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Effects of Sunlight on Bacteriophage Viability and Structure†

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Current estimates of viral abundance in natural waters rely on direct counts of virus-like particles (VLPs), using either transmission or epifluorescence microscopy. Direct counts of VLPs, while useful in studies of viral ecology, do not indicate whether the observed VLPs are capable of infection and/or replication. Rapid decay in bacteriophage viability under environmental conditions has been observed. However, it has not been firmly established whether there is a corresponding degradation of the virus particles. To address this question, viable and direct counts were carried out employing two Chesapeake Bay bacteriophages in experimental microcosms incubated for 56 h at two depths in the York River estuary. Viruses incubated in situ in microcosms at the surface yielded decay rates in full sunlight of 0.11 and 0.06 h^{-1} for CB 38Φ and CB 7Φ, respectively. The number of infective particles in microcosms in the dark and at a depth of 1 m was not significantly different from laboratory controls, with decay rates averaging 0.052 h^{-1} for CB 38Φ and 0.037 h^{-1} for CB 7Φ. Direct counts of bacteriophages decreased in the estuarine microcosms, albeit only at a rate of 0.028 h^{-1}, and were independent of treatment. Destruction of virus particles is concluded to be a process separate from loss of infectivity. It is also concluded that strong sunlight affects the viability of bacteriophages in surface waters, with the result that direct counts of VLPs overestimate the number of bacteriophage capable of both infection and replication. However, in deeper waters, where solar radiation is not a significant factor, direct counts should more accurately estimate numbers of viable bacteriophage.

Viruses are now widely recognized as the most abundant member of aquatic planktonic communities. Recognition of an abundant viroplankton community was made possible through the use of the transmission electron microscope (TEM). Electron microscopy for direct examination of aquatic microbial communities (33, 41) or specific marine bacteriophages (42, 43) has been used for some time. However, quantitative use of TEM for the purpose of obtaining total (direct) counts of viruses in natural water samples is a relatively recent development (1). Through utilization of this technique, it is now known that numbers of viruses in natural waters can range from 10^4 to 10^8 virus-like particles per ml and are typically 3 to 10 times greater than numbers of bacteria (1, 31, 48). Enumeration of viruses in natural waters has also been accomplished by epifluorescence microscopy (11, 14) and pulsed-field gel electrophoresis (19). TEM has been widely used and has the important advantage of yielding useful morphological information (5, 9, 36, 48).

By direct enumeration, without prefiltration of natural water samples, it was shown that viral abundance can change over very short time scales, e.g., during spring phytoplankton blooms (5), over longer-term seasonal scales in estuarine (48) and marine (17) waters, spatially along coastal to offshore transects (7, 31), and along a trophic gradient in the northern Adriatic Sea (45). In each study, viral numbers exceeded bacterial numbers by a factor of at least 3 and usually by a factor of 10. With the recognition that large dynamic populations of marine viruses are present in seawater, research on marine viral ecology has begun to focus on the capacity of bacteriophages to limit bacterial productivity and to investigate mechanisms for maintaining large aquatic viral communities and analyze the composition and stability of natural viroplankton communities (see reference 10 for a review).

Questions concerning the ecological role of viruses in microbial communities have prompted a resurgence of interest in the persistence of free viruses in marine waters. Traditionally, due to public health concerns about the safety of recreational and drinking waters, research on the fate of viruses in natural waters has focused directly on enteroviruses or on coliphages, which are routinely used as indicators of enteroviral pollution. In these studies, experimental treatments have concentrated on chemical or biological virus-inactivating agents. Possible chemical inactivating agents examined have been salinity (21, 23, 49), sewage pollution (2, 44), filtrates of bacterial cultures (40), and the presence of proteins and amino acids (22). Studies on biological inactivating agents have focused on the presence of specific bacteria or the natural bacterial community (6, 24, 26, 32, 39) or lysates of bacterial cultures (23). From these studies the most significant factor associated with inactivation of viruses in water is the presence of a natural bacterial community. The effect of bacteria on numbers of viruses has been shown to be offset by the protective effect of viral adsorption to sediment and particulates suspended in the water column (3, 4, 13, 20, 34). Even heat- or UV-killed bacterial cells can protect viruses through adsorption (25).

In a comprehensive review of viral inactivation in seawater, Kapuscinski and Mitchell (18) concluded that the level of solar radiation penetrating surface waters is not important in the degradation of enteric viruses discharged into natural waters. In more recent studies, utilizing various indigenous marine bacteriophages (37, 39), cyanophage (37), and a virus of *Micromonas pusilla*, a cosmopolitan marine phytoplankter (8), Suttle and coworkers have examined several processes implicated in the loss of infectivity of viruses in seawater. In all studies sunlight was determined to be the dominant factor controlling decay of viral infectivity in seawater. In light of the conclusion by Kapuscinski and Mitchell (18), the discovery of sunlight as a major factor in the decay of viruses in natural

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waters has been innovational (10, 38, 39). To evaluate the effects of solar radiation on survival of viruses in surface waters, in situ effects of natural sunlight on infectivity and destruction of viral capsids of two estuarine bacteriophages were examined.

MATERIALS AND METHODS

Phage host systems and preparation of bacteriophage stocks. Isolation, propagation, and characteristics of the two phage host systems used in this study, CB 7 and CB 38, are described elsewhere (46, 47). Previous examination of the carbon substrate utilization profiles of phage hosts CB 38 and CB 7, using the Biolog system (Biolog, Hayward, Calif.), revealed that CB 38 had a weak identity to *Flavobacterium meningosepticum*, while host CB 7 showed no significant relationship to any strains in the Biolog database (47). Upon retesting, with an updated version of the Biolog database, both bacterial hosts were judged most closely related to *Aeromonas* DNA group 11 (16) strains, with identities of 88% (host CB 7) and 52% (host CB 38).

Bacteriophage were purified by elution from soft agar overlays, using sterile four-salts buffer (1% NaCl, 3.8 mM $CaCl_2$, 3.8 mM KCl, 18 mM $MgSO_4 \cdot 7H_2O$). The soft agar was separated from the phage-containing buffer by centrifugation (3,000 rpm, 1 h), filtered through a 0.22-μm-pore-size Sterivex syringe filter (Millipore, Bedford, Mass.), and dialyzed against sterile four-salts buffer at 4°C. The titer of the final dialyzed phage solution was determined by plaque assay, and the solution was stored at 4°C.

Preparation of estuarine microcosms and experimental conditions. Experimental microcosms consisted of small (9 by 3 cm) plastic bags prepared by sealing two sheets of plastic, Cultu-Sac (Becton-Dickenson, Baltimore, Md.) (27), with a heat sealer. The percentages of light transmitted by the plastic film for the following ranges of wavelengths are given in parentheses: UV-C, 200 to 290 nm (3 to 23%); UV-B, 290 to 320 nm (23 to 26%); UV-A, 320 to 400 nm (26 to 32%); and photosynthetically active radiation (PAR), 400 to 700 nm (32 to 55%). Each bag was filled with 15 ml of filtered (0.22-μm-pore-size filter) 15‰ artificial seawater containing 10^7 PFU of either CB 38Φ or CB 7Φ ml^{-1}. Once filled, the bags were heat sealed so that no air bubbles were trapped in the microcosm. At the beginning of the experiment, microcosms were attached horizontally with rubber bands to an experimental apparatus which held the bags at either the surface or a depth of 1 m in the York River estuary off the Virginia Institute of Marine Science pier (37°15′15″ N, 76°30′00″ W). To achieve dark incubation, microcosms were covered with black plastic. Microcosms were incubated at the surface, light and dark; and at 1 m, light and dark. There was a laboratory control incubated in the dark at 18°C. Replicate samples for each treatment were collected at selected times to 56 h. Data on total PAR were taken from daily climate records kept at the Virginia Institute of Marine Science. PAR was measured with an Epply model PSP Precision Spectral Pyranometer at 6-min intervals. Available PAR in the water column was measured at noon and 1600 h daily, using a submersible Li-Cor quantum sensor, model LI-192S (Li-Cor, Inc., Lincoln, Neb.).

Bacteriophage counts of estuarine microcosms. At selected intervals, two replicate bags were collected and transported to the laboratory for processing. Each bag was wiped with 80% ethanol and aseptically opened. Two milliliters of the phage suspension was taken from each bag for plaque assay, and the remaining 13 ml was fixed with glutaraldehyde (2% [wt/vol] final concentration) and stored at 4°C. Direct phage counts of the glutaraldehyde-fixed water samples was done with a TEM (47).

The titers of viable bacteriophages in the microcosms were determined from the number of PFU in microcosm samples. Host cells were prepared by inoculating 50 ml of LBES broth (45) with a bacterial host picked from an LBES agar plate with a sterile loop. Broth cultures were grown overnight at 25°C with shaking (250 rpm). For determining titers, 100 μl of phage suspension was added to 100 μl of susceptible host cells in a 13-ml disposable culture tube. The mixture was incubated at 25°C in the dark for 30 min. After the attachment period, 3 ml of molten (45 to 47°C) LBES agar (0.6% agar) was added to the phage-host mixture, the tube was vortexed, and the contents were poured onto a solid LBES agar plate. Plaques were counted after incubation for 16 to 24 h at 25°C. For CB 7Φ, plating efficiency had been determined to be maximum on exponential growth stage cells (46); therefore, a 1:100 dilution of overnight cells was added to 50 ml of fresh LBES broth, and the mixture was incubated for 4 to 5 h prior to determination of titers. Viable counts of CB 38Φ were obtained with cells from a culture inoculated overnight.

RESULTS AND DISCUSSION

Bacteriophage viability. Experiments were conducted over a 56-h period from 0800 h on 17 October 1994 to 1600 h on 19 October 1994. The water temperature was 18°C, and weather conditions were clear and sunny on 17 and 18 October with noon irradiance values of 720 and 690 W m^{-2}, respectively. On 19 October, conditions were partly sunny and noon irradiance was 380 W m^{-2}, with high cirrus clouds. Sunlight conditions are shown in Fig. 1A and B in which solar data have been provided along with corresponding plaque count data. Attenuation of PAR through the water column was measured at noon on 18 and 19 October. Light levels at 1-m depth were 10 and 38% of surface sunlight, respectively, on those days.

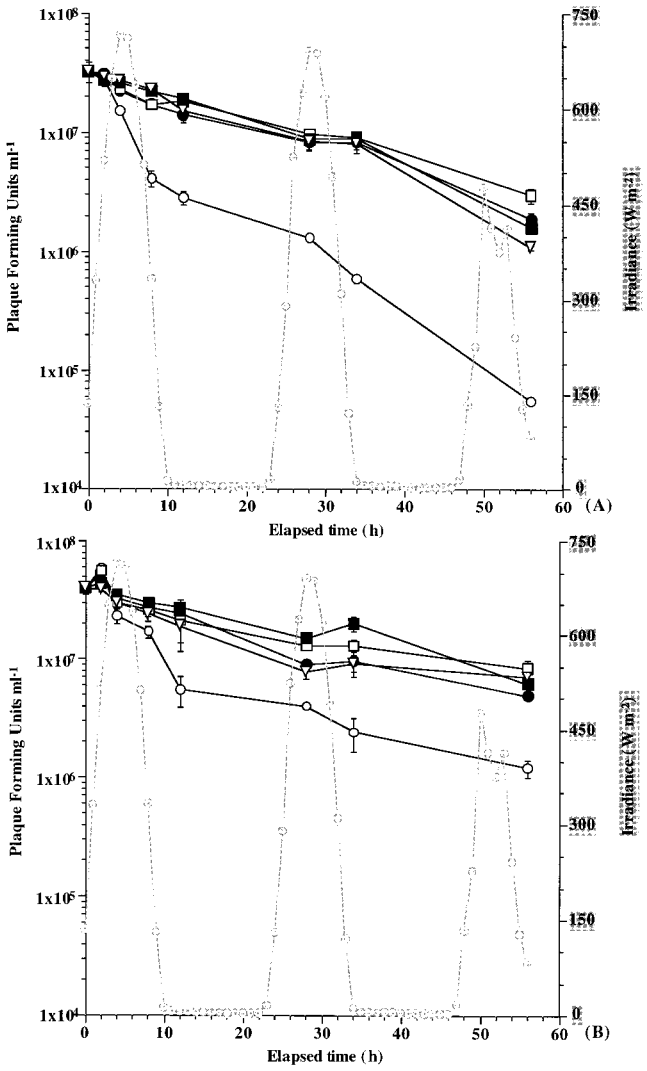

FIG. 1. Viable counts of CB 38Φ (A) and CB 7Φ (B) in microcosms in full sunlight at the surface (○) and at 1 m (□); in the dark at the surface (●) and at 1 m (■); and of a laboratory control (▼). Irradiance data (shaded) have been superimposed over viable count data. Error bars are standard errors of duplicate determinations.

Viable phage counts declined with time in all experimental treatments (Fig. 1). Analysis of variance indicated that both treatment and time elapsed had a significant effect on viable counts of CB 38Φ and CB 7Φ in estuarine microcosms ($P < 0.01$). The combined effect of treatment and time elapsed had a significant effect only on viable counts of CB 7Φ ($P < 0.05$). Retesting of the effects of treatment and time elapsed after removal of viable counts from the surface light treatment showed a significant effect of time elapsed ($P < 0.01$), indicating that compared with results for the laboratory control, only the surface light treatment had a significant effect on the viable counts of both CB 38Φ and CB 7Φ in the estuarine microcosms.

TABLE 1. Direct counts of CB 38Φ and CB 7Φ for each microcosm

Phage	Time (h)	Counts (10^6) ml^{-1} ± SD[a]			
		Control	Surface light	Surface dark	1 m dark
CB 38Φ	0	35 ± 5.1			
	2	ND[b]	ND	ND	45 ± 5.8
	12	46 ± 6.3	17 ± 5.4	14 ± 6.3	15 ± 6.3
	34	12 ± 6.3	6.2 ± 5.0	7.1 ± 6.3	17 ± 5.2
	56	7.4 ± 5.1	8.4 ± 5.1	7.4 ± 5.2	8.9 ± 8.0
CB 7Φ	0	51 ± 4.6			
	2	ND	ND	ND	84 ± 8.7
	12	42 ± 3.9	40 ± 4.9	33 ± 4.5	35 ± 3.1
	34	6.7 ± 1.0	12.8 ± 0.9	17 ± 2.8	37 ± 3.0
	56	6.0 ± 0.4	9.4 ± 0.8	9.9 ± 1.3	15 ± 1.6

[a] Standard deviations of duplicate determinations are given.
[b] ND, not determined.

Examination of the surface light treatment alone indicated that viable counts of CB 38Φ and CB 7Φ in the microcosms were significantly different from one another ($P < 0.01$), while initial concentrations of viable CB 38Φ and CB 7Φ were identical ($P > 0.01$). The differential responses of CB 38Φ and CB 7Φ to surface light were evident by comparison of their decay rates (see Table 2). The decline in viable count of CB 38Φ was greater than that of CB 7Φ ($P < 0.05$). Comparisons of experimental treatments indicated that the decline in viable count was most rapid for the surface light treatment ($P < 0.01$), whereas decay rates from dark and control treatments were identical ($P > 0.01$).

Virus particle persistence. Direct counts of virus particles in estuarine microcosms were conducted at five of the eight time points of the experiment. Summary data of direct counts of CB 38Φ and CB7Φ phage particles are given in Table 1. Direct counts of CB 38Φ and CB 7Φ declined over the course of the experiment. This decline can be entirely attributed to the singular effect of time elapsed ($P < 0.001$). Neither treatment nor the combined effect of treatment and time had a significant effect on the loss of phage particles ($P > 0.05$).

Direct counts of viruses in water have been made by most investigators by counting virus-like particles, using a TEM, sedimented via ultracentrifugation onto an electron microscopy grid. In order to test if sampling error contributes significantly to experimental error estimated from replicate microcosms, replicate electron microscopy grids were placed at the bottom of the ultracentrifuge tube. It is important to note that electron microscope grids were placed at nearly the same distances relative to the center of the centrifuge tube. This avoids possible sampling errors due to the taper effect, which causes greater deposition of viruses at the edges of the centrifuge tube (35). No significant difference was observed in direct counts of CB 38Φ or CB 7Φ obtained from replicate grids ($P > 0.05$). However, in the case of CB 38Φ, mean squares obtained in analysis of variance test results indicated that precision was gained through the counting of replicate grids for a single water sample.

Phage viability and virus particle counts. Direct counts of virus-like particles were greater than or equal to the corresponding viable count ($P < 0.001$) (Fig. 2). Direct counts were, with the exception of those obtained at time zero, significantly greater than corresponding viable counts for microcosms incubated at the surface and in the light. The difference between decrease in numbers of the viral capsids and loss of infectivity

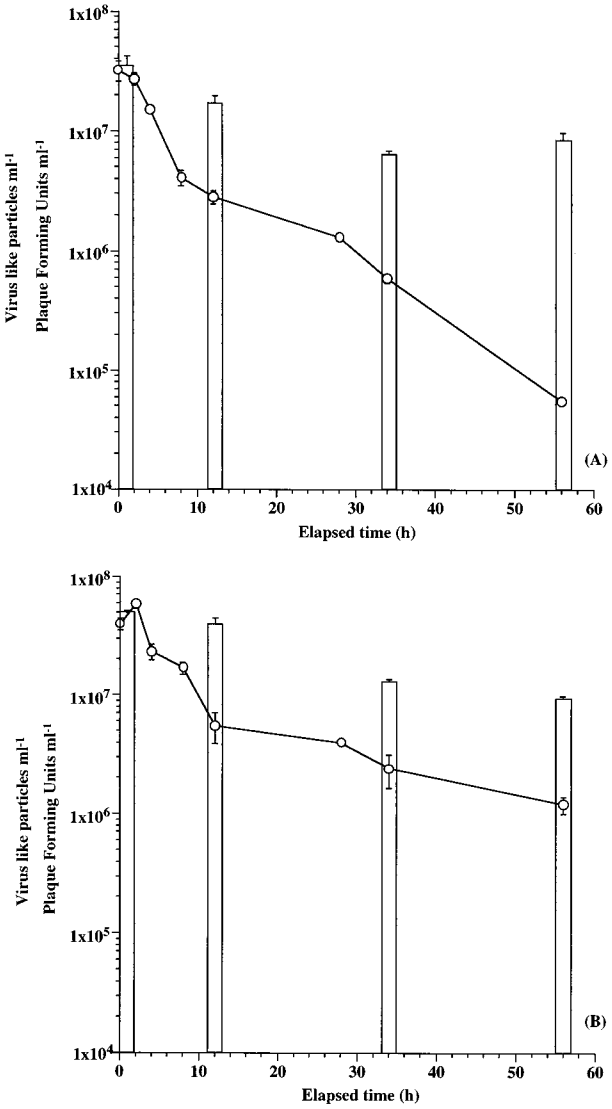

FIG. 2. Viable (○) and direct counts (bars) of CB 38Φ (A) and CB 7Φ (B) in estuarine microcosms exposed only to surface light conditions. Error bars are standard errors of duplicate determinations.

was demonstrated also by the difference between the loss rates of phage viability and virions (Table 2). For CB 38Φ, the decay rate of infectivity was significantly greater than the loss of virus particles for each treatment ($P < 0.001$). For CB 7Φ, only the surface light treatment caused a significantly greater rate of decay of phage infectivity than loss of virus particles ($P < 0.001$).

Previous studies with radiolabeled enteroviruses have examined destruction of viral particles and loss of infectivity. Destruction of viruses in experimental microcosms has been inferred from loss of radiolabel within the virus fraction (29, 44). From these studies, it was concluded that inactivation of enteroviruses coincides with the cleavage or damage of viral RNA. With this approach, neither study could implicate the destruction of virus particles in the decay process.

Rates of decline in the number of phage particles ranged from 0.016 to 0.031 h^{-1}, with an average value of 0.028 h^{-1}, and were statistically identical regardless of experimental treatment (Table 2). This range compares well with results of other

TABLE 2. Decay rates of viable and direct counts of CB 38Φ and CB 7Φ in estuarine microcosms

Phage	Counting method	Decay rate (10^{-2} h^{-1} ± SD) for each treatment[a]				
		Surface light	Surface dark	1 m light	1 m dark	Control
CB 38Φ	Viable count[b]	−11.4 ± 0.02	−5.1 ± 0.34	−4.2 ± 3.4	−5.3 ± 0.0	−6.1 ± 1.1
	Direct count[c]	−2.6 ± 0.1	−2.8 ± 0.1	ND[d]	−2.7 ± 0.3	−2.6 ± 0.9
CB 7Φ	Viable count	−6.3 ± 0.42	−3.8 ± 0.13	−2.8 ± 0.44	−3.3 ± 0.2	−4.8 ± 0.42
	Direct count	−3.1 ± 0.54	−2.9 ± 0.17	ND	−1.6 ± 0.9	−3.8 ± 0.7

[a] Decay rates were determined according to the following formula: $(\ln C_t - \ln C_0)/t$, where C_t is the cell concentration at time t; C_0 is the cell concentration at time 0; and t is the number of hours elapsed between C_t and C_0. Standard deviations of duplicate determinations are given.
[b] Determined as PFU per milliliter ± standard deviation.
[c] Determined as virus-like particles per milliliter ± standard deviation.
[d] ND, not determined.

studies done to examine reduction in phage viability in the absence of sunlight. Suttle and Chen (39) found that without sunlight the range of decay rates for viable phage counts of two marine bacteriophages tested was 0.008 to 0.028 h^{-1}. Similarly, in studies examining viral inactivation using coliphages, reported ranges of phage decay under no-sunlight conditions have been 0.01 to 0.086 h^{-1} for bacteriophage T2 (2, 12) and 0.01 to 0.03 h^{-1} for T7 (4). The close correlation of loss rates of CB 38Φ and CB 7Φ phage particles in experimental microcosms and decay rates of infectivity for coliphages and other marine bacteriophages suggests that similar processes are responsible for the loss of infectivity and destruction of virus particles in the absence of sunlight. Further evidence is achieved by comparison of average direct counts from all treatments, except the surface light treatment, and average viable counts (Fig. 3). At low levels of sunlight or no sunlight, direct and viable counts were in close agreement. This observation indicates that in the absence of strong sunlight direct counts more closely estimate the actual number of infective viruses in natural waters.

Using an in situ experimental approach, Heldal and Bratbak (15) monitored viral abundance in bottle incubations of coastal seawater which had been treated with cellular poisons to halt virus production. This approach allowed assessment of viral decay through changes in direct counts of virus-like particles. From these experiments an average viral loss rate of 0.5 h^{-1}, with a high value of 1.1 h^{-1}, was detected (15). These loss rates are 18 to 39 times the loss rates of CB 38Φ and CB 7Φ phage particles in estuarine microcosms and are generally inconsistent with those observed in other studies of marine viruses (10, 39). The large discrepancy between our observed rates of loss of phage particles and those of Heldal and Bratbak (15) could be a result of differences in experimental approach. It is possible that through the use of artificial seawater and controlled experimental conditions we have eliminated other factors responsible for the destruction of virus particles in natural waters. Despite the disparity of actual rates, both studies demonstrate that virus particles are labile in natural waters.

Suttle and coworkers (38) incubated suspensions of a natural bacteriophage isolate, LB1VL (39), in UV transparent bags. After a 12-h incubation at various depths, direct and viable counts of LB1VL in the experimental microcosms were assayed. As in the present study, viable counts declined much faster than direct counts at each treatment depth. However, from the data presented, reductions in direct counts were inversely related to in situ incubation depth (vis-à-vis sunlight levels) of the microcosm. Although no statistical analysis was performed, it appears that the level of sunlight had an effect on the loss (destruction) of LB1VL virions in experimental microcosms (38). In the present study, low levels (ca. 25 to 55% of ambient) of sunlight appeared to have no significant effect on the loss of virus particles, suggesting that natural destruction of CB 38Φ and CB 7Φ viral capsids occurs through other mechanisms.

By demonstrating a strong effect of sunlight on the survival of bacteriophage in water, we support the contention of Suttle and Chen (39) that natural solar radiation is the principal factor responsible for the decay of viral infectivity in surface waters. In an extensive study involving 10 marine bacteriophage host systems, Moebus (26) found no appreciable effects of sunlight on loss of phage infectivity. However, in his experimental system, phage suspensions were incubated in glass containers not transparent to UV-B, the most biologically damaging wavelengths of solar radiation (30, 39). In examining survival of coliphage T2 in seawater, Berry and Norton (2) found that sunlight enhanced phage decay; however, because phage suspensions were held in dialysis bags and incubated directly in situ in coastal waters, the authors could not separate the effect of sunlight from possible effects of dialyzable virucidal substances in seawater. Use of dialysis bags to assay the in situ effects of the chemical environment on virus suspensions is also problematic as the cellulose dialysis membrane can promote bacterial growth, thus altering diffusion through the membrane (18).

Despite attenuation from the plastic film, the strong effect of sunlight on viral decay, even at low levels of UV-B, is clearly shown. In a theoretical model simulating the interaction of UV and virus survival in seawater, Murray and Jackson (28) propose a range of decay rates for viruses from sensitive (4.6 h^{-1}) to tolerant (≤0.51 h^{-1}). The few marine bacteriophages studied to date have all been sunlight tolerant. In their experiments utilizing a natural marine phage host system, PWH3a-P1, Suttle and Chen (39) found decay rates of infectivity in full sunlight of 0.4 to 0.8 h^{-1}. These rates are 3.5 to 7 times greater than our fastest decay rate of 0.11 h^{-1} for CB 38Φ in the surface light treatment; however, our rates compare well with the decay rate of 0.17 h^{-1} for PWH3a-P1 found in the absence of UV-B (39) and compiled from sunlight decay data on five different marine viruses (37). Multiplying the decay rates of CB 38Φ and CB 7Φ by a factor of 4 to account for UV-B absorbance by the plastic film would give rates of 0.44 and 0.25 h^{-1}, respectively. These adjusted rates are probably a closer estimate of the actual sensitivities of CB 38Φ and CB 7Φ to solar radiation and demonstrate that the sunlight tolerance of CB 38Φ and CB 7Φ falls between that of PWH3a-P1 at ca. 0.6 h^{-1} (39) and that of the extremely tolerant North Sea bacteriophage H40/1 (9), with a full-sunlight decay rate of 0.05 to 0.11 h^{-1} (10).

As data on the sunlight-mediated decay of specific marine viruses have become available, it is increasingly evident that

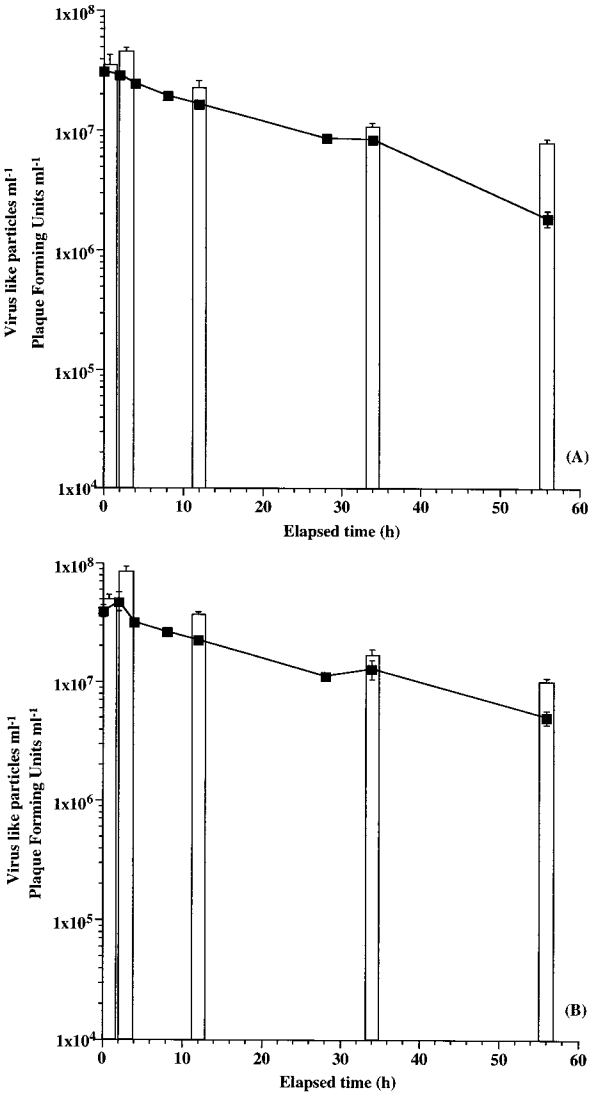

FIG. 3. Comparison of viable (■) and direct counts (bars) of CB 38Φ (A) and CB 7Φ (B) in estuarine microcosms. Direct counts are mean values from all treatments at each time point. Viable counts are mean values from all treatments except the surface light treatment. Error bars are standard errors of duplicate determinations.

within viroplankton communities there may exist a wide range of susceptibilities to the destructive effects of natural sunlight on viral infectivity (8, 10). Our study has shown that two marine bacteriophages having substantially different tolerances to the effects of sunlight on infectivity have similar rates of destruction of viral capsid structure. This suggests that natural destruction of viral capsids in the aquatic environment may be more consistent between different viral taxa than is the loss of viability. A more thorough understanding of the factors responsible for the loss of virus particles in natural waters will assist in the interpretation of viral direct counts from natural water samples.

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