EFFECTS OF SUNLIGHT AND SEASONAL TEMPERATURE ON PERSISTENCE OF FOUR BACTERIOPHAGES IN AN ESTUARINE ENVIRONMENT

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In Partial Fulfillment
Of the Requirements for the Degree of
Master of Arts

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by
Terri A. Muller
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This thesis is submitted in partial fulfillment of

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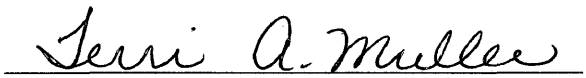

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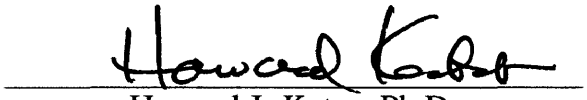

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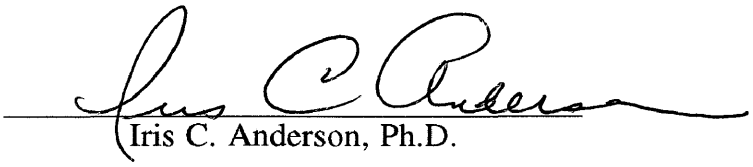

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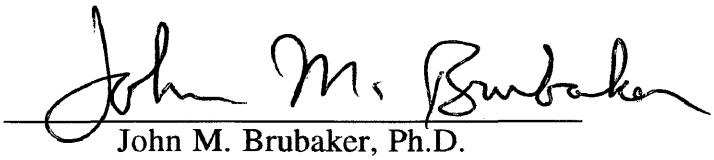

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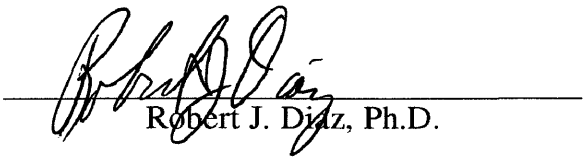

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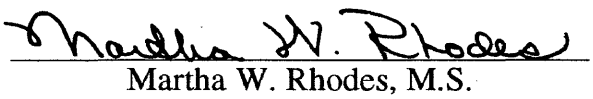

Martha W. Rhodes, M.S.

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ABSTRACT

Recent interest in bacteriophages has focused on their use as indicators of sewage pollution and their role in bacterioplankton population dynamics. Understanding how environmental factors affect bacteriophage persistence and infectivity is essential to understanding their ecology. This study examined the effects of natural sunlight and ambient temperature on four bacteriophages of contrasting origin by in situ exposure in an estuary. Bacteriophages included two autochthonous dsDNA tailed phages isolated from the upper Chesapeake Bay, a dsDNA *Salmonella* somatic phage, and an FRNA coliphage, both isolated from sewage discharge. Phages were diluted in membrane filtered (0.22 μm) artificial seawater (15 psu) and sealed in transparent plastic Cultu-Sak® bags. Bags were deployed in the estuarine water column either exposed to sunlight or covered with dark opaque plastic and sampled over time intervals ranging from hours to days for infective phages by plaque assay. Phages were exposed once in summer and once in winter.

All bacteriophage titers decreased for all treatments over the exposure periods, which varied by phage from 24 hours in summer to 7 days in winter. Tests for homogeneity of slopes (ANCOVA) revealed significant sunlight inactivation in summer of the indigenous and *Salmonella* somatic phages, and the *Salmonella* somatic phage in winter . Inactivation was seen over time intervals as short as 2 and 4 hours. The FRNA coliphage showed no inactivation due to sunlight. All phages exhibited significantly increased survival during winter (temperature range 3-8°C) compared with summer (23-25°C). Estuarine phages were not as sensitive to temperature as sewage derived phages. The *Salmonella* somatic phage showed the greatest temperature sensitivity, with persistence during winter almost ten times greater than during summer.

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INTRODUCTION

Significance of marine bacteriophages and bacteriophage indicators of sewage contamination

Bacteriophages and other viruses may be important regulators of microbial populations in marine environments (Fuhrman and Suttle, 1993). Human pathogenic viruses can enter estuarine environments via sewage treatment systems, and may pose a risk of illness if they are present in shellfish that are consumed uncooked. Presently the potential risk of bacterial and viral sewage contamination in estuarine waters and shellfish beds is assessed by determining coliform densities; however, this method may not accurately reflect the occurrence of enteric viruses. Coliphages, particularly male-specific FRNA coliphages, have been proposed as potential indicators of enteric viral persistence in estuarine environments. The fate of viruses indigenous to the estuary and allochthonous viruses associated with sewage in a controlled estuarine environment was the focus of this study.

Factors affecting phage survival in the marine environment

Microbes in the marine environment are subject to environmental factors that influence their survival. These include physical, chemical and biological parameters that can have significant direct or interactive effects. There is relatively little information available quantifying the effects of environmental parameters such as solar radiation, seasonal temperature, and salinity on viral persistence, particularly for autochthonous phages, in natural waters. Bacteriophage inactivation is believed to occur in two ways: through damage to either the capsid protein coat or to the nucleic acid. Damage to the capsid results in an inability to adsorb to a host, and damage to the nucleic acid inhibits transcription of the virus (Bitton, 1978).

F-specific RNA (FRNA) coliphages share many properties with human enteroviruses, and have been suggested as candidate indicators of sewage pollution in aquatic systems. Understanding the influence of environmental factors on FRNA coliphage persistence is necessary to assess its suitability as an indicator of enteric viral contamination. In addition, knowledge of environmental effects on autochthonous phage persistence would increase understanding of the dynamic role of viruses in microbial food webs. This study focused on two physical factors affecting virus survival in the estuarine environment- sunlight and seasonal temperature. The following is a discussion of these factors based on available literature on the survival of indigenous and non-indigenous viruses in the marine environment.

Solar radiation

Solar radiation can either directly or indirectly damage organisms (Block, 1983). For example, (Block, 1983) radiation can directly affect an organism, usually by impacting a molecule such as a nucleic acid or protein. An indirect effect results when the radiation changes the environment surrounding the organism in a detrimental way, e.g. production of oxygenated radicals. Until recently solar radiation was not thought to be an important factor influencing microorganisms in environmental waters (Kapuscinski and Mitchell, 1983). It was believed that harmful ultraviolet radiation (UV-A and UV-B) did not penetrate beyond a few centimeters in seawater (Hendricks, 1978). However, this idea has been challenged by studies showing evidence that ultraviolet and visible light wavelengths can cause mortality in both bacteria and bacteriophages (Kapuscinski and Mitchell, 1983; Karentz and Lutze, 1990; Rhodes and Kator, 1990). Regan et al. (1992) created a solar dosimeter from DNA molecules for use in the ocean. One source of DNA was ssDNA coliphage øX174. The plaque forming ability of coliphage øX174 was reduced 96

percent after exposure to sunlight at the ocean surface after day in clear tropical water, compared with a 17% reduction seen after one day at a depth of 3 meters.

The ultraviolet component of solar radiation is considered the most biologically harmful, and is comprised of wavelengths between 1 and 400 nm. Within this range, UV-B (280-320 nm) is the most harmful, compared with UV-A (320-400 nm). UV-B is strongly absorbed by DNA and proteins, causing structural changes in these molecules (Ananthaswamy and Eisenstark, 1976; Block, 1983; Sutherland, 1983; Karentz and Lutze, 1990). Gameson and Gould (1975) determined that fifty percent of sunlight lethality to bacteria in seawater is due to wavelengths shorter than 370 nm, 25 percent to 370-400 nm (near visible UV), and 25 percent to 400-500 nm (blue/green). Lethality from wavelengths longer than 500 nm was determined to be negligible. Within the UV-B range there appears to be an exponential gradient of lethality, with damage increasing with decreasing wavelength (Karentz and Lutze, 1990). The damaging effects of UV radiation have been known for some time (Bitton, 1978), and UV-B is routinely used as an agent of disinfection for water supplies and sewage effluent, as well as for shellfish depuration. UV-A is known to penetrate farther into coastal estuarine water columns than UV-B (Calkins, 1982) because dissolved organic materials, chlorophyll and particulates selectively absorb shorter wavelengths. Thus, it is reasonable to conclude that viruses in an estuarine water column would be exposed to more UV-A and visible light rather than UV-B.

Temperature

Temperature is believed to be one of the most important factors affecting viral persistence (Bitton, 1980). Block (1983) summarized the known mechanisms of viral inactivation which appear to vary according to temperature. Between 37°C and 50°C oxidation of the nucleic acid may be the primary inactivating factor, whereas at 60°C

viral protein denaturation appears to the most important factor. The primary inactivating mechanisms of temperature below 37°C are not discussed, and are assumed to be complex and not understood. The critical temperature necessary for nucleic acid denaturation depends in part on the composition of the molecule (Brock and Madigan, 1988). RNA is more resistant than DNA to heat denaturation because it does not contain the weak hydrogen bonds between base pairs of a double helix. DNA varies in resistance to temperature depending upon its base pair composition, since guanine-cytosine bonds are stronger than adenine-thymine bonds (Brock and Madigan, 1988).

Research has shown that within a viral population there is not always uniformity in heat resistance, and thermal inactivation curves usually display tailing, showing that a small fraction of the initial population is often highly resistant to thermal influence (Bitton, 1980). Different kinds of viruses show varying degrees of sensitivity to heat, however marine and enteric bacteriophages appear to persist better at colder temperatures. Moebus (1992a) found larger rates of inactivation in natural marine phages at 20°C compared to 5°C. Studies using enteric viruses consistently show increased survival at lower temperatures (Metcalf and Stiles, 1967; Akin et al., 1971). Akin et al. (1971) found enteric virus survival in seawater to be better at 4-6°C compared with 20-25°C. Metcalf and Stiles (1967) observed an inverse relationship between the rate of poliovirus 1 inactivation in autoclaved and natural seawater and temperature.

Composition of natural seawater

Because inactivation of viruses in natural seawater is affected by independent and interacting physical, chemical and biological factors (Block, 1983), quantification of the effect of a single factor is difficult. The effects of light and temperature on virus

survival have been documented in experiments using filtered and unfiltered natural seawater (Metcalf and Stiles, 1967; Akin et al. 1971; Moebus 1992a; Suttle and Chen, 1992), however, the direct effects of the physical parameters are not distinguished from the indirect effects mediated via organisms, dissolved organics, and particles. The use of artificial seawater has been proposed as a way to isolate the direct effects of specific physical or chemical parameters on viral survival (Akin et al., 1976). Artificial sea salts, purified to be "free" of organics (Aquarium Systems, Mentor, OH), when dissolved and sterilized should in theory provide a seawater to facilitate controlled studies of viral inactivation.

Importance of marine phage ecology

At one time marine viruses were thought to be relatively unimportant in relation to the marine planktonic food web (Fuhrman and Suttle, 1993). Recent literature suggests that this assumption is false and that viruses are important factors influencing both population densities and genetic composition of marine microorganisms (Fuhrman, 1992). There are several suggested means by which viruses influence marine bacterial populations (Fuhrman and Suttle, 1993):

1. Marine viruses cause microbial mortality, thereby controlling bacterial populations. However, studies reveal short viral residence times in marine waters (Suttle and Chen, 1992; Moebus, 1992b), and if viral inactivation is fast, then cycles of bacterial lysis and phage generation must also be rapid to support continuous populations of infective viruses in the environment.

2. The presence of marine viruses may help maintain diversity of microbial communities by controlling the specific composition of bacterial populations. Most viruses are species specific, therefore a high population of a specific host would

increase the encounter rate for phage infection. As the population density of a bacterial species increased, there would be a concomitant increased rate of phage infection.

3. Viruses promote cycling of organic matter in the microbial food web, providing a food source for grazing heterotrophs (Block, 1983; Fuhrman and Suttle, 1993) and through the release of dissolved and particulate organic carbon from lysed bacterial cells (Fuhrman and Suttle, 1993).

4. Viruses provide a mechanism for genetic interchange between bacteria in the environment (Miller et al., 1992).

Importance of coliphages as indicators of sewage contamination

Contamination of molluscan shellfish growing waters by sewage and animal fecal wastes not only impacts the ecological health of the affected marine waters but can reduce the yield of harvestable shellfish. Enteric diseases may be transmitted through consumption of shellfish containing enteric pathogens. Management decisions regulating the harvesting of shellfish from growing waters are based upon an assessment of actual and potential shoreline pollution sourcesas well as an assessment of sanitary water quality based on densities of coliform indicator bacteria.

Coliform indicators now used were chosen because of their presence in the intestines of warm blooded animals, sewage and domestic waste and because they allow for relatively cost efficient detection methods (NSSP, 1992). However, it has been shown that coliform bacteria are not predictive of enterovirus persistence (Fattal et al, 1983; Elliot and Colwell, 1985) and that certain coliphages and enteroviruses are more persistent in natural waters than coliform bacteria (Berry and Noton, 1976;

Borrego et al., 1983; Fattal et al., 1983). Shellfish in beds approved for harvesting have been found to contain enteric viruses (Fugate et al., 1975; Goyal et al., 1979). The studies cited found no correlation between bacterial indicator counts and viral concentrations.

Enteroviruses, e.g. poliovirus, echovirus, Coxsackievirus, hepatitis A and Norwalk virus are human pathogens transmitted by shellfish (Fattal et al., 1983). There is an effort to find viral indicators with persistence characteristics similar to these pathogens. An ideal indicator (Dutka, 1973; Goyal, 1983) should be consistently and exclusively associated with the same source as the pathogens, and should respond to environmental conditions the same as pathogens. Male-specific FRNA coliphages share similar traits with human enteroviruses, such as an RNA genome, physical size, and gross capsid morphology. Kott (1981) and Keswick et al. (1985) cite evidence that male-specific FRNA coliphages and hepatitis A and Norwalk agent have similar resistance to chlorination, a desirable characteristic for viral indicators indicative of treated sewage contamination.

Given the reasons listed above, it has been proposed (Havelaar et al., 1991) that use of FRNA coliphages could facilitate improved assessment of the sanitary quality of drinking water, shellfish, and public beaches. Use of these viruses as indicators of sewage pollution in shellfish growing waters will require evaluation of their persistence characteristics.

Hypotheses

The following hypotheses were addressed in this study.

•**H_1: Autochthonous viruses are less susceptible to inactivation by sunlight than viruses isolated from sewage.** As autochthonous viruses are

normally found in a sunlit environment, it is believed they would be less affected by sunlight than the viruses isolated from sewage which generally occur in a dark environment.

•H_2: Viruses are inactivated faster during the summer than in other seasons. Solar insolation and water temperatures are both higher in summer than winter, and previous studies indicate that bacteriophages survive longer at colder temperatures.

•H_3: Viral inactivation from irradiation decreases with depth in the water column. This is based on knowledge that light wavelengths attenuate in productive waters like estuaries.

Experimental Design

The studies were conducted between August 1994 and March 1995. An in situ exposure technique using light "transparent" virus-impermeable bags was used to expose phage suspensions to ambient light and temperature conditions. Each phage tested was exposed once each under summer and winter ambient conditions to compare seasonal effects. During each experiment phages were exposed at depths of 0.05 m, 0.5 m, and 1 m in an estuarine water column to provide a gradient of light intensity. Of the four phages selected, two were indigenous to the Chesapeake Bay estuary and two were non-indigenous phages typically associated with sewage and septage, an FRNA coliphage and a *Salmonella typhimurium* somatic phage. At selected intervals phage suspensions were sampled and enumerated by plaque assay using appropriate hosts to determine inactivation rates.

MATERIALS AND METHODS

Microorganisms

Bacteriophages used in this study included two isolated on bacterial hosts indigenous to the Chesapeake Bay and two associated with domestic sewage and septage. Phages CB7 and CB38 were isolated by K.E. Wommack (Center of Marine Biotechnology, University of Maryland) from viral concentrates of Chesapeake Bay water, following methods outlined by Wommack et al. (1995). Propagation and maintenance were done by agar overlay employing Luria broth estuarine salts (LBES) agar (Wommack et al., 1995). Wommack et al. (1995) were unable to identify the hosts using the Biolog System (Biolog, Hayward, CA). Based on morphological characteristics, phages CB7 and CB38 are believed to be dsDNA tailed phages (Wommack, personal communication).

Bacteriophages associated with wastewater were isolated using selected *Salmonella typhimurium* (Havelaar and Hogeboom, 1984) hosts, and were purified, propagated and maintained using methods described by Kator and Rhodes (1993). A tailed somatic phage was isolated from post-chlorinated effluent and propagated on the host *S. typhimurium* WG45 (Havelaar and Hogeboom, 1984). The somatic phage has the morphological characteristics of a dsDNA phage (as determined by electron microscopy). The FRNA coliphage used was isolated from septage by M. Rhodes (personal communication) and characterized as a Type II serogroup FRNA phage (Furuse, 1987). A phage suspension maintained at -89°C in the laboratory was propagated on the host *Salmonella typhimurium* WG49, genetically altered to produce the *Escherichia coli* sex pilus (F+) (Havelaar and Hogeboom, 1984).

Phage enumeration methods

Enumeration methods were consistent for all phages studied. Water samples or dilutions thereof were enumerated for phage using single agar overlay (SAL) and double agar overlay (DAL) techniques (Adams, 1959). DAL assays involved mixing 1 ml of sample with 0.5 ml host and 5 ml molten agar medium held at 47°C in a water bath. The resulting suspension was gently mixed and poured over a base agar layer in a petri dish (100 mm x 15 mm) and allowed to cool to room temperature. SAL assays involved mixing 10 ml sample, 0.75 ml host and 10 ml of appropriate agar medium containing double concentrations of all ingredients. This mixture was distributed into 3 petri dishes and cooled to room temperature. Inverted plates containing *Salmonella* hosts and indigenous hosts were incubated overnight at 35°C and 25°C, respectively. Plaques were counted at appropriate dilutions and were a direct measure of the number of bacteriophages present and capable of infecting the host. Counts were expressed as plaque forming units (PFU) per unit sample volume.

Growth media

Phage hosts *S. typhimurium* WG45 and WG49 were grown on tryptone yeast extract glucose (TYG; Havelaar and Hogeboom, 1984) agar and broth. TYG broth [10 g tryptone, 1 g yeast extract, 8 g NaCl, distilled water to 1000 ml]. Media were formulated to yield a final concentration of 5 g/l agar for the overlay (DAL or SAL) and 15 g/l agar for the base layer. After autoclaving, the agar medium was cooled to 47°C in a water bath, and the following membrane sterilized ingredients added at the time of assay. Stock solutions of glucose and $CaCl_2 \cdot 2H_2O$ were added to final concentrations of 1 g/l and 0.3 g/l, respectively. Nalidixic acid was added to a final concentration of 0.1 g/l, and 2,3,4-triphenyltetrazolium chloride dye was added for plaque visualization (0.085g/l final concentration). Kanamycin was added to the medium for *S. typhimurium* WG49 to a final concentration of 0.02 g/l.

FRNA and *Salmonella* somatic phages were diluted when necessary in phage buffer (Tartera and Jofre, 1987) [7g Na_2HPO_4, 3g KH_2PO_4, 5g NaCl, 10 ml of 0.1M $MgSO_4$, 10 ml of 0.01M $CaCl_2$, distilled water to 1000 ml, pH= 6.8-7.0].

Indigenous bacterial hosts CB7 and CB38 were assayed using LBES broth and agar described by Wommack et al. (1995). LBES [10 g NaCl, 10 g tryptone, 5 g yeast extract, 10 ml of 0.38M KCl, 10 ml of 0.38M $CaCl_2$, 10 ml of 1.8M $MgSO_4 \cdot 7H_2O$, distilled water to 1000 ml]. Agar concentrations for assays were the same as those used for *Salmonella* hosts.

Dilutions of indigenous phage were made in four salts buffer (Wommack et al., 1995), composed of 10 g NaCl, 10 ml of 0.38M KCl, 10 ml of 0.38M $CaCl_2$, 10 ml of 1.8M $MgSO_4 \cdot 7H_2O$, distilled water to 1000 ml]. All media were sterilized by autoclaving at 121° C, 15 psi.

Seawater medium

Exposure experiments were done using an artificial seawater (Instant Ocean®, Aquarium Systems, Mentor, OH) containing the major ions of natural seawater in the following concentrations (per liter): Cl^- 18.7 g; Na^+ 10.5 g; $SO4^=$ 2.6 g; Mg^{++} 1.3 g; Ca^{++} 0.4 g; K^+ 0.4 g; HCO_3^- 0.2 g; B 0.006 g; Sr^{++} 0.008 g at 34 psu. For the experiments the artificial seawater was diluted to 15 psu in order to match the local estuarine salinity.

In situ exposure experiments

Each phage was tested for sunlight-induced inactivation and response to temperature during two seasons, once in late summer (1994) and once in winter (1995). Experiments were initiated between 9 and 10 AM to maximize the duration of

sunlight exposure. A titered suspension of given purified phage was inoculated into light transparent plastic bags (Cultu-Sak®, Becton-Dickinson) containing 25 ml sterile artificial seawater (15 psu) for an approximate final concentration of 10^4 PFU per ml. The bags were impermeable to viruses and transmitted radiation as shown in Figure 1. Before heat sealing, zero time samples from inoculated bags were collected to determine initial phage concentrations. Bags were exposed at average depths of 0.05 m, 0.5 m and 1 m by deploying a floating array (Figure 2) designed to orient bags parallel to the water surface. At each treatment depth half of the bags prepared were covered with opaque plastic for use as dark controls. Bags were exposed for time intervals corresponding to 1, 2, 4, 6 and 24 hours in summer. Exposures were modified for each phage in winter when viral inactivation was slower, with total exposure times lasting from 48 hours to 7 days. At each sampling interval three bags corresponding to each treatment at each depth were removed under cover, and from each bag two replicate determinations of phage titer were made. Samples from individual bags were enumerated for phage as described and discarded.

Supplemental experiments

Because some experiments showed measurable rates of phage inactivation in the dark controls, experiments were conducted to compare survival of phages in artificial seawater and buffer in the dark, and to assess the possibility that the Cultu-Sak® bags inactivated phages. Accordingly, survival of phage CB7, the *Salmonella* somatic, and the FRNA phage was compared in phage buffer (Tartera and Jofre, 1987) and in sterile artificial seawater. Incubations were made in glass milk dilution bottles as controls against the Cultu-Sak® bags. Titered suspensions of each phage were dispensed into four bottles, each containing 100 ml buffer or artificial seawater, and were incubated in the dark. One bottle for each media treatment was incubated at 25°

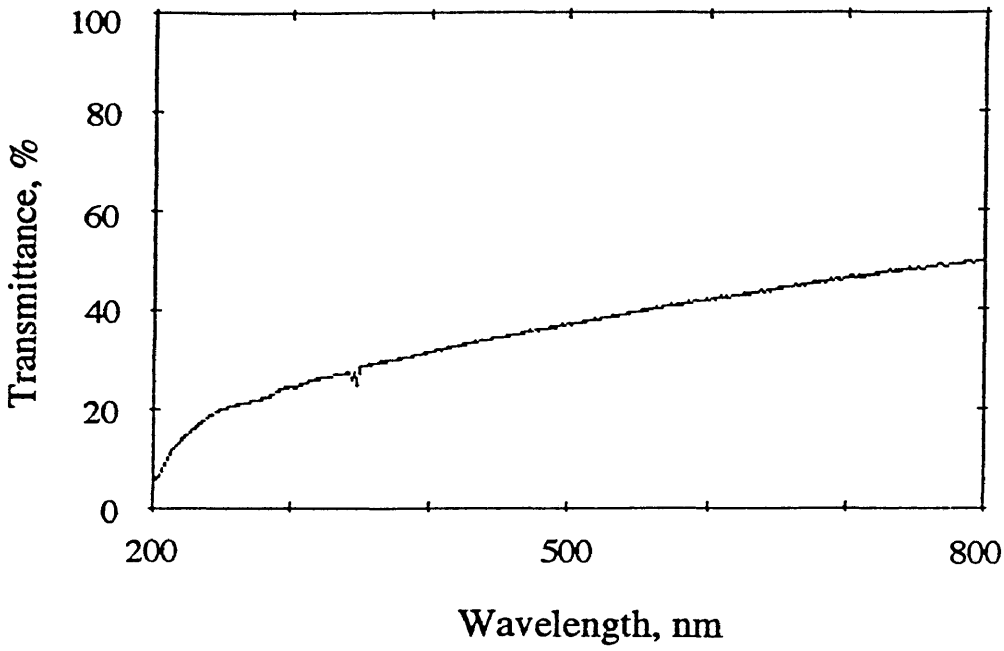

Figure 1. Transmittance of light through Cultu-Sak® bags. Percent transmittance is based on comparison of light transmittance through air.

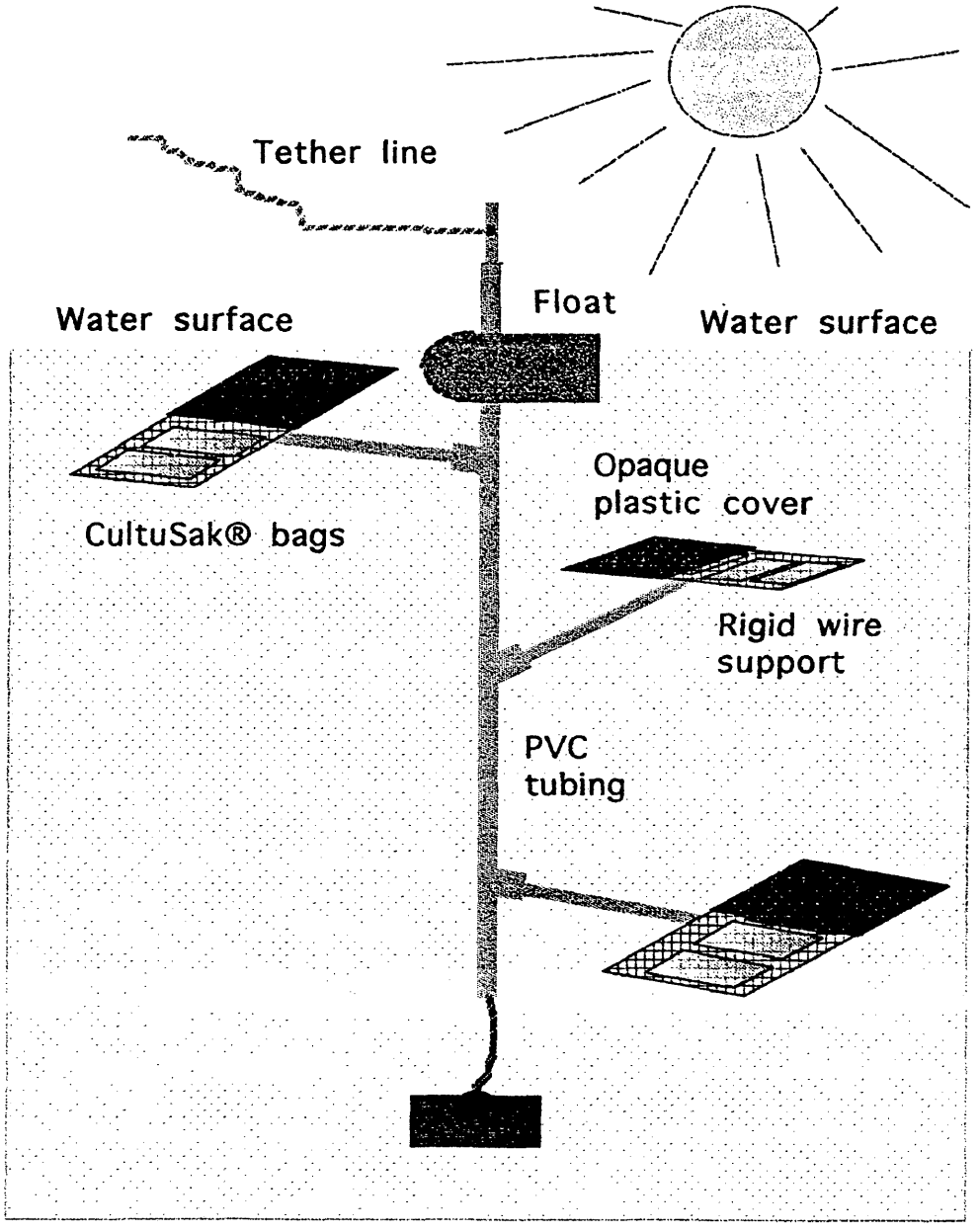

Figure 2. In situ exposure apparatus.

C, and one at 11° C. Two replicate samples were taken for analysis at 0, 2, 6, 12 and 26 hours from each of the 4 bottles for each phage (12 total) and assayed by DAL.

FRNA coliphage was used to test the effect of the Cultu-Sak® bag on phage survival. Titered suspensions of phage were dispensed into a glass milk dilution bottle and a Cultu-Sak® bag, each containing 100 ml artificial seawater (15 psu), and incubated in the dark in the laboratory at 25° C. Two replicate samples were taken at 0, 2, 5, 10 and 24 hours, and assayed as in the above experiment.

Physical measurements

During each field experiment light extinction was determined using a photosynthetically active radiation (PAR) meter (Li-Cor quantum sensor, model LI-185B) sensitive to wavelengths between 400-700 nm. Attenuation coefficients of PAR were calculated using the formula

$$-k = [\ln (I_d/\ln_{o)} /d]$$

where I_o and I_d are the intensities of light measured at depths 0 and d, respectively, and -k is the extinction coefficient. Salinity measurements were made during each experiment using a refractometer, and temperature was measured using a standard thermometer. Incident solar radiation (300-3000 nm) was measured with an Eppley pyranometer (Model PSP, Eppley Laboratory, Inc., Newport, RI) located on the roof of Byrd Hall at VIMS.

Data analysis

Plaque counts were natural log transformed for statistical analysis. Data were plotted to show changes in infectivity over time, and linear regressions (Nester et al., 1990) of ln PFU counts against time were used to calculate the slope, -k, interpreted as an overall inactivation rate for each treatment according to the formula

$$-k = [\ln C_t - \ln C_o)]/t$$

where C is the PFU concentration at zero time (C_o) and after t hours (C_t), respectively. r^2 values were calculated to measure the tightness of fit of the data to the regression line, and indicate the proportion of the total sum of squares explained by the regression. Analysis of covariance (ANCOVA) using light treatment as a factor and time as a covariate was used to determine homogeneity of slopes. ANCOVAs were performed according to methods described by Nester et al. (1990) to identify significant ($p<0.05$) differences between treatments at the different depths. Data sets for ANCOVAs involving summer experiments did not include PFU counts taken at the 0 hour time point. Comparisons of inactivation rate coefficients for each phage between seasons were made using Student's *t* tests (Zar, 1984).

RESULTS

Summer exposure experiments

Physical data

Environmental parameters for summer experiments are summarized in Table 1. Daily incident solar radiation varied between 580 Langleys (during CB7 exposure) and 664 Langleys (during CB38 exposure). Water temperatures ranged between 23° and 25° C for all experiments. PAR extinction coefficients ranged from 1.21 m^{-1} during exposure of the FRNA phage to 1.52 m^{-1} during exposure of phage CB38.

Inactivation of phages

Examination of inactivation curves using ln transformed data (Figures 3a-3h) suggested that inactivation rate coefficients calculated by regression were, to varying degrees, under-estimates because the largest inactivation rates sometimes occurred during the first 4 to 6 hours of exposure. For comparability, the data from all time points were used in analyses, although r^2 values (Table 2) would have been larger if the 24 hour data point had been eliminated.

Analysis of inactivation rates for phages exposed to light compared with the dark controls at 0.05 m revealed that three phages showed sensitivity to sunlight (Table 2). The *Salmonella* somatic phage exposed to sunlight at 0.05 m showed an inactivation rate coefficient almost twice that of the dark control, with ANCOVA giving a strong level of significance ($p<0.001$). The two indigenous phages, CB7 and CB38, showed inactivation rate coefficients when exposed to light at 0.05 m that were 2.8 and 0.5 times greater than the dark controls, respectively. Only the FRNA phage did not exhibit sensitivity to sunlight at a depth of 0.05 m in summer.

Table 1. Physical measurements taken during seasonal exposure experiments.

Season		Phage origin			
		Estuary		Sewage	Septage
		CB 7	CB 38	FRNA	SOMATIC
Summer					
	Incident solar radiation, Langleys/day	580	664	592	661
	PAR extinction coefficient, m^{-1}	1.48	1.52	1.21	1.38
	Water temperature, °C	25	25	23	26
Winter					
	Incident solar radiation, Langleys/day	329	341	99	320
	PAR extinction coefficient, m^{-1}	1.08	0.93	0.89	1.06
	Water temperature, °C	3	5.5	8	6

Total incident solar radiation and water temperatures were estimated from measurements taken every 6 minutes. PAR attenuation coefficients were calculated using the formula $I_d = I_o e^{-kd}$.

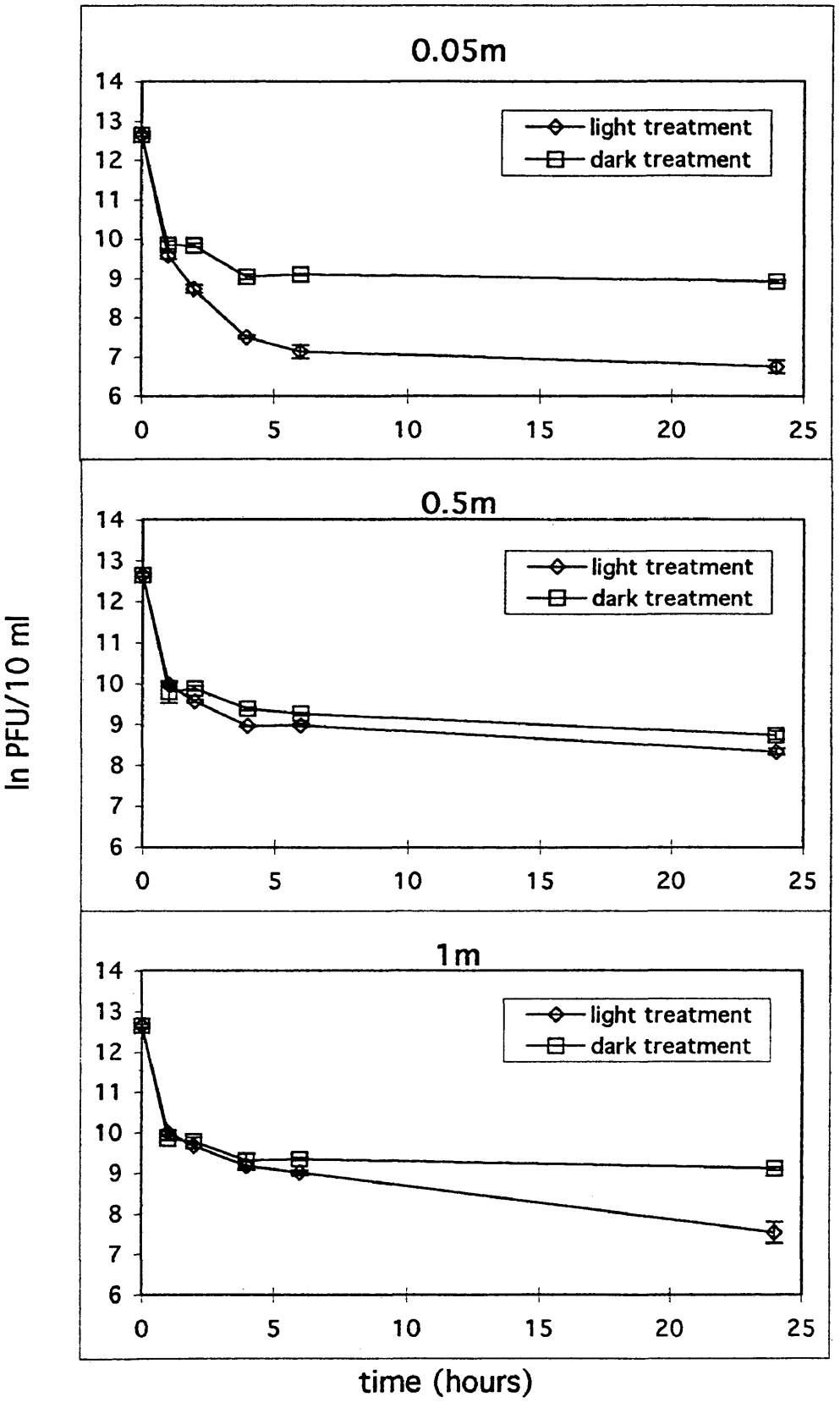

Figure 3a. Loss of phage CB7 infectivity in summer at 25° C.

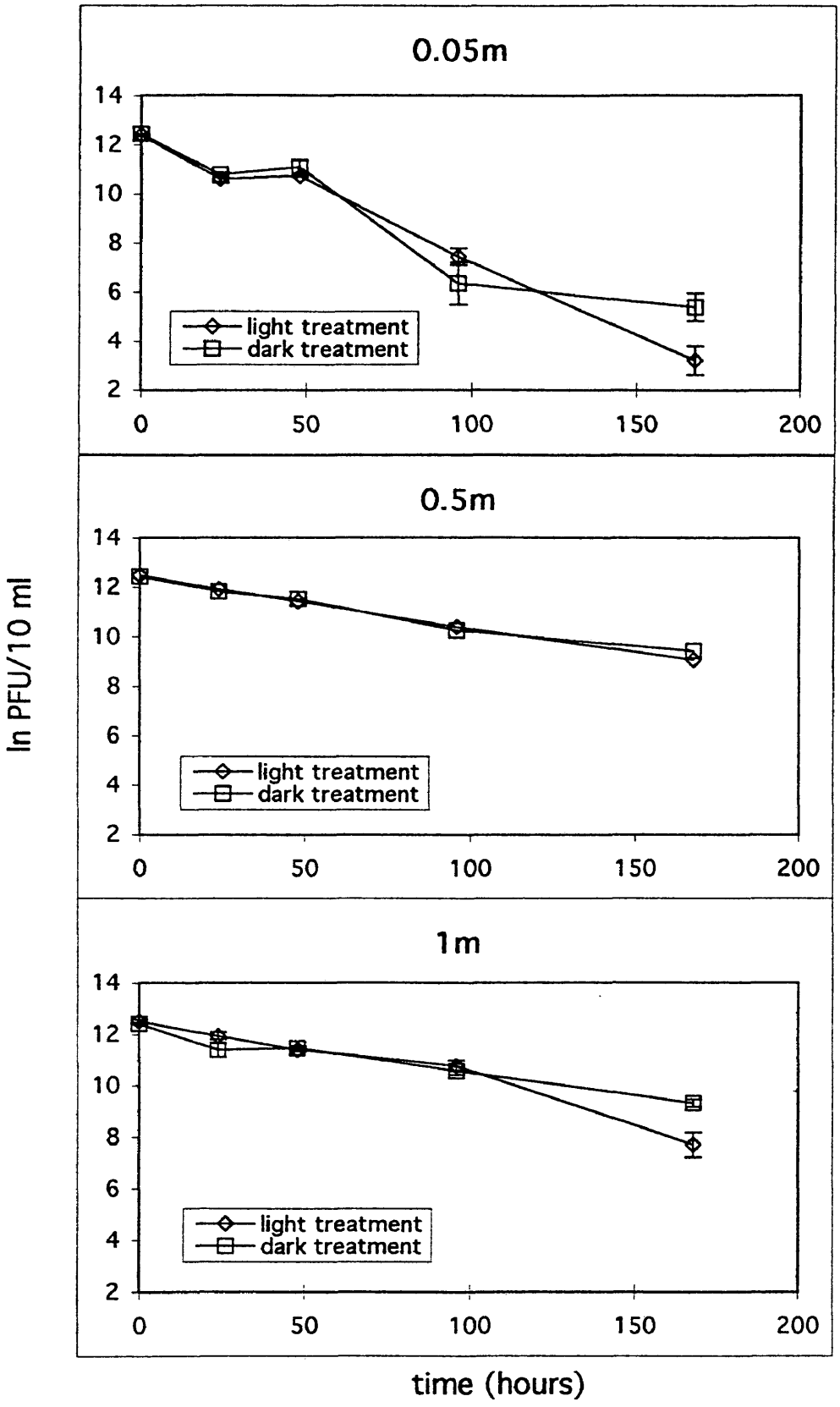

Figure 3b. Loss of phage CB7 infectivity in winter at 3° C.

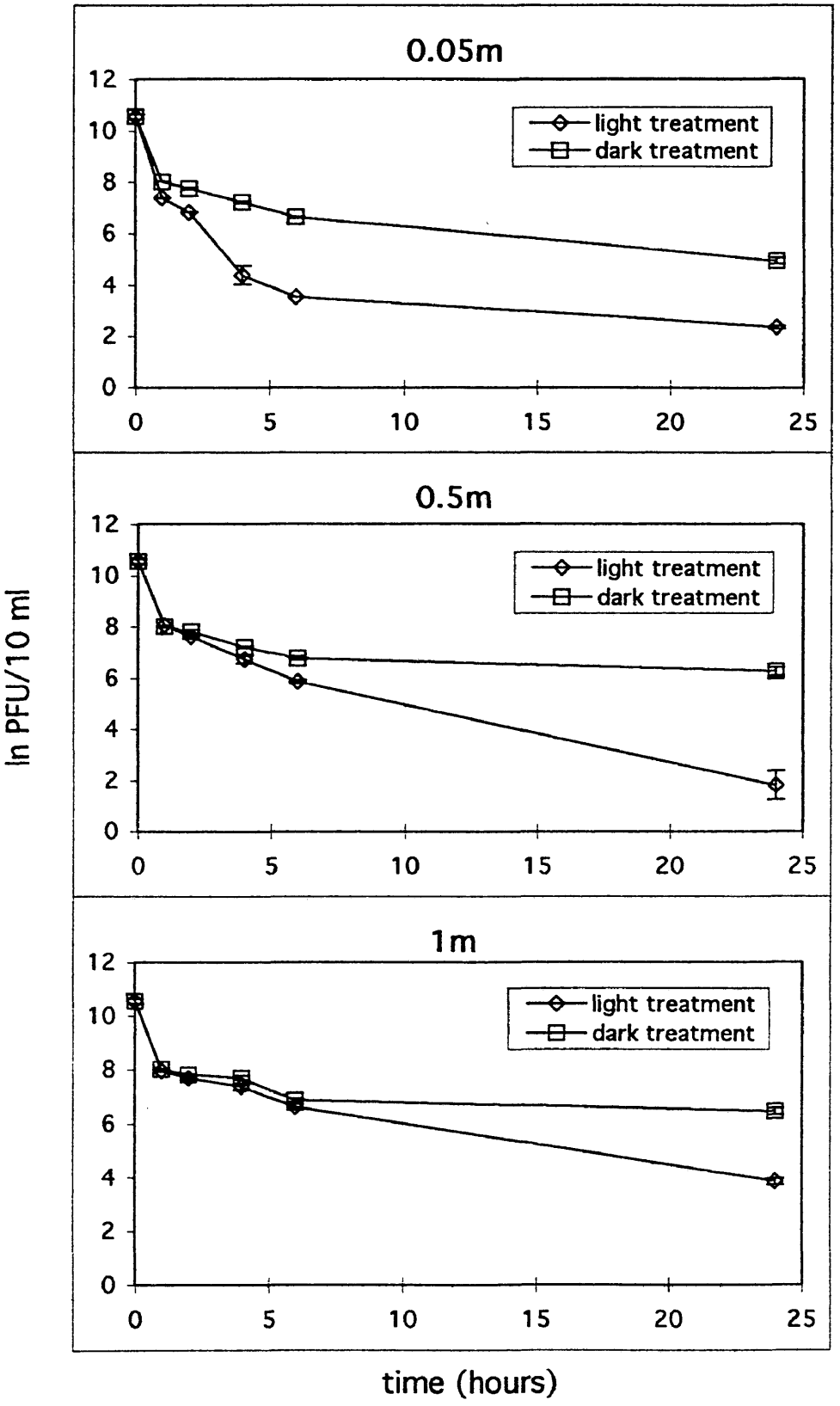

Figure 3c. Loss of phage CB38 infectivity in summer at 25° C.

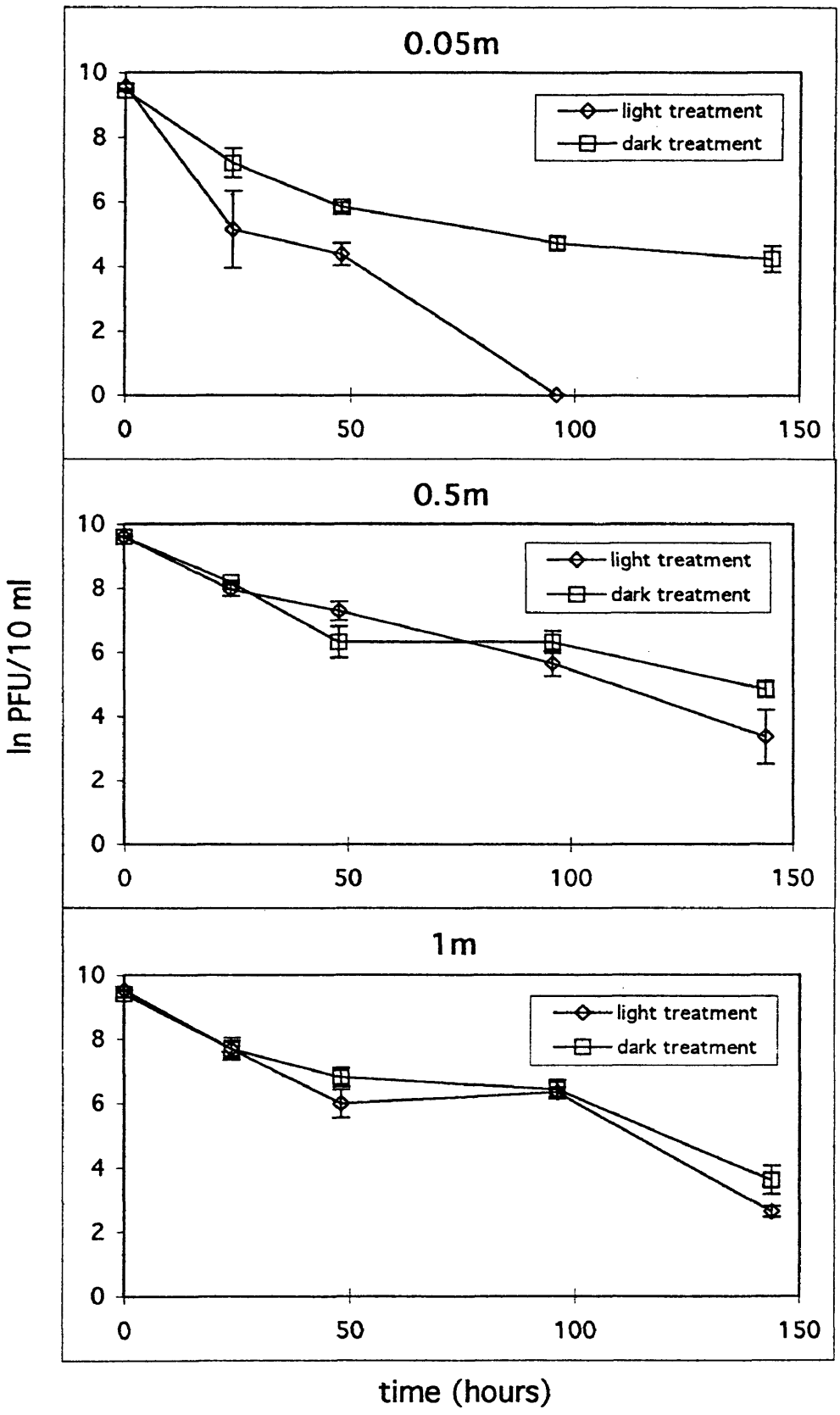

Figure 3d. Loss of phage CB38 infectivity in winter at 5.5° C.

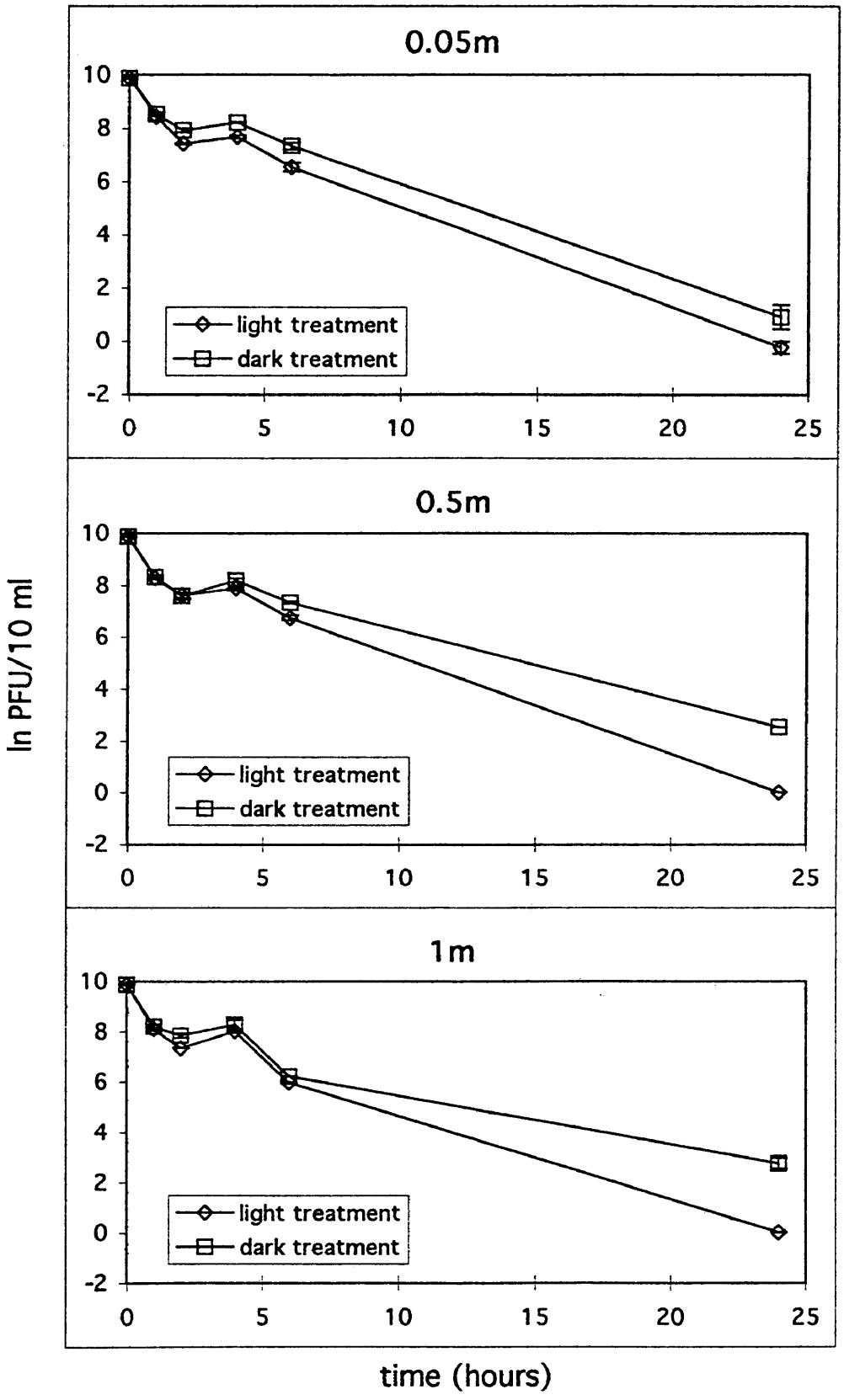

Figure 3e. Loss of FRNA phage infectivity in summer at 23° C.

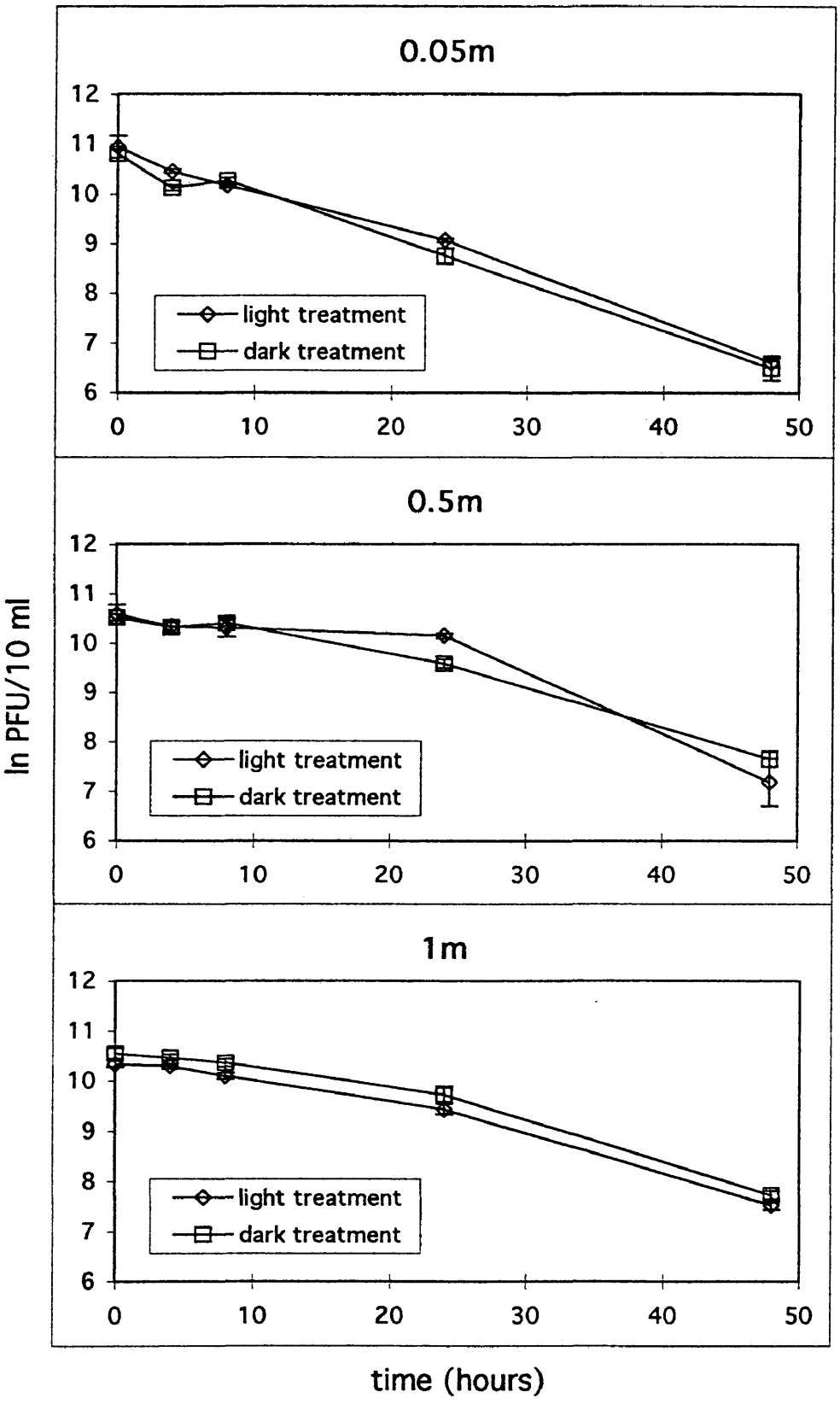

Figure 3f. Loss of FRNA phage infectivity in winter at 8° C.

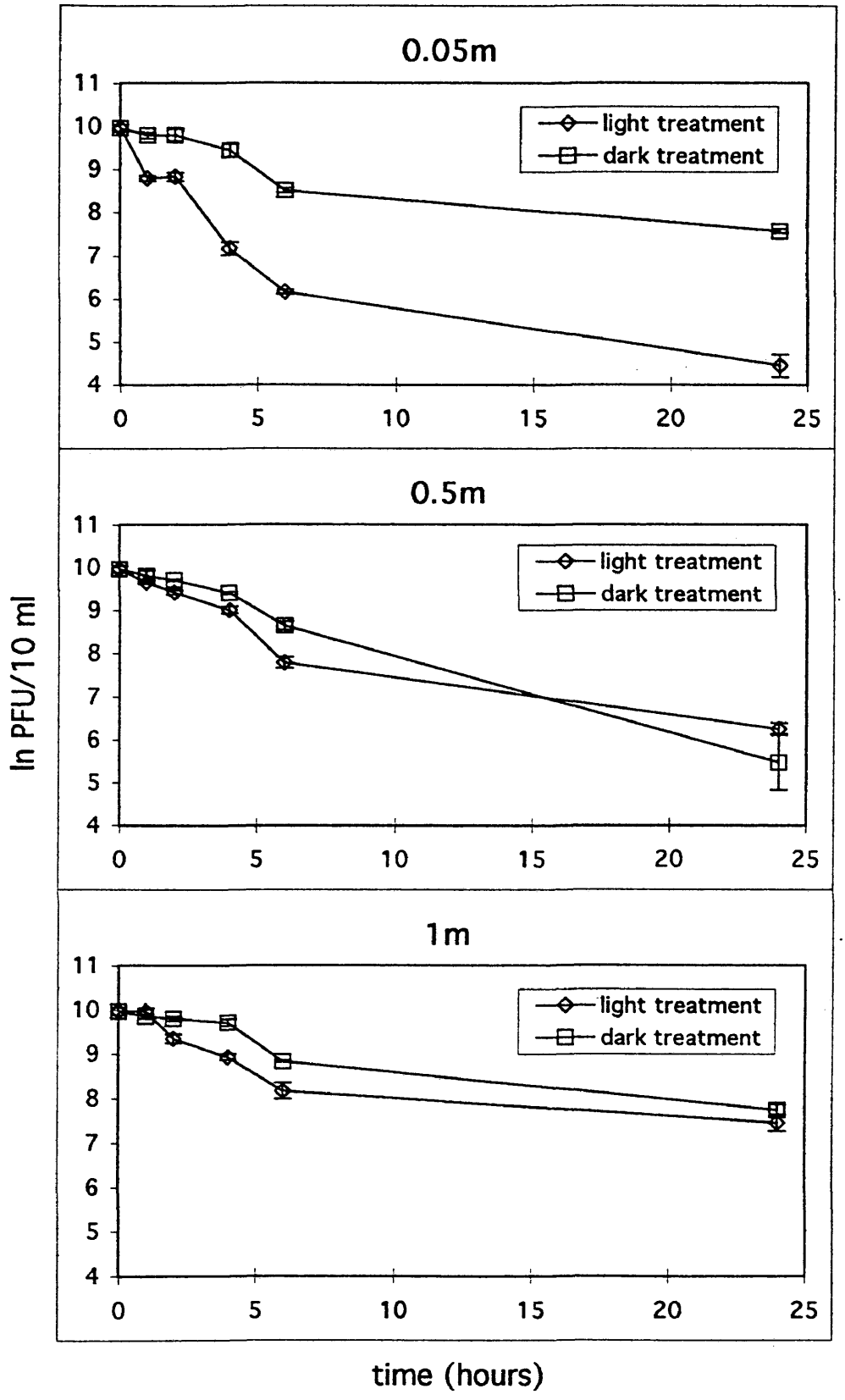

Figure 3g. Loss of *Salmonella* somatic phage infectivity in summer at 26° C.

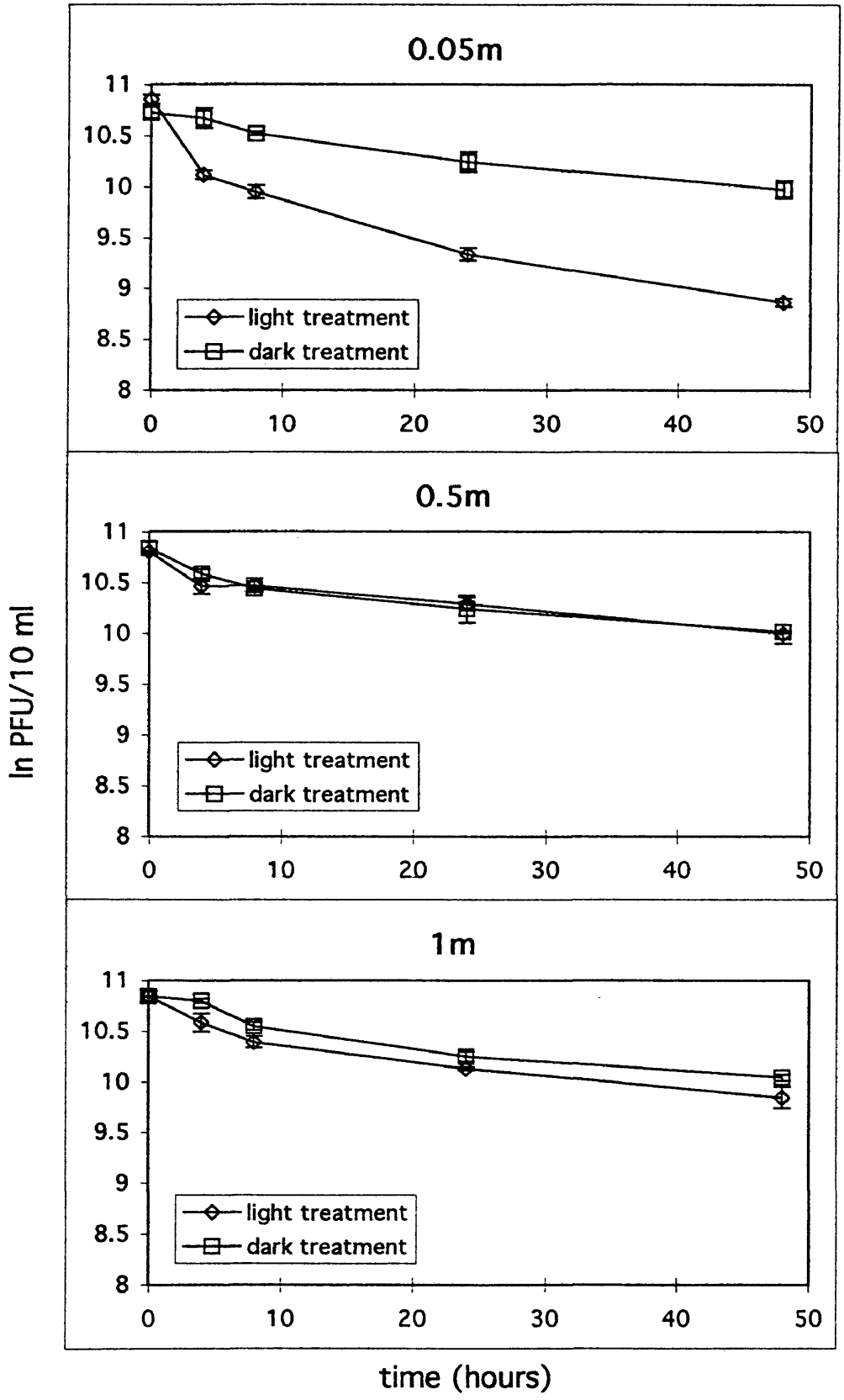

Figure 3h. Loss of *Salmonella* somatic phage infectivity in winter at 6° C.

Table 2. Inactivation rate coefficients and r^2 values for the phage exposure experiments in summer.

Depth	Treatment	Phage origin							
		Estuary				Sewage		Septage	
		CB 7		CB 38		FRNA		SOMATIC	
		Inactivation rate coefficient	r^2	Inactivation rate coefficient	r^2	Inactivation rate coefficient	r^2	Inactivation rate coefficient	r^2
0.05 m	Light	0.089 h^{-1}	0.493	0.195 h^{-1}	0.65	0.370 h^{-1}	0.986	0.176 h^{-1}	0.792
	Dark	0.032 h^{-1}	0.416	0.127 h^{-1}	0.921	0.335 h^{-1}	0.974	0.094 h^{-1}	0.835
	p value	0.003		0.007		0.122		0.0002	
0.5 m and 1.0 m	Light	0.077 h^{-1}	0.786	0.218 h^{-1}	0.898	0.357 h^{-1}	0.971	0.115 h^{-1}	0.768
	Dark	0.035 h^{-1}	0.588	0.064 h^{-1}	0.707	0.243 h^{-1}	0.947	0.141 h^{-1}	0.786
	p value	0.874		0.252		0.797		0.021	

No significant differences were found between 0.5m and 1m depth treatments so data were combined. P values were calculated using one factor analysis of covariance, and compared light and dark treatment inactivation rate coefficients at the specified depth.

Because there was no significant difference within each light treatment between inactivation coefficients at 0.5 m and 1 m for any experiments, the data from these two depths were combined for statistical analysis. Inactivation rates for all four phages were not affected significantly by sunlight at these depths (Table 2). However, CB7 and CB38 inactivation rates appeared larger in the light exposed bags and resulting phage densities (Figures 3a and c) were 1-2 ln units lower. Low r^2 values (Table 2) for the somatic phage, CB7 and CB38 indicated that the data did not fit a linear natural log transformed model. Light extinction data indicated that over 45% of the photosynthetically active radiation at the surface had attenuated at 0.5 m (Table 3).

Inactivation rate coefficients for the *Salmonella* somatic phage at 0.5 m and 1 m were significantly different as a function of light ($p=0.02$), with a greater inactivation rate in the dark. This statistical difference was considered invalid because of an interaction effect between treatment factors, as discussed later in this paper.

Winter exposure experiments

Physical data

Duration of winter exposure experiments varied from 48 hours for the *Salmonella* somatic and FRNA phages, to 6 and 7 days for phages CB38 and CB7. At lower seasonal temperatures the phage inactivation rates decreased, requiring longer exposure periods to observe inactivation. Exposure times of 7 and 6 days for phages CB7 and CB38 compared with 48 hours for the FRNA and *Salmonella* somatic phages produced higher total solar exposures, although total daily solar incident radiation (Table 1) was similar for all winter experiments except the FRNA phage experiment, which exhibited low values because of extended cloud cover. PAR

Table 3. Percent of PAR light remaining at 0.5 m below the estuarine surface of the estuary.

Season	Phage exposure experiment			
	CB7	CB38	FRNA	Somatic
Summer	48%	47%	55%	50%
Winter	61%	63%	64%	59%

Values are based on PAR light attenuation measurements determined as discussed in the methods section of this paper.

extinction coefficients (Table 1) varied from 1.08 m^{-1} during CB7 exposure to 0.89 m^{-1} during FRNA phage exposure.

The range of water temperatures (Table 1) for the winter experiments was larger than in summer, because the experiments were conducted over a 6 week period from mid-January through February. Minimum and maximum exposure temperatures were 3°and 8°C for experiments involving CB7 and FRNA phages, respectively.

Inactivation of phages

Data presented in Table 4 show that during the winter exposure experiments only the *Salmonella* somatic phage showed inactivation from sunlight, with a light inactivation rate coefficient at 0.05m over twice that in the dark control. Although phage CB38 exposed to light at 0.05 m showed a reduction of PFU eleven times larger than the dark control (Figure 3d), a statistically significant light effect was not seen (ANCOVA, $p=0.67$). Neither phage CB7 nor the FRNA phage showed inactivation from sunlight.

Effect of depth on phage inactivation

Analysis of covariance comparing phage inactivation rates indicated absence of sunlight effects at 0.5 and 1 m ($p>0.05$), even when a significant effect was apparent at 0.05 m. This observation held for both the summer and winter seasons, which differed in light attenuation, temperature, and solar insolation. Analysis of covariance did not show a significant difference in phage inactivation between 0.5 and 1 m in any experiment.

Table 4. Inactivation rate coefficients and r^2 values for the phage exposure experiments in winter.

Depth	Treatment	Phage origin							
		Estuary				Sewage		Septage	
		CB 7		CB 38		FRNA		SOMATIC	
		Inactivation rate coefficient	r^2	Inactivation rate coefficient	r^2	Inactivation rate coefficient	r^2	Inactivation rate coefficient	r^2
0.05 m	Light	0.054 h^{-1}	0.961	0.097 h^{-1}	0.819	0.088 h^{-1}	0.982	0.036 h^{-1}	0.849
	Dark	0.044 h^{-1}	0.843	0.034 h^{-1}	0.815	0.088 h^{-1}	0.972	0.016 h^{-1}	0.842
	p value	0.556		0.669		0.219		0.027	
0.5 m and	Light	0.023 h^{-1}	0.92	0.041 h^{-1}	0.875	0.063 h^{-1}	0.852	0.016 h^{-1}	0.791
1.0 m	Dark	0.018 h^{-1}	0.897	0.033 h^{-1}	0.841	0.059 h^{-1}	0.94	0.016 h^{-1}	0.824
	p value	0.056		0.522		0.168		0.763	

No significant differences were found between 0.5m and 1m depth treatments so data were combined. P values were calculated using one factor analysis of covariance, and compared light and dark treatment inactivation rate coefficients at the specified depth.

Even under winter conditions of minimal turbidity, the phages at 0.5m were always exposed to 64% or less of surface incident PAR (Table 3). That amount was further attenuated through the Cultu-Sak® bags. Relative to air, Cultu-Sak® bags transmit approximately 35% of the radiation at 400 nm, with transmission directly related to wavelength (Figure 1).

Effect of seasonal temperature on phage inactivation

There was an approximate 20°C difference between the average summer and winter exposure temperatures for each phage tested (Table 1). Seasonal differences in persistence were observed for all phages, with decreased persistence in summer (Figure 4). The inactivation rate coefficients for dark controls at 0.5 and 1 m in summer and winter for each phage were significantly affected by seasonal temperature (Student's *t* test, $p = 0.05$)

Estuarine phages were least affected by temperature in the dark, with winter inactivation rates only about half those observed in summer (Figure 4).

In contrast, the FRNA coliphage showed a four-fold decrease in inactivation rate at 8°C compared with 23° C. *Salmonella* somatic phage showed the greatest temperature sensitivity with a summer inactivation rate almost ten times larger than in winter.

Effect of seawater and Cultu-Sak® bags on phage inactivation

As shown in Tables 2 and 4, all phages exhibited significant inactivation over time regardless of treatment. In all ANCOVAs, time (as the covariate) had a p value significance of less than 0.0001. In order to validate the experimental design, follow-

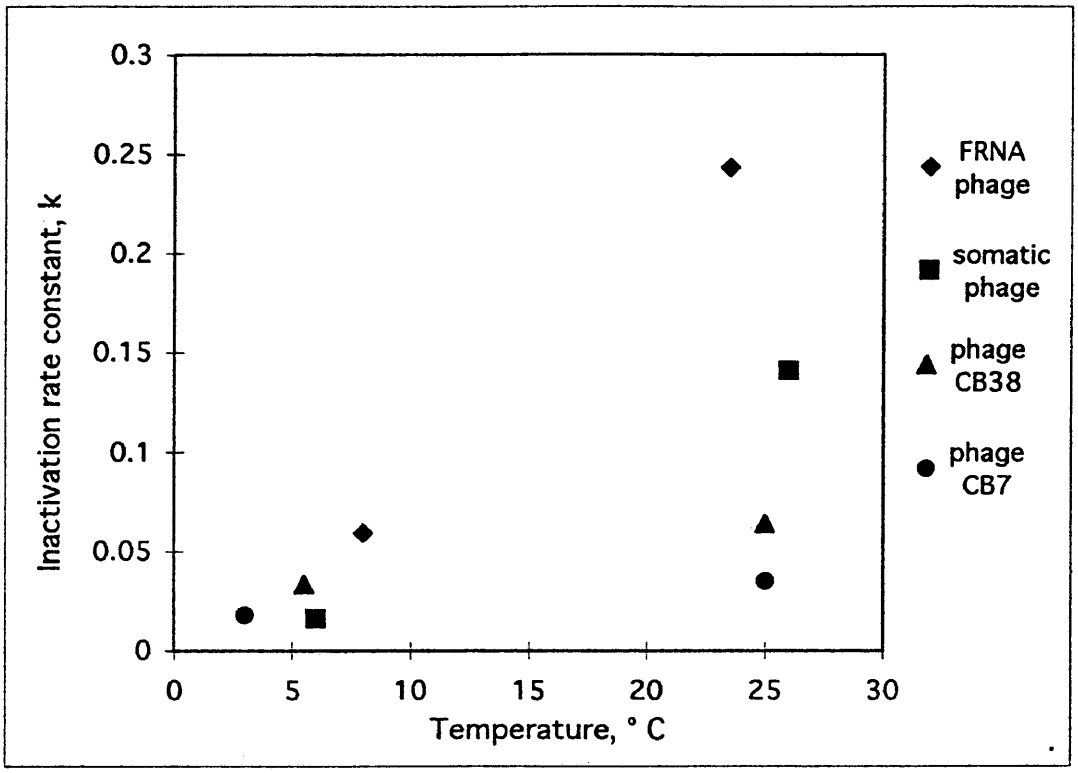

Figure 4. Plot of inactivation rate coefficients for dark controls below the water surface versus seasonal water temperature. Tests for differences between the inactivation coefficient rates for each phage made using Student's *t* tests revealed that phage inactivation rates were significantly different between summer and winter (p=0.005).

up laboratory experiments were performed to investigate possible sources of artifacts and experimental bias.

The first possible factor explored was the effect of artificial seawater. A 26 hour laboratory experiment was conducted to determine if phage survival differed in artificial seawater compared with buffer. With one exception there was no measurable loss of PFU (Figure 5), leading to the conclusion that artificial seawater was not the cause of inactivation over this time period.

The second factor tested for effects on phage inactivation potential was the Cultu-Sak® bag itself. The bags were tested against glass bottles which are known from previous experiments conducted at 5 and 22°C not to effect FRNA coliphage survival in buffer during four days esposure (H. Kator, personal communication). Incubations at 25°C showed no difference in PFU counts after 24 hours between the two exposure containers (Figure 6).

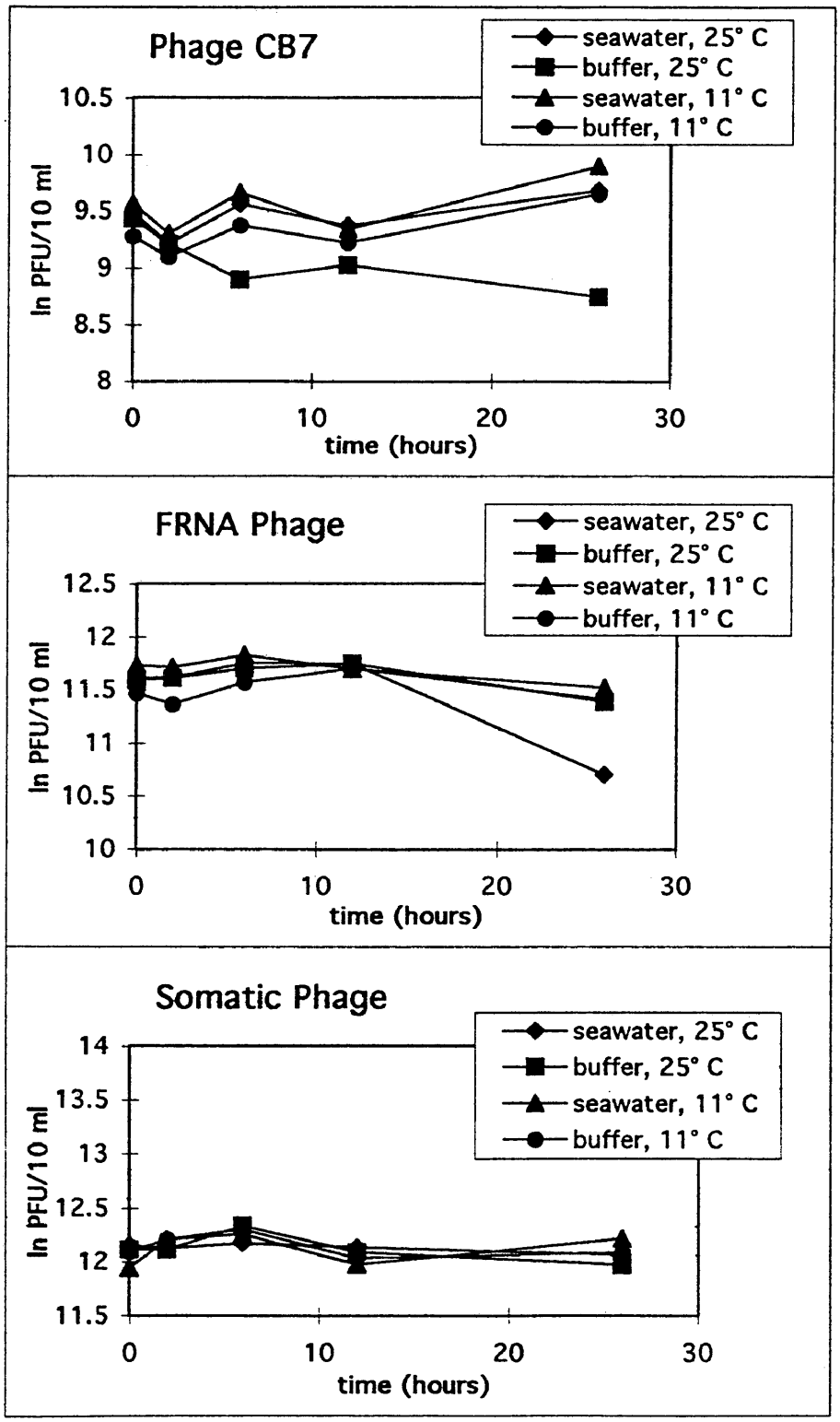

Figure 5. Results of laboratory experiments comparing survival of three phages in artificial seawater (15 psu) and buffer at two temperatures.

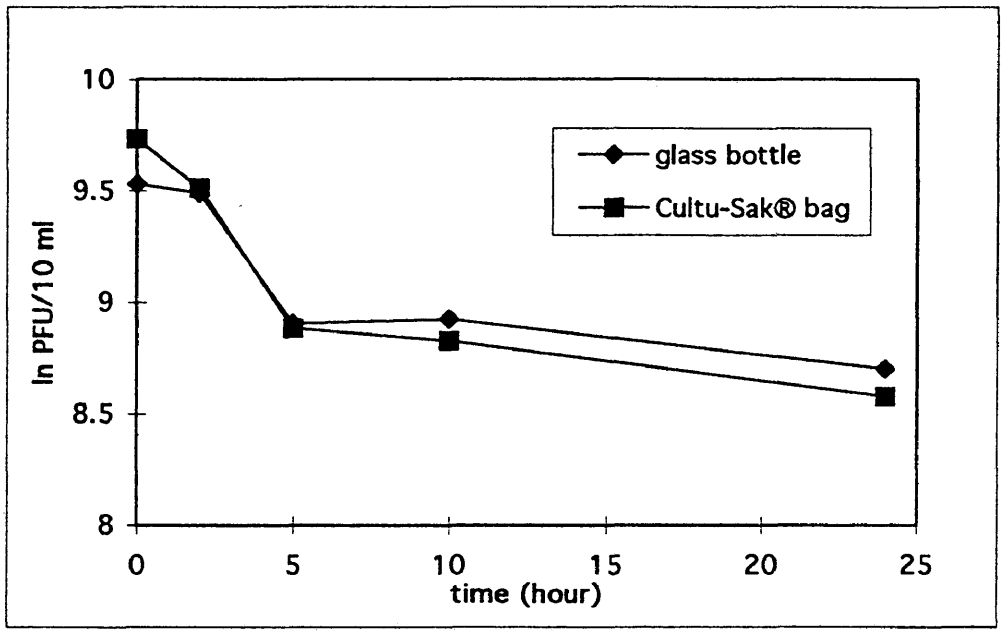

Figure 6. Results of laboratory experiment comparing survival of the FRNA coliphage in a glass milk dilution bottle and a Cultu-Sak® bag. Incubations were done at 25°C in artificial seawater (15 psu).

DISCUSSION

Phage survival in the estuarine environment

Effect of sunlight

In contrast to results predicted by the first hypothesis, the estuarine phages tested were as or more sensitive to inactivation by sunlight than were the allochthonous phages (Table 1, Figures 3a and c). Suttle and Chen (1992) presented a conceptual model which partitioned marine viral decay in the top 10 m of a hypothetical water column among many different mechanisms, based on results from experiments with indigenous viruses characterized as double stranded DNA viruses. The average inactivation rate coefficient attributed to solar radiation was 0.016 $hour^{-1}$ (25° C) for the model viruses. My experiments showed rates of phage inactivation in summer attributed to sunlight of 0.06 $hour^{-1}$ (CB7) and 0.07 $hour^{-1}$ (CB38). These results and those of Suttle and Chen both suggest that sunlight can be a factor causing viral inactivation. My results also support the idea that a large proportion of free viruses in seawater are not infective (Fuhrman and Suttle, 1993).

Since Suttle and Chen (1992) first introduced their model, more information concerning the survival of indigenous marine phages has become available. Marine viruses have been found that are less sensitive to sunlight than those studied earlier (Fuhrman and Suttle, 1993). Fuhrman and Suttle (1993) suggested that sensitivity is likely related to the differences in the DNA repair abilities of the hosts, or to the ability of virus encoded nucleic acid polymerases to function on radiation damaged phage DNA.

In my study, PFU counts at 0.5 m and 1 m were analyzed separately from those at 0.05 m because of interactions among depths in summer which complicated the interpretation of the main treatment effects in the ANCOVA. The source of

interaction is conjectured to be an increase in water temperature in the bags at the 0.05 m depth, caused by solar warming of the dark controls wrapped in the black plastic cover. Although holes were punched in the bags to promote water entry, it was noted at the time of deployment that air and water were often trapped within the black wrapping.

Results of my study show a definite effect of depth on the inactivation of phages by sunlight. This was expected for the turbid and productive waters of the Chesapeake Bay estuary. In natural waters, selective adsorption of shorter wavelengths by dissolved organic material, chlorophyll and particulate organic material allows the longer wavelengths of sunlight to penetrate farther than shorter wavelengths. Because of this phenomenon, bacteria and viruses are exposed primarily to visible and some near UV light rather than UV-B in the euphotic zone of the estuary (Calkins, 1982). Rhodes and Kator (1990) found that the survival of *E. coli* deployed in situ was maximized at a depth of 1 meter in the estuary and that survival decreased as the bacteria were placed closer to the water surface. Incubation in the dark resulted in the best survival of bacteria.

Peak and Peak (1982) discussed the lethal lesion effect of UV light on DNA molecules. The lethal lesion in DNA caused by exposure to UV of less than 300 nm is the thymine dimer and they determined that the lethality of this radiation is a result of direct photochemical alteration of the genetic material itself. When bacteria were exposed to near UV light (wavelengths greater than 320 nm), genetic damage observed was not as severe because the repair systems appeared able to fix the lesions more efficiently. Peak and Peak (1982) observed single strand breaks in bacterial DNA after exposure to UV light of wavelengths greater than 300 nm.

Kapuscinski and Mitchell (1983), studying the effects of sunlight on microbial survival in coastal water, found that mortality of bacteria and viruses in naturally sunlit water was much higher than in water stored in the dark. The sunlight dose lethal to a male-specific phage, MS2, (and *E. coli*) was not reduced by a poly-acrylic filter with a transmission cutoff at 370 nm. The Cultu-Sak® bags used in my experiments transmitted 40% of light at 500 nm, but only 22% of light at 300 nm. Therefore UV-A and UV-B were attenuated more than PAR (visible light). Results of my study indicated that sunlight inactivation still occurred even when UV-A and UV-B were reduced with respect to the visible spectrum. Sieracki (1980) also found no significant difference between inactivation of bacteria and coliphage exposed to unfiltered light and light passed through a 325 nm cutoff filter which allowed for transmission of UVA and visible light.

The FRNA phage used in this study was not sensitive to sunlight, an observation consistent with studies showing FRNA phages to be resistant to inactivation by ultraviolet radiation (Havelaar et al., 1991). On the other hand, the *Salmonella* somatic and the two indigenous phages, all containing dsDNA, were sensitive to inactivation by sunlight. This apparent instability of the double stranded nucleic acid may be due to an effect of sunlight on base pair structure of the molecule.

The inactivation rate for the dark control of the *Salmonella* somatic phage in winter below the surface is significantly greater than that in the light exposed bags (ANCOVA $p=0.02$). However, this appeared to be a possible interaction of treatment (light and dark) and time. It appears that through time, the light treatment curve crossed over the dark treatment curve as it decreased (Figure 3g, 0.5 m).

Many viral decay studies in the estuarine environment have focused on the fates of human enteric viral pathogens and coliphages. Kapuscinski and Mitchell (1980) suggested that as much as 90% of the infectious bacteriophages in the marine environment are inactivated in 1 to 10 days , and more recent studies focusing on sunlight as a factor in viral inactivation support this statement. Experiments involving poliovirus 1 and coliphage T4 (Bitton et al., 1978) revealed that under laboratory conditions viruses exposed to sunlight have a larger inactivation rate than those in the dark. Hill et al. (1970) exposed poliovirus 1 in estuarine water to artificial UV radiation and also found significant inactivation. Borrego and Romero (1985) found that in situ coliphage survival in seawater varied by season and duration of exposure to sunlight. Fattal et al. (1983), working with enteric viruses and bacteria in the Mediterranean Sea, found decreasing concentrations of enteric viruses and bacteriophages at beach sites near a sewage outfall during summer months. He attributed reductions in microbial levels to increased hours of dayllight and solar intensity in summer. My results agree that solar insolation levels do have an effect on inactivation rates, evidenced by the reduced inactivation effect of sunlight in winter when solar insolation levels were low compared with summer.

Effect of temperature

Phages isolated from sewage were more sensitive to seasonal temperature changes than the estuarine phages, with the *Salmonella* somatic phage the most temperature sensitive (Figure 4). Comparisons of the inactivation rates for the dark controls using the combined data at 0.5 m and 1 m showed the inactivation rates for the FRNA and *Salmonella* somatic phage in summer were four and nine times greater than in winter, respectively. Similar comparisons made for the indigenous phages CB7 and CB38 showed that inactivation rates were two times greater in summer than winter. It can

be speculated based on these results that the indigenous phages have adapted to survival in the changing seasonal temperatures of the Chesapeake Bay estuary.

Other studies investigating the survival of indigenous marine phages under ambient conditions show better survival at 5° C than at 20° C (Moebus, 1992a). The results for the autochthonous viruses presented here agree with these findings, indicating that colder temperatures enhance survival. Metcalf and Stiles (1967) found inactivation of three enteroviruses to be influenced by temperature. Studies conducted using autoclaved and UV radiated seawater at 5°, 24° and 37° C showed greater survival at the lowest temperature.

Effect of artificial seawater

Inactivation of viruses in natural waters is influenced by biological factors such as antiviral activity by bacteria and algae (Block, 1983). Antiviral oxygenated particles also impact viruses (Borrego et al., 1983; Block, 1983; Suttle and Chen, 1992). To simplify study of the effects of physical parameters like sunlight and temperature, viral survival studies have been conducted using filter sterilized or autoclaved natural seawater (Metcalf and Stiles, 1967; Akin et al, 1971; Moebus 1992a; Suttle and Chen, 1992) to exclude microbial interactions. Another way to remove the biological and organic effects is to use filter sterilized artificial seawater, as was done in this experiment, prepared with organic-free salts. Artificial seawater salts combined in proportions to equal those in natural seawater at 30 psu (Aquarium Systems, Mentor OH) can be diluted to any salinity desired (Akin et al., 1976). Although supplemental experiments (Figure 5) did not find an inactivation effect of artificial seawater on the phages used in my study, sterile artificial seawater apparently has an inactivating effect on some viruses (Metcalf and Stiles, 1967; Akin et al., 1975 and 1976; Kapuscinski and Mitchell, 1980). Although documented, this effect has not

been well studied and the mechanisms are not understood. Trace heavy metals (Kapuscinski and Mitchell, 1980) have been proposed as antiviral agents. Several investigators reported that dilution of artificial seawater reduces the effect (Akin et al., 1976; Kapuscinski and Mitchell, 1980). However, Akin et al. (1976) were unable to explain increased inactivation at 10 psu compared with high (20 psu) and low (1 psu) salinity using artificial seawater.

Effect of exposure time

One important observation made in the summer experiments was the apparent large rate of inactivation that occurred within the first few hours of incubation for the somatic, CB7 and CB38 phages (Figures 3a, c, and g). At the time of this writing there are no published reports demonstrating sunlight induced viral inactivation within a few hours. Akin et al. (1976) found similar patterns over several days when studying loss of poliovirus infectivity in artificial seawater. Their studies revealed two-component inactivation curves when incubations were made in artificial seawater with salinity between 5 and 20 psu. They found a maximum effect at 10 psu (Akin et al., 1976) but did not observe this pattern when using filtered natural seawater. Akin et al. (1976) at first believed a genetically more resistant population within the initial stock of viruses could be the explanation, but after further studies they concluded this was not the case. Further studies also discounted a theory of viral aggregation to explain the phenomenon. Although investigations did not reveal the nature of the inactivation, it was noticed that agitation seemed to increase the effect, and that when left stationary the phage survived better. However, Akin et al, (1976) were not able to demonstrate that the loss of infectivity was due to viral adsorption to the container. The two component curves observed in my experiments may have been due to an absence in incident sunlight during nighttime hours (Figures 7-9). At the time of this writing, I was unable to find published literature describing

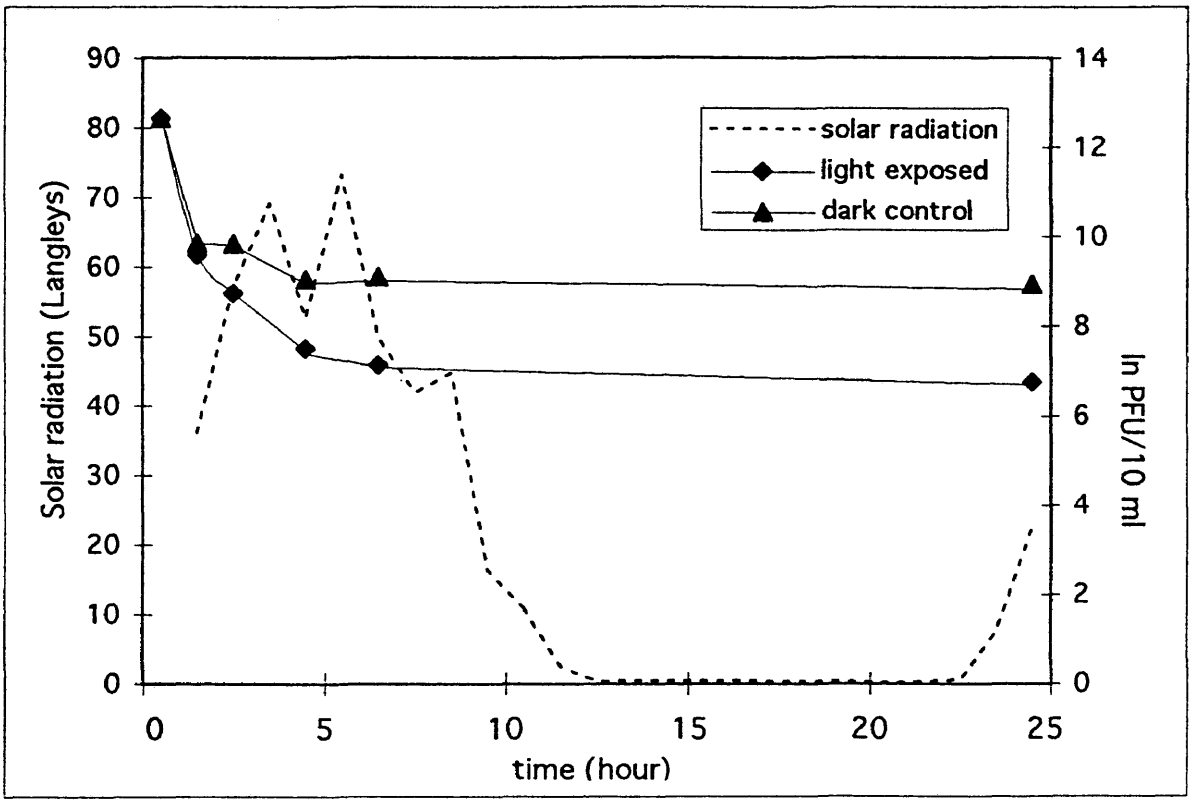

Figure 7. Inactivation curves of phage CB7 at 25°C and a depth of 0.05 m in the estuary. Solar radiation was measured above the water surface.

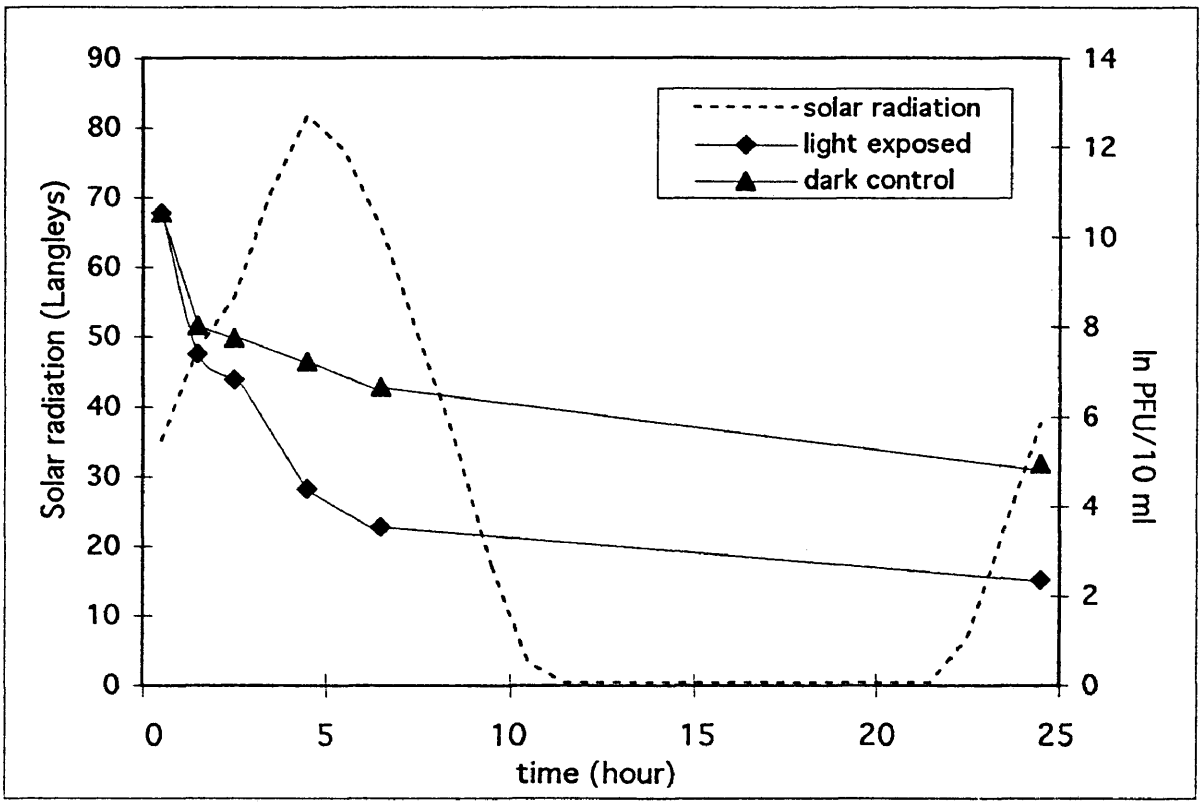

Figure 8. Inactivation curves of phage CB38 at 25°C and a depth of 0.05 m in the estuary. Solar radiation was measured above the water surface.

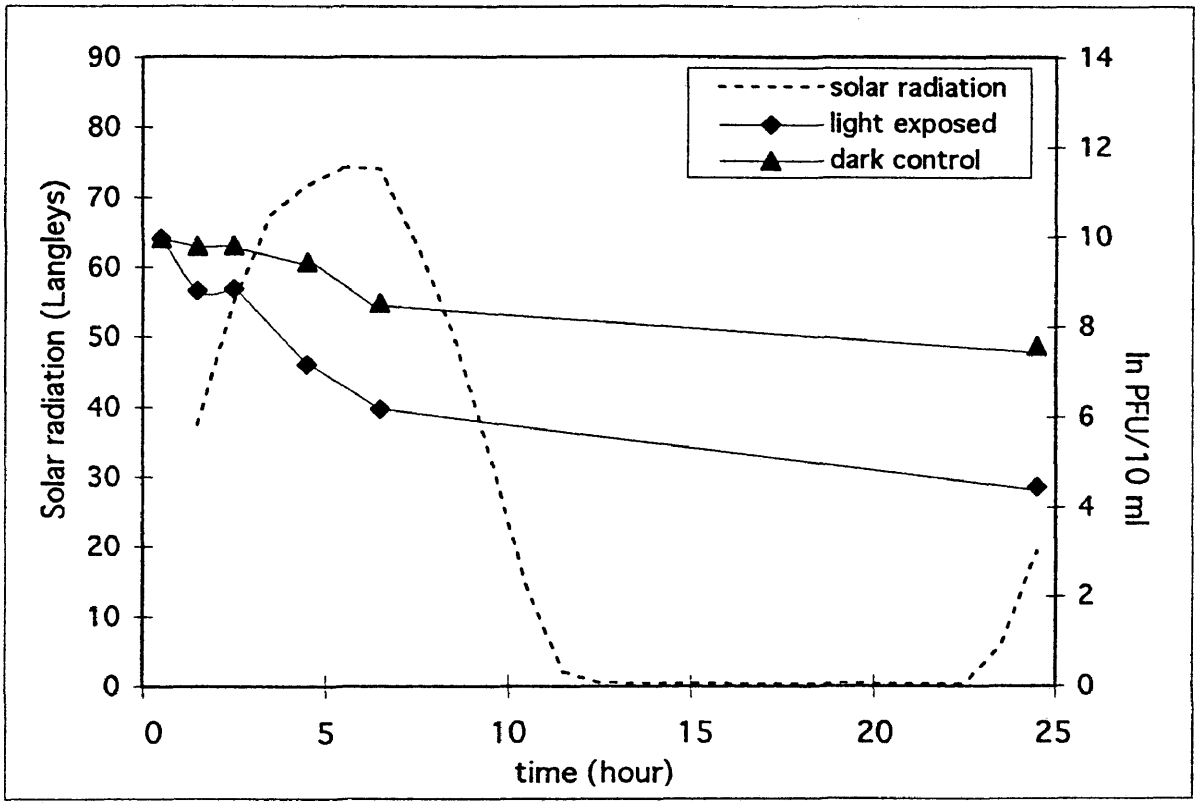

Figure 9. Inactivation curves of the *Salmonella* somatic phage at 26°C and a depth of 0.05 m in the estuary. Solar radiation was measured above the water surface.

in situ light exposure experiments sampled over hourly time increments with which to compare my results.

Implications of my results

Based upon the results of this study, I conclude that the bacteriophages examined display different intrinsic light and temperature sensitivity characteristics. Inactivation by sunlight in sensitive phages is seen only at a depth of 0.05 m, not at 0.5 m and 1 m. The estuarine phages were inactivated by sunlight in summer, and the *Salmonella* somatic phage was inactivated by sunlight in both summer and winter. The FRNA phage was not affected by sunlight in either season. The allochthonous phages were more sensitive to elevated temperatures than the indigenous phages, although all were affected by seasonal temperature differences.

The observations in this study have ecological, microbiological and water quality implications. The persistence characteristics of autochthonous viruses in the marine environment are under current scrutiny, as viral persistence has obvious implications to the role of viruses in microbial food webs. Moebus (1992b) suggests that "free" indigenous virions suspended in the water column appear to be short lived. My results do not contradict this statement. If Moebus is correct, then it may be that maintenance of environmental phage-host systems depends mainly on the survival of the phage within their hosts. This could be achieved through lysogeny, the carrier state, or delayed reproduction or release (Moebus, 1992b).

The implication of temperature inactivation of the FRNA coliphage (and *Salmonella* somatic phage) is that it would not be an effective indicator of viral pathogens from sewage in summer when water temperatures are elevated unless these pathogenic viruses share the same sensitivity to temperature. Viruses may also persist within

sediments and animals, which must be considered when evaluating persistence in the natural environment. However, the apparent insensitivty of the FRNA phage to sunlight reinforces its candidacy as a possible viral indicator of sanitary water quality during the colder months when shellfish are normally harvested for direct consumption.

Further studies should be done to address the questions raised regarding temperature-mediated inactivation. Experiments involving selective filtration of portions of the solar spectrum and the effects of wavelength on phage survival could resolve the notion that wavelengths greater than 325 nm have a significant inactivating effect on viruses (Sieracki, 1980). Studies similar to those described in this thesis could be performed using fractionated samples of estuarine water but would introduce some secondary factors influencing bacteriophage inactivation. Additional studies might also address the relationship between viras structure and nucleic acid type on persistence in natural (nonfiltered) estuarine waters.

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