large proportion of the viruses are bacteriophages. The high virus counts obtained in this study suggest that viruses may be an important factor affecting bacterial populations in the Chesapeake Bay, with implications for gene transfer in natural aquatic bacterial populations and release of genetically engineered microorganisms to estuarine and coastal environments.

Viruses in natural waters have recently become a subject of renewed interest, owing to results of direct electron microscopic observations showing abundances of 10^3 to 10^8 viruses ml⁻¹ in marine waters (2, 16). The high viral counts reported within the last 2 years suggest that viral infection may be an important factor controlling bacterial populations in natural waters. The results of Børsheim et al. (3) indicate this possibility, based on the demonstration of high viral proliferation rates in marine water samples. Also, the presence of mature phage particles in marine bacteria and cyanobacteria has been directly demonstrated (16). There is, therefore, a growing body of evidence that viruses may have an important trophic role in aquatic ecosystems.

The presence in seawater of viruses capable of infecting marine bacteria was demonstrated in 1955 by Spencer (19), whose findings were followed by isolations of bacteriophages from marine sediment (18, 25) and shellfish (1). The structure of a marine bacteriophage was studied in detail by Valentine et al. (24), and parameters associated with replication of two marine bacteriophages were reported by Chen et al. (6). Isolation of marine bacteriophages and their distribution have been reviewed by Moebus (14). The recent resurgence of interest in marine viruses is primarily a result of the large numbers of marine viruses reported to be present in natural waters. Enumeration of viruses in marine ecosystems has taken on special significance because of problems associated with pollution of the marine environment by ocean dumping of domestic and industrial wastes. High viral abundance in natural waters also has important implications for the release of genetically engineered microorganisms (7). Prior information on viral abundance, reviewed by Moebus (14), and the conclusion drawn by Wiggins and Alexander (26) that bacteriophages are unlikely to have any impact on bacteria in natural environments if concentrations of individual bacterial populations are low were consistent with the prevailing view that the role of viruses in exchange of genetic material in aquatic ecosystems was insignificant. However,

the high viral counts in freshwater and marine ecosystems that have been reported now strongly indicate that genetic transduction by bacteriophages may be very important in natural waters, notably in gene transfer between autochthonous bacteria and genetically engineered microorganisms released to the estuarine, coastal, and marine environments.

The Chesapeake Bay is a well-characterized aquatic ecosystem, where an understanding of factors affecting bacterial populations is critical to effective environmental management. Bacterial production rates (11) and the relationships between bacteria and organic carbon (10) and nitrogen (9) transformations have been well studied in the Chesapeake Bay. In addition to the role of heterotrophic bacteria in nutrient cycling and secondary production and the role of cyanobacteria in primary production, bacteria are considered to be responsible for most of the oxygen consumption that results in destructive seasonal deep-water oxygen deficits (23).

There have been no investigations on the effects of bacteriophages on bacterial populations, and the abundance of viruses in the Chesapeake Bay has, heretofore, not been studied in detail. There is a single report of high virus numbers, 1.0×10^7 virus particles ml⁻¹ (2), from a Chesapeake Bay surface water sample. The objective of the study reported here was to investigate the presence of bacteriophages in the Chesapeake Bay and their role in that ecosystem. Viruses could be an important and previously neglected factor controlling bacterial populations in the Chesapeake Bay. In addition, knowledge of the interactions between bacteriophages and bacteria in natural waters is essential to understanding the importance of transduction as a mechanism of exchange of genetic material in aquatic ecosystems and to evaluate the risks of genetic transduction of genetically engineered microorganisms, a subject of extensive study in our laboratory.

As a first step in elucidating the role of viruses in the Chesapeake Bay, the number of virus particles present in Chesapeake Bay water samples was determined. Also, variations in virus counts related to sampling depth, degree of stratification of the water column, and season were deter-

---

* Corresponding author.
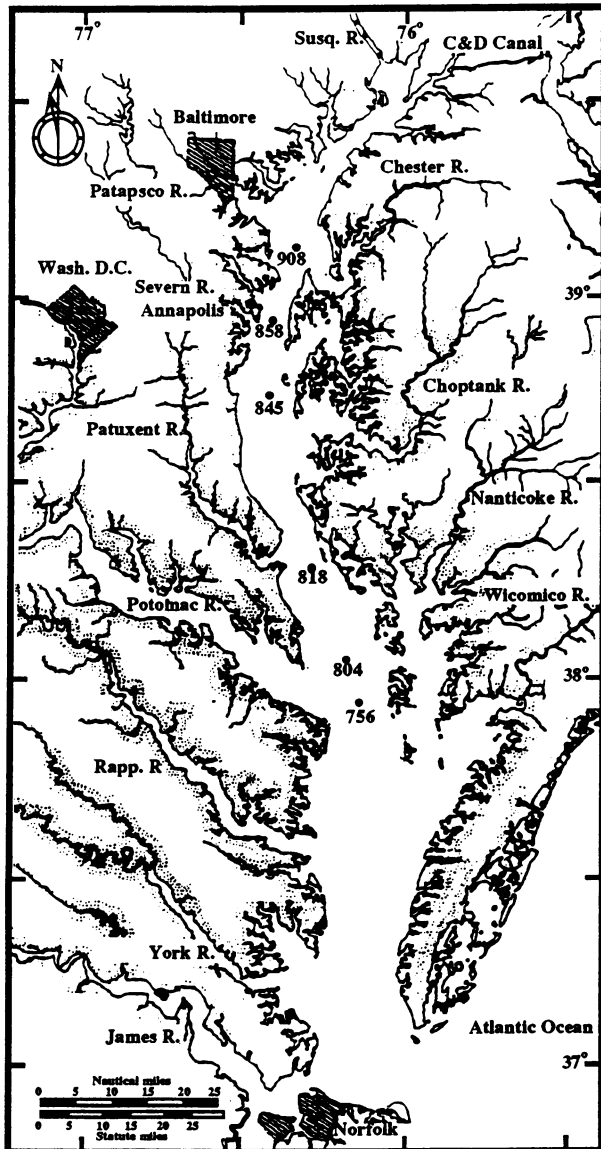† Contribution no. 194 from the Center of Marine Biotechnology.

FIG. 1. Locations of sample stations in Chesapeake Bay.

mined. From this study, a relationship between viral and bacterial numbers was able to be determined.

MATERIALS AND METHODS

Sample collection. Water samples were collected on 19 September 1990, 4 April 1991, 1 June 1991, 10 August 1991, and 8 October 1991 at six stations along the midstem axis of the Chesapeake Bay at latitudes 39°08′N, 38°58′N, 38°45′N, 38°18′N, 38°04′N, and 37°56′N. These stations are designated 908, 858, 845, 818, 804, and 756, respectively, and their locations are shown in Fig. 1. Water samples were collected 1 m below the surface and 1 m above the bottom at all stations, using 10-liter Niskin bottles attached to a General Oceanics rosette (General Oceanics, Miami, Fla.). In all cases, samples from the surface are designated T and samples from the bottom are designated B.

Water samples were fixed immediately after collection with glutaraldehyde (final concentration, 2%) for electron

microscopy and formaldehyde (final concentration, 2%) for epifluorescence microscopy and stored at 4°C until processing. Vertical profiles of temperature, salinity, dissolved oxygen, and fluorescence were determined by using an EG&G Neal Brown NK III CTD system (EG&G, Calaumet, Mass.). Dissolved oxygen was measured with a Beckman polarographic oxygen probe (Beckman Instruments, Palo Alto, Calif.). Fluorescence and attenuation were measured with a SeaTech fluorometer and a 25-cm path length transmissometer (SeaTech, Corvallis, Oreg.). These profiles established the presence or absence of stratification in the water column.

Enumeration of viruses. Viruses in the water samples were enumerated as described by Børsheim et al. (3) and Bratbak et al. (5), with slight modification as follows. Viruses were collected by ultracentrifugation onto a 400-mesh, Formvar-coated Cu electron microscopy grid (Bio-Rad Microscience Division, Cambridge, Mass.). Ten ml of each fixed water sample was centrifuged at 100,000 × g for 2.5 h in a Beckman L8-80M ultracentrifuge in an SW 41 swing-out rotor (Beckman Instruments, Palo Alto, Calif.). Grids were stained for 2 to 3 min with 2% uranyl acetate. The grids were photographed at a magnification of ×14,000 and an accelerating voltage of 80 kV with a JEOL 100CX transmission electron microscope (JEOL USA Inc., Peabody, Mass.). Negatives of the photomicrographs were enlarged and printed to give a final magnification of ×36,600.

Viruses, enumerated on the enlarged prints, were identified on the basis of size and shape. Grids were photographed randomly; however, in a few instances view fields were obstructed by large, amorphous, dark-staining material and were omitted. Five to 13 fields were counted for each sample. The number of viruses counted in each case exceeded 200, except for samples 818, 845B, and 804B from 19 September 1990, in which the number of viruses counted exceeded 150.

Enumeration of bacteria. Bacteria were counted by epifluorescence microscopy of acridine orange-stained preparations by the method of Hobbie et al. (8). Formaldehyde-fixed water samples (1 ml) were mixed with 100 μl of 0.025% (wt/vol) acridine orange, held for 3 min, and filtered onto dyed 0.2-μm-pore-size filters (Poretics Corp., Livermore, Calif.). Acridine orange direct counts were done by using an Olympus AH-2 microscope at ×1,000 magnification (Olympus Optical Co. Ltd., Lake Success, N.Y.). Six fields, each containing 80 to 200 cells, were counted on each filter. In all cases, six counts were adequate to obtain a standard deviation within 15% of the mean.

Statistical analysis. Log transformed viral and bacterial count data were analyzed by analysis of variance by using Statview II and Super ANOVA software packages (Abacus Concepts, Inc., Berkeley, Calif.).

RESULTS

Hydrographic conditions. The hydrographic data for each sampling station are presented in Fig. 2. The general trends found were as follows. During the September 1990, April 1991, and June 1991 cruises, the water column was noticeably stratified with respect to salinity. In August 1991, the water column was similarly stratified by salinity for the three mid-bay sites. The three upper-bay sites in August and all sites in October 1991 showed little change in salinity through the water column.

September and June results showed marked stratification in dissolved oxygen with nearly anoxic conditions near the
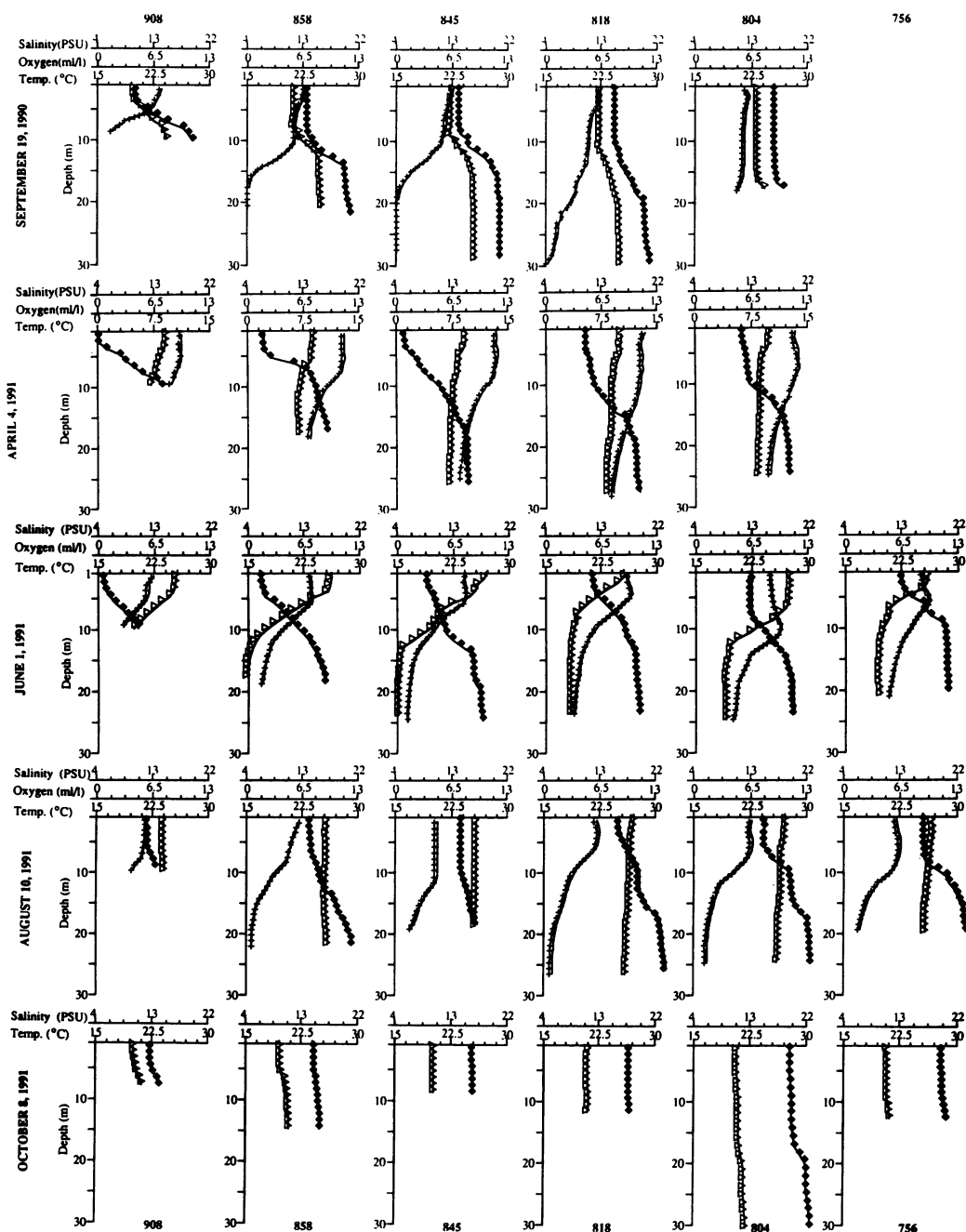
FIG. 2. Vertical profiles of salinity (◆), dissolved oxygen (+), and temperature (△) of sampling stations in Chesapeake Bay.

bottom. The reduction of dissolved oxygen with depth was less dramatic in April and August. Dissolved oxygen data were not available for the October 1991 cruise. Temperature stratification was widespread during September and June. In contrast, during April, August, and October, water temperature changed little with respect to depth.

Viral distributions. The mean virus counts for the entire period of the study and for all stations ranged between 2.6 × 10^6 and 1.4 × 10^8 viruses ml^{-1}, with a mean of 2.5 × 10^7 viruses ml^{-1} (7.1 × 10^5 standard error). The virus count data are presented in Fig. 3. Statistical analysis of the virus count data showed that viral abundance varied only seasonally.

Variability in viral abundance could not be attributed to either the water column or the sample site. Viral counts were greatest in August and October and lowest in April and September.

Bacterial distributions. Direct bacterial counts ranged from 2.5 × 10^5 to 1.4 × 10^7 cells ml^{-1} and are presented in Fig. 3. The overall mean of the mean bacterial counts was 3.1 × 10^6 cells ml^{-1} (1.2 × 10^5 standard error). Bacterial numbers during September 1990 and June 1991 samplings were at similar levels; however, both September and June counts were significantly greater than counts obtained during April 1991 ($P < 0.05$). April counts were greater than August 1991
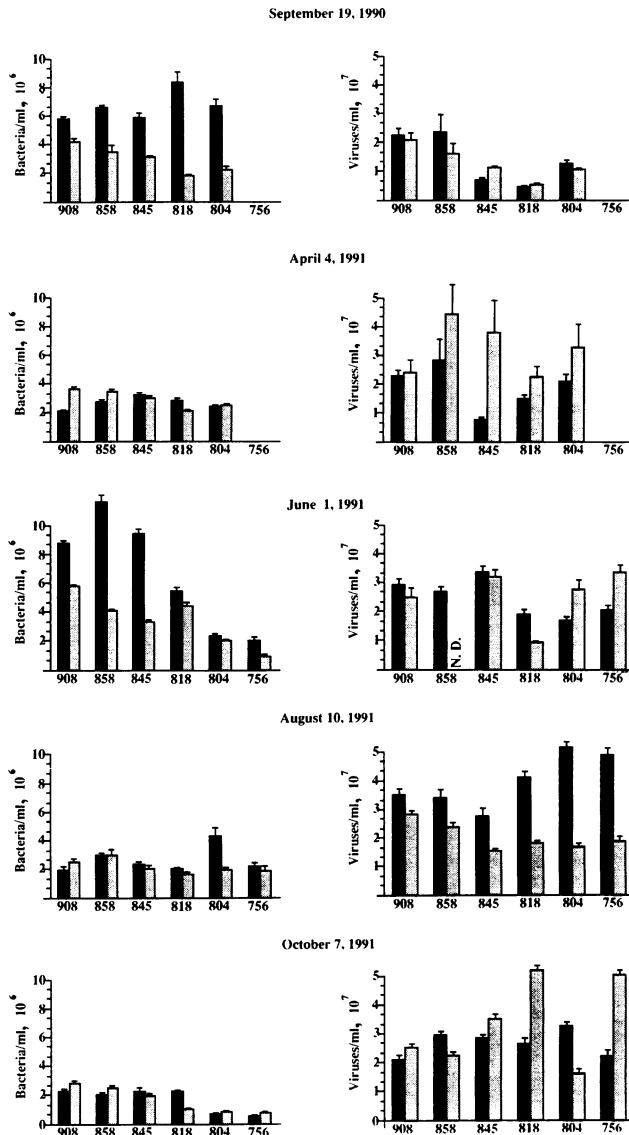
FIG. 3. Bacterial and viral abundances in water samples from Chesapeake Bay (N.D., not determined; ■, top water sample; ▣, bottom water sample). Error bars represent standard errors.

TABLE 1. VBR for each site and date of this study

Site	VBR for sample date				
	Sept. 19, 1990	April 4, 1991	June 1, 1991	Aug. 10, 1991	Oct. 8, 1991
908T	3.9	10.9	3.4	17.8	9.0
908B	4.9	6.6	4.3	11.4	9.0
858T	3.6	10.3	2.3	11.2	14.4
858B	4.6	12.8	ND^a	7.9	9.0
845T	1.2	2.4	3.6	11.8	12.5
845B	3.7	12.6	9.7	7.4	18.3
818T	0.6	5.3	3.5	20.6	11.7
818B	3.1	10.7	2.1	11.1	51.4
804T	1.9	8.6	7.1	11.9	47.8
804B	4.8	13.0	13.8	8.5	19.1
756T	ND	ND	10.2	22.3	41.0
756B	ND	ND	36.7	10.1	64.3
Total mean VBR	3.2	9.2	8.8	12.6	25.6

^{a} ND, not determined.

ratio and was statistically greater than all the other sampling dates ($P < 0.05$).

Morphology of viruses. Viruses were separated into three groups based on the diameter of the phage head: 30 nm and less, 30 to 60 nm; and 60 nm and greater. The most abundant size class was the 30- to 60-nm group. The percentages of viruses in each size group and percentages of tailed and nontailed viruses are given in Table 2. No distinction was made between bacteriophages and other viruses. Particles in the micrographs were counted as viruses on the basis of staining properties, size, and icosahedral morphology.

DISCUSSION

Early investigations of marine bacteriophages were dependent on the detection of plaque-forming units for the enumeration of bacteriophages and, although numerous marine phage host systems were isolated during the 1960s and 1970s (see the work of Moebus [14] for a review), there were very few estimates of bacteriophage concentrations. Factors affecting marine bacteriophage replication and stability were examined by Chen et al. (6). Spencer (20) detected four phage strains in concentrations of between 0.1 and 10 PFU ml^{-1} in water from the North Sea. Torrella and Morita (22) presented the first semiquantitative evidence from electron microscopy for the high incidence of viral particles in

counts, and August counts were greater than October 1991 counts ($P < 0.05$). Samples from September 1990, June 1991, and August 1991 showed significantly greater bacterial counts in surface water samples than bottom water samples ($P < 0.05$). Bacterial counts from April 1991 and October 1991 showed no variation between surface and bottom water ($P < 0.05$). Bacteria were significantly more numerous at upper-bay stations during April, June, and October. September and August samplings showed no site variation ($P < 0.05$).

VBR. On each date, the ratio of mean virus count to mean bacterial count for each site was computed; these numbers are presented in Table 1 as the virus-to-bacterium ratios (VBRs). The VBRs for each sampling date were compared. The VBRs for September 1990, April 1991, and June 1991 were not statistically different ($P < 0.05$). The VBR ratio of 25.6 (5.7 standard error) during October 1991 was the highest

TABLE 2. Size and morphology data of virus particles in Chesapeake Bay water samples

Size class	% of total count^a	% Nontailed	% Tailed
<30 nm	4.5	77	23
30 to 60 nm	64.7	66	34
>60 nm	31.8	38	62
Total	100	57	43

^{a} Total count = 33,086.

seawater and indicated that the number of phage particles observed in their seawater samples was at least 10^4 ml^{-1}. The presence of large numbers of viruses in a number of aquatic environments was confirmed a decade later (2, 16).

Results of this study show that virus counts in the Chesapeake Bay are consistently high and usually at least three times higher than total direct bacterial counts in corresponding samples. The virus particles observed by electron microscopy were morphologically similar and grouped into size classes similar to those observed by Bergh et al. (2). Many of the viruses demonstrated polygonal heads with tail structures typical of double-stranded DNA bacteriophages and were similar to the marine phage described by Valentine et al. (24). In the largest size category of viruses, where tails were most easily discernible, 62% of the viruses were tailed. Viruses with no discernible tail structure could be either bacteriophages that have lost their tails during the preparation procedure, prior to electron microscopic visualization, or bacteriophages without tail structures, such as those in Bradley groups C, D, and E (4).

It cannot be concluded, however, that all the virus particles visualized are bacteriophages. Some of the viruses may have eukaryote hosts, such as phytoplankton, or represent human and animal viruses. Eukaryotic viruses in Chesapeake Bay most probably represent a mixture of phytoplankton viruses, animal viruses (either of autochthonous origin or derived from agricultural waste), and human viruses, entering Chesapeake Bay via domestic waste discharge, either directly or from tributaries.

Suttle et al. (21) have shown that viruses which are pathogens of marine phytoplankton can be readily isolated from marine ecosystems. Phytoplankton densities during April 1991 were generally below 5×10^3 cells ml^{-1}, comprising mainly dinoflagellates and flagellates (17). It is likely that some of the viruses present in the Chesapeake Bay infect phytoplankton, but because of the large numbers of viruses present relative to phytoplankton numbers and the morphology of these viruses, we conclude that the majority of these viruses are likely to be bacteriophages. Further enumeration of viruses, bacteria, and phytoplankton is being done to elucidate those virus-host relationships.

It is interesting to speculate that bacteriophages were responsible, at least in part, for the lower direct bacterial counts measured during the April, August, and October cruises, i.e., coinciding with higher virus numbers. This relationship of lower bacterial counts and higher viral counts was observed during the spring (April), midsummer (August), and early fall (October), when changes associated with mixing of the water column are known to occur. Hydrographic data indicate that the water column was only slightly stratified on these cruise dates.

Ogunseitan et al. (15) demonstrated establishment of an equilibrium between phage and bacteria in freshwater microcosm experiments with *Pseudomonas aeruginosa* as the host. It is possible that the equilibrium between viruses and bacteria fluctuates in response to changing seasonal conditions and the formation and breakdown of water column stratification. Equally likely is the possible relationship of viruses to blooms of planktonic bacterial species occurring in the late summer months, as observed for *Vibrio parahaemolyticus* in Chesapeake Bay (12). Additional sampling is required to determine whether the increase in virus numbers observed in spring, midsummer, and early fall represents an annual cycle of abundance or transiently elevated counts.

There were no significant differences in bacterial abundance within the water column, i.e., top versus bottom

water, on the spring and early-fall sampling dates, whereas the late-spring and late-summer samplings (June and September, respectively) showed appreciable water column differences in bacterial abundance, with surface counts being greater than subpycnocline counts. These data are in general agreement with the findings of Jonas and Tuttle for seasonal fluctuations in bacterial abundance (10). Virus counts did not vary with depth of collection or sample site, indicating that viral populations may not be as strongly affected as bacterial populations by changing water column conditions.

Replicate counts of water samples from sites 818 and 804 on the April 1991 cruise demonstrate the difficulty of counting viruses in turbid estuarine water samples. Two separate transmission electron microscopy preparations of water samples 818T, 818B, 804T, and 804B were counted. The means of these counts were compared by using the Fisher's protected least-significant-difference technique. Three pairs were identical ($P < 0.05$) (818B, 818T, and 804T), and one (818B) was significantly different ($P < 0.01$). We are investigating various modifications of the method used in this study to obtain consistency in virus counts from water samples containing significant amounts of particulate matter, e.g., cellular debris and silt. We are presently unable to obtain reliable virus counts in samples collected from the northern reaches of Chesapeake Bay, near the mouth of the Susquehanna River, because of the silt content of this water.

The large numbers of viruses and the indication of seasonal changes in virus numbers suggest that viruses may, indeed, be an important trophic factor in Chesapeake Bay. Microcosm studies by Kokjohn et al. (13), employing environmental isolates of *Pseudomonas aeruginosa*, have shown that efficient attachment and replication of bacteriophages are possible at host densities as low as 10^2 culturable cells ml^{-1} and phage concentrations of 10^2 ml^{-1}. Such concentrations should be exceeded for many naturally occurring phage host systems in Chesapeake Bay, given the consistently large numbers of bacteria and phage found to be present in Chesapeake Bay water samples collected during different times of the year. There is no question but that the revitalized interest in viruses in the marine environment triggered by recent reports in the literature (3) will engender fascinating new discoveries about these microorganisms in the near future.

REFERENCES

1. **Baross, J. A., J. Liston, and R. Y. Morita.** 1978. Incidence of *Vibrio parahaemolyticus* bacteriophages and other *Vibrio* bacteriophages in marine samples. Appl. Environ. Microbiol. **36:** 492–499.
2. **Bergh, Ø., K. Y. Børsheim, G. Bratbak, and M. Heldal.** 1989. High abundance of viruses found in aquatic environments. Nature (London) **340:**467–468.
3. **Børsheim, K. Y., G. Bratbak, and M. Heldal.** 1990. Enumeration and biomass estimation of planktonic bacteria and viruses by transmission electron microscopy. Appl. Environ. Microbiol. **56:**352–356.
4. **Bradley, D. E.** 1967. Ultrastructure of bacteriophages and bacteriocins. Bacteriol. Rev. **31:**230–314.

5. **Bratbak, G., M. Heldal, S. Norland, and T. F. Thingstad.** 1990. Viruses as partners in spring bloom microbial trophodynamics. Appl. Environ. Microbiol. **56:**1400–1405.

6. **Chen, P. K., R. V. Citarella, O. Salazar, and R. R. Colwell.** 1966. Properties of two marine bacteriophages. J. Bacteriol. **91:**1136–1139.

7. **Colwell, R. R., and D. J. Grimes.** 1985. Genetic modification of microorganisms occurring in the natural environment. Proc. American Society for Testing and Materials, Philadelphia, 15 April 1985.

8. **Hobbie, J. E., R. J. Daley, and S. Jasper.** 1977. Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. **33:**1225–1228.

9. **Horrigan, S. G., J. P. Montoya, J. L. Nevins, J. J. McCarthy, H. Ducklow, R. Goericke, and T. Malone.** 1990. Nitrogenous nutrient transformations in the spring and fall in the Chesapeake Bay. Estuarine Coastal Shelf Sci. **30:**369–391.

10. **Jonas, R. B., and J. H. Tuttle.** 1990. Bacterioplankton and organic carbon dynamics in the lower mesohaline Chesapeake Bay. Appl. Environ. Microbiol. **56:**747–757.

11. **Jonas, R. B., J. H. Tuttle, D. L. Stoner, and H. W. Ducklow.** 1988. Dual-label radioisotope method for simultaneously measuring bacterial production and metabolism in natural waters. Appl. Environ. Microbiol. **54:**791–798.

12. **Kaneko, T., and R. R. Colwell.** 1978. The annual cycle of *Vibrio parahaemolyticus* in Chesapeake Bay. Microb. Ecol. **4:**135–155.

13. **Kokjohn, T. A., G. S. Sayler, and R. V. Miller.** 1991. Attachment and replication of *Pseudomonas aeruginosa* bacteriophages under conditions simulating aquatic environments. J. Gen. Microbiol. **137:**661–666.

14. **Moebus, K.** 1987. Ecology of marine bacteriophages, p. 136–156. *In* S. M. Goyal, C. P. Gerba, and G. Bitton (ed.), Phage ecology. John Wiley & Sons, Inc., New York.

15. **Ogunseitan, O. A., G. S. Sayler, and R. V. Miller.** 1990. Dynamic interactions of *Pseudomonas aeruginosa* and bacteri-

ophages in lake water. Microb. Ecol. **19:**171–185.

16. **Proctor, L. M., and J. A. Fuhrman.** 1990. Viral mortality of marine bacteria and cyanobacteria. Nature (London) **343:**60–62.

17. **Sellner, K. (Benedict Estuarine Research Laboratory).** 1991. Personal communication.

18. **Sklarow, S. S., R. R. Colwell, G. B. Chapman, and S. F. Zane.** 1973. Characterization of a *Vibrio parahaemolyticus* bacteriophage isolated from Atlantic coastal sediments. Can. J. Microbiol. **19:**1519–1520.

19. **Spencer, R.** 1955. A marine bacteriophage. Nature (London) **175:**690.

20. **Spencer, R.** 1960. Indigenous marine bacteriophages. J. Bacteriol. **79:**614.

21. **Suttle, C. A., A. M. Chan, and M. T. Cottrell.** 1991. Infection of phytoplankton by viruses and reduction of primary productivity. Nature (London) **347:**467–469.

22. **Torrella, F., and R. Y. Morita.** 1979. Evidence by electron micrographs for a high incidence of bacteriophage particles in the waters of Yaquina Bay, Oregon: ecological and taxonomical implications. Appl. Environ. Microbiol. **37:**774–778.

23. **Tuttle, J. H., R. B. Jonas, and T. C. Malone.** 1987. Origin, development and significance of Chesapeake Bay anoxia, p. 442–472. *In* S. K. Majumdar, L. W. Hall, Jr., and H. M. Austin (ed.), Contaminant problems and management of living Chesapeake Bay resources. Pennsylvania Academy of Sciences Press, Philadelphia.

24. **Valentine, A. F., P. K. Chen, R. R. Colwell, and G. B. Chapman.** 1966. Structure of a marine bacteriophage as revealed by the negative-staining technique. J. Bacteriol. **91:**819–822.

25. **Wiebe, W. J., and J. Liston.** 1968. Isolation and characterization of a marine bacteriophage. Mar. Biol. **1:**244–249.

26. **Wiggins, B. A., and M. Alexander.** 1985. Minimum bacterial density for bacteriophage replication: implications for significance of bacteriophages in natural ecosystems. Appl. Environ. Microbiol. **49:**19–23.