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Effect of Sunlight on Survival of Indicator Bacteria in Seawater†

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The stability of the natural populations of fecal coliforms and fecal streptococci in raw sewage diluted 1:1,000 in seawater or phosphate-buffered water at 24 ± 2°C was markedly affected by the absence or presence of sunlight. In the absence of sunlight, these bacteria survived for days, whereas in the presence of sunlight 90% of the fecal coliforms and fecal streptococci were inactivated within 30 to 90 min and 60 to 180 min, respectively. The bactericidal effect of sunlight was shown to penetrate glass, translucent polyethylene, and at least 3.3 m of clear seawater, suggesting that the visible rather than the ultraviolet light spectrum of sunlight was primarily responsible for the observed bactericidal effect. However, these same sewage-borne bacteria were relatively resistant to the bactericidal effect of sunlight when diluted in fresh mountain stream waters. These results indicate that the presence of sunlight is a major factor controlling the survival of fecal coliforms and fecal streptococci in seawater.

As a result of selecting the coliform group of bacteria to assess fecal contamination of marine waters, many studies have been conducted to determine the survival rate and the factors controlling the stability of these bacteria in the marine environment. A review of the published reports (3, 12, 14) reveals that in most studies the coliform bacteria were reported to survive for days in seawater at moderate temperatures of 15 to 25°C. However, in some in situ studies (2, 7) these same bacteria have been reported to be effectively inactivated within a few hours. Moreover, high salinity, heavy metals, sunlight, temperature, competition for nutrients, predation by other microorganisms, lysis by bacteriophage, aggregation, and adsorption to particulate matter have all been reported to be the primary mechanism by which coliform bacteria are killed or their numbers are reduced in the marine environment (5, 9, 11). These discrepancies with regard to the expected survival rates and the factors controlling the stability of coliform bacteria in the marine environment are especially disturbing to water quality analysts and to engineers who design ocean sewage outfalls based partially on the reported stability of coliform bacteria in the marine environment.

The purpose of this study was to determine the stability of the populations of fecal coliforms (FC) and fecal streptococci (FS) naturally present in raw sewage after the sewage was diluted 1:1,000 in seawater to simulate the discharge of sewage into the marine environment, a practice still commonly used throughout the world. By carefully controlling the experimental conditions to simulate the variable field conditions, the survival rates and the major factors controlling the survival of these bacteria in the marine environment were determined, and the results were evaluated in light of previously published reports.

MATERIALS AND METHODS

Sewage, feces, and natural water samples. Seawater was obtained from Black Point Beach, located on the southeastern coast of Oahu, at a depth of 1.2 m. This beach did not receive obvious sources of contamination such as stream or storm drain runoff and was not extensively used for swimming. Freshwater was obtained from Nuuanu Stream at an elevation of 800 ft (ca. 236.4 m) above sea level close to the forest reserve area to ensure that the water was not significantly contaminated with the by-products of human activities. Raw sewage was obtained from the Ala Moana Pump Station, which collects the sewage from a major portion of the city of Honolulu. To obtain populations of indicator bacteria of animal origin, fresh feces from chicken and cattle were obtained from animals used by the University of Hawaii. All samples were collected on the day of the experiment.

Bacteriological analysis. Procedures described previously (1) were used for the preparation of the phosphate-buffered water (PBW) in distilled water (pH 7.2) as the sample diluent and for the recovery of bacteria by filtering 25 ml of the diluted water samples through GN-6 (Gelman Sciences, Inc.) or HC (Milli-

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pore Corp.) membrane filters (47 mm). FC bacteria were selectively grown by incubating the membrane on mFC agar (Difco Laboratories) at 44.5 ± 1°C, whereas the FS bacteria were grown on KF or m-Enterococcus Agar (Difco) at 35 ± 1°C. Purified cultures of FC and FS were obtained by picking isolated and characteristic colonies of these bacteria from either mFC or KF agar and streaking for purity on the same media. These cultures were washed and resuspended in PBW.

Experimental design. Sewage samples were routinely clarified of large particulate matter by filtration through an AP-25 prefilter (Millipore) before being used. Feces from the animals were dispersed well in PBW and centrifuged at 2,000 rpm for 20 min, and the resulting supernatants were clarified by filtration through the AP-25 prefilter. These clarified samples were then diluted 1:1,000 in seawater, PBW, or freshwater, and the survival of the populations of FC and FS in these samples was determined before and after incubation under laboratory or simulated daylight field conditions. Under laboratory conditions the samples (in 1- to 4-liter beakers or flasks) were continually mixed by a nonheating magnetic stirrer and incubated in the laboratory at room temperature (24 ± 2°C) under overhead fluorescent lighting for 10 to 12 h/day, but in the absence of sunlight. Under simulated field conditions the samples were added to borosilicate glass containers ranging in volume from 2 to 20 liters which were placed into styrofoam boxes filled with cool (15 to 20°C) water to maintain the temperature in the experimental water at 24 ± 2°C. Each styrofoam box was fitted with a cover to allow sunlight to enter only through the open top of the glass containers, and magnetic stirrers were placed under the box to keep the samples mixed. These samples were exposed to sunlight at the top of Holmes Hall, located on the campus of the University of Hawaii, between the hours of 1000 and 1500. In a separate study (15), hourly sunlight measurements were taken at this same location with an Eppley precision radiometer (model PSP). Since the amount of sunlight could not be controlled and thus varied significantly from day to day, routine experiments were conducted only on bright, predominantly sunny days when solar radiation measurements varied from 415 to 703 cal (ca. 1.738 to 2.944 kJ) per cm^2 per day. The average solar radiation at this Holmes site for 1978 was 482 cal (ca. 2.018 kJ) per cm^2 per day, with a minimum and maximum range of 13 to 780 cal (ca. 0.054 to 3.266 kJ) per cm^2 per day. The rates of bacterial inactivation under the various conditions were compared by calculating the T_{90}, or the time required for 90% of the populations of bacteria to be inactivated under a given set of conditions.

RESULTS

Comparative stability of FC and FS in seawater. To determine the stability of sewage-borne populations of FC and FS in seawater, clarified sewage was diluted 1:1,000 in seawater or PBW and incubated under laboratory (no sunlight, 24 ± 2°C) or simulated field (sunlight, 24 ± 2°C) conditions as described above. The results of eight experiments conducted under laboratory conditions revealed that after the sewage was diluted in seawater, the T_{90} for the FC ranged from 21 to 48 h, whereas the T_{90} for the FS ranged from 36 to 84 h. Since the same populations of bacteria were stable for 4 days when similarly diluted in PBW, it is clear that seawater is an unfavorable environment for enteric bacteria, but that these bacteria can be expected to remain viable for 1 to 3 days in seawater. The results of one such typical experiment are shown in Fig. 1. However, when the

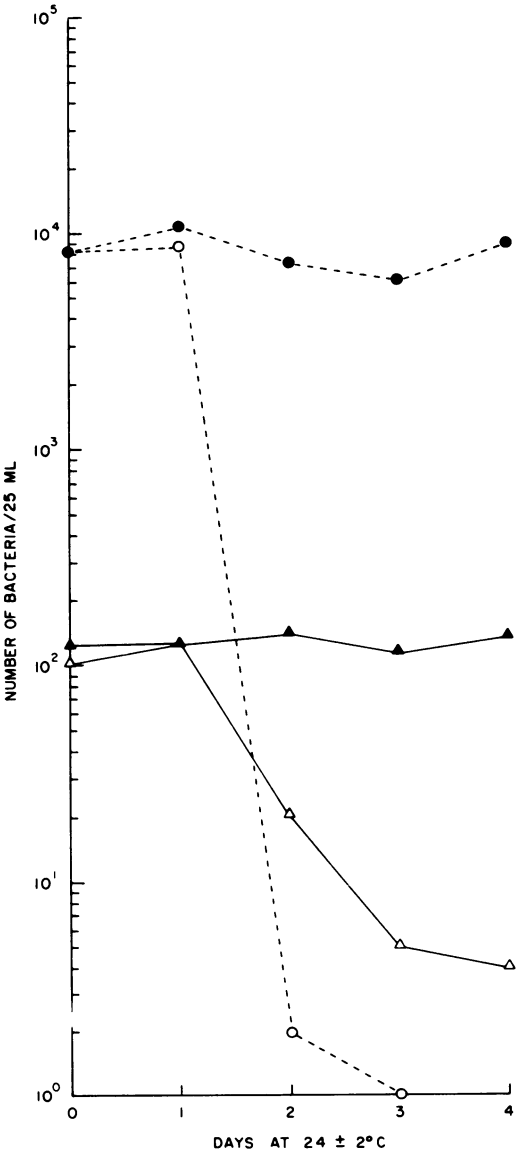

FIG. 1. *Comparative survival of FC suspended in PBW (●) or seawater (○) versus FS suspended in PBW (▲) or seawater (△) in the absence of sunlight.*

same experiment was repeated on 10 different days in the presence of sunlight (simulated field conditions) the T_{90} for FC diluted in seawater ranged from 30 to 90 min, whereas the T_{90} for FS ranged from 60 to 180 min. Figure 2 shows the results of one such typical experiment and furthermore shows that the FC and the FS diluted in PBW were inactivated to nearly the same extent as when diluted in seawater, indicating that the high salt content and stressful environment of seawater is not a requirement for the inactivation of bacteria by sunlight.

To demonstrate that the rapid inactivation of enteric bacteria by sunlight truly reflected the activity of sunlight and was not dependent on some particular variable of our experimental design, the following parameters were evaluated: (i) sewage was not clarified before use; (ii) sewage dilution in seawater was adjusted to 1:10, 1:100, and 1:1,000; (iii) sewage from another source was used; (iv) the sample was not mixed during incubation; (v) the volume of the sample was varied between 1 to 20 liters; (vi) the seawater was filter sterilized through a membrane filter (0.22-μm pore size) before using; (vii) another source of natural seawater or artificial seawater (10) was used; and (viii) the temperature of the reaction mixture exposed to sunlight was maintained between 15 to 20°C. None of these variables significantly changed the basic reaction that in the presence of sunlight, the populations of FC and FS suspended in seawater were drastically inactivated within 1 to 4 h, and in any given experiment the FS was more stable than the FC. To demonstrate that the bactericidal effect of sunlight required the constant presence of sunlight, seawater containing a 1:1,000 dilution of filter-sterilized sewage was first exposed to sunlight for 2 h. Sewage at a 1:1,000 dilution was then immediately added to this mixture, and the sample was incubated for 2 h in the absence of sunlight. Under these conditions, the populations of FC and FS were not inactivated.

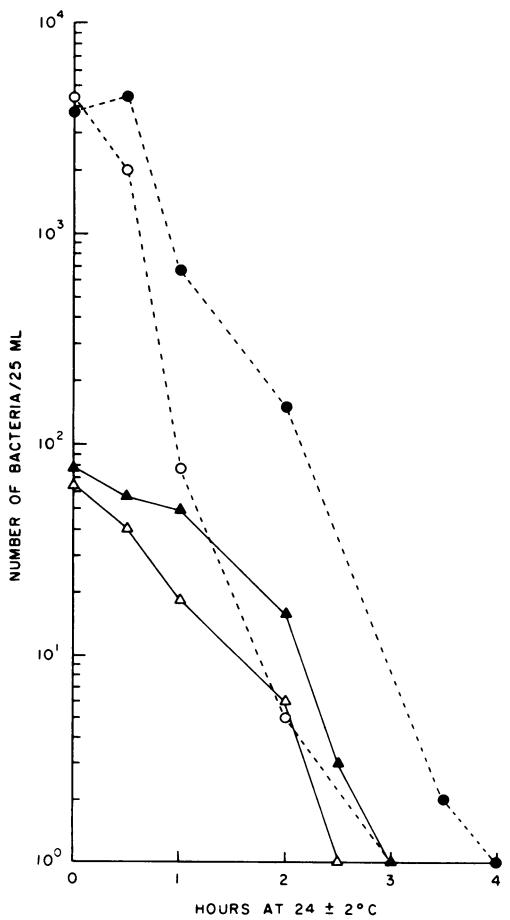

FIG. 2. *Comparative survival of FC suspended in PBW (●) or seawater (○) versus FS suspended in PBW (▲) or seawater (△) in the presence of sunlight.*

Stressful or bactericidal effect of direct and indirect sunlight. To determine whether the sunlight-induced reduction of colony-forming units was due to stress or actual die-off of the indicator bacteria, the sewage was diluted into seawater and exposed to sunlight as described above. Samples were then assayed by standard techniques as well as by the resuscitation technique of Rose et al. (13). For any sample, the number of FC colony-forming units as determined by the resuscitation technique did not increase by more than a factor of 2 even when 99 to 99.9% reduction of the colony-forming units was observed. Furthermore, after a 4-h exposure to sunlight, the FC counts were zero whether the standard or resuscitation technique was used, indicating that sunlight has a killing rather than a stressful effect on the indicator bacteria.

Since the rate of bacterial inactivation was correlated with the presence of sunlight, and since the amount of direct sunlight varies from day to day, the question was raised as to the bactericidal nature of indirect sunlight as observed on cloudy days. To answer this question, the same simulated field experiment was conducted on a very dark, cloudy day when only 106 cal (ca. 0.444 kJ) per cm^2 per day of solar radiation was measured. This day was characterized by no obvious sunlight or shadow casting, indicating that only indirect or diffuse sunlight was present. Under these conditions, the FS diluted in seawater were stable even after 4 h of exposure, whereas the FC diluted in seawater were stable for 1 h followed by gradual inactivation, reaching a maximum of nearly 99% in-

activation after 4 h. These results indicate that indirect sunlight can still be expected to inactivate FC, but probably not the FS.

Spectrum and penetrability of sunlight. To determine the spectrum of sunlight responsible for the bactericidal effect and the penetrability of these wavelengths, four beakers were initially filled with 2-liter samples of sewage diluted 1:1,000 in seawater. The top of one beaker was left uncovered, and the tops of the remaining three beakers were covered with either 5-mm-thick cardboard wrapped with a black 1.5-mil (ca. 38.1-μm-thick) polyethylene trash bag to completely block out the sunlight, 3-mm-thick clear window glass to allow most of the visible light to penetrate, or 5-mm-thick white linear polyethylene which was translucent. These samples were placed into styrofoam boxes and exposed to sunlight as described above to allow sunlight to enter only through the tops of the beakers. Samples were taken and assayed for residual concentrations of FC and FS after a 5-h incubation period. The results (Table 1) show that extensive inactivation of both FC and FS was observed in all of the samples, except the sample covered with the cardboard, indicating that the bactericidal effect of sunlight will penetrate clear glass as well as translucent polyethylene. To provide direct evidence that ultraviolet light was incapable of penetrating glass and the polyethylene, purified cultures of FC and FS were suspended in seawater, and 10-ml samples were placed into four 100-mm petri dishes. One of the samples was left uncovered, and the other three samples were covered with either the same clear window glass and polyethylene used earlier as well as the pyrex glass (2-mm-thick) cover of the petri dish. All samples were then exposed to 5 min of ultraviolet light from a germicidal lamp (General Electric G30T8) which was placed 18 cm above the samples. The concentration of bacteria before and after ultraviolet light treatment was determined by streaking 0.1 ml of the proper dilution of the samples onto EMB (Difco) or KF agar. The results (Table 2) show that greater than 99.99% of the populations of FC and FS were destroyed in the dishes without a cover, whereas the populations of bacteria in the dishes covered with both kinds of glasses and the translucent polyethylene remained unaffected.

To assess the penetrability of the bactericidal effect of sunlight through a column of water, 75-ml samples of sewage diluted 1:1,000 in seawater were added to dialysis bags. The ends of these dialysis bags were stretched and fastened between two parallel aluminum bars which were anchored to an automobile tire rim. The test dialysis bags were left uncovered to allow sunlight to penetrate and react with the sample. As controls some dialysis bags were covered with a 1.5-mil black polyethylene bag to prevent sunlight from reacting with the sample and yet allow these samples to be subjected to the same conditions as the test samples. The entire apparatus was then immersed in the ocean at various depths, and after various incubation periods samples were recovered and assayed for FC and FS. Experiments were conducted only during sunny periods and on days when the ocean water was clear and the immersed samples could be observed from the surface of the water to be bathed in sunlight. Initially, two experiments conducted at a depth of 0.9 m showed that greater than 99% of both FC and FS in the test dialysis bags were inactivated after 4 h of immersion in the ocean environment, whereas the same bacterial populations in the control dialysis bags were unaffected. The results (Table 1) of a single experiment conducted at the maximum depth of 3.3 m show that after 5 h of immersion, greater than 99% of the FS and about 99.9% of the FC had been inactivated, whereas these

TABLE 1. *Penetrability of the bactericidal effect of sunlight*[a]

Experimental design	Bacterial counts/25 ml	
	FC	FS
Samples in beakers covered with:		
Cardboard	2,400	250
Clear glass	0	1
Translucent polyethylene	1	6
Uncovered	0	1
Samples in dialysis bags immersed 3.3 m below ocean surface:		
Uncovered bags	2	3
Bags covered with black trash bag	3,100	560

[a] Samples consisted of sewage diluted 1:1,000 in seawater with the following initial bacterial concentrations (per 25 ml): in beakers, 2,200 FC and 340 FS; in dialysis bags, 1,300 FC and 600 FS. Counts were made after 5 h of exposure to direct sunlight.

TABLE 2. *Penetrability of the bactericidal effect of ultraviolet light*[a]

Petri dish covered with:	Bacterial counts/ml	
	FC	FS
Clear Pyrex glass	4.5×10^6	5.0×10^6
Clear window glass	4.2×10^6	5.7×10^6
Translucent polyethylene	3.9×10^6	4.2×10^6
Uncovered	<10	<10

[a] Initial bacterial concentrations (per ml) were 4.4×10^6 FC and 5.7×10^6 FS. Counts were made after 5 min of exposure to ultraviolet light.

bacteria in the control dialysis bags remained almost unaffected. These results show that the bactericidal effect of sunlight is capable of penetrating through at least 3.3 m of clear seawater. The accumulated data suggest that the observed bactericidal effect of sunlight may be due to its penetrating visible light spectrum and is not restricted to its ultraviolet light spectrum.

Comparative sensitivities of FC and FS of animal origin. One of the major problems in environmental monitoring of indicator bacteria is to determine whether the source of pollution is human or animal. To resolve this predicament, Geldreich and Kenner (8) proposed that since human feces contain a higher ratio of FC to FS (>4) than is found in most animal feces (<0.7), the ratio of FC to FS can be used to determine whether the source of pollution is animal or human. However, the results of the present study indicated that in marine waters, FC are much more sensitive to sunlight inactivation than are FS; therefore, the high ratio of FC to FS in domestic sewage may be reversed within a few hours after the sewage is discharged into the marine environment. These results also raise the question of whether animal strains of FC and FS are as sensitive as the human strains of these bacteria to sunlight inactivation. To answer this question, indicator bacteria obtained from two common domestic farm animals representing the avian group (chicken) and bovine group (cattle) were selected. The feces from these two animals were dispersed in PBW and centrifuged at 2,000 rpm for 20 min to remove large debris, and the supernatant was clarified through an AP-25 prefilter. These animal fecal samples and a sample of domestic sewage representing the source of human feces were diluted 1:1,000 in seawater and incubated in the presence of sunlight (simulated field conditions), and hourly samples were taken and assayed for FC and FS. Two such experiments were conducted, and the T_{90} for FC and FS from each of the three different sources was calculated. The results (Table 3) show that FC and FS from humans, cattle, and chickens were inactivated to a similar extent by sunlight. Although the human strains of FC and FS appear to be more resistant than the animal strains it is recognized that the sources of human and animal strains of these bacteria are not the same. Based on these results it was concluded that human and animal strains of FC and FS are similarly susceptible to the inactivating effect of sunlight, and that the FS of all species are more resistant than the FC to the bactericidal effect of sunlight.

TABLE 3. *Comparative sensitivity of human and animal strains of FC and FS to sunlight*

Source of bacteria	Calculated T_{90} (min)			
	FC		FS	
	Expt 1	Expt 2	Expt 1	Expt 2
Human	40	30	180	105
Chicken	45	20	80	90
Cattle	50	20	70	90

Comparative stability of FC and FS in seawater versus freshwater. The observation that sunlight inactivated FC and FS to nearly the same extent whether suspended in seawater or PBW indicated that the stressful high salt content of seawater was not a requirement for the bactericidal action of sunlight. Therefore, it follows that the same phenomenon should occur even when the suspending medium is freshwater. To confirm this reasoning, sewage was initially diluted 1:1,000 in seawater, deionized water, or freshwater obtained from a mountain stream (Nuuanu Stream). Samples of these mixtures were then incubated under laboratory and simulated field conditions, and the concentrations of FC and FS were determined after various periods of incubation. Under laboratory conditions, the populations of FC and FS suspended in freshwater were determined to remain stable for up to 3 days, whereas the same populations of bacteria suspended in seawater were drastically inactivated during the second and third days of incubation (Fig. 2) indicating that the freshwater environment was much more favorable to the bacteria than was the seawater. Under simulated field conditions, the populations of FC and FS suspended in seawater were determined to have a T_{90} of 36 and 98 min, respectively, and to be completely inactivated within 3 h (Fig. 3). The rate of bacterial inactivation in the deionized water was only slightly slower than that in seawater and is not plotted in Fig. 3. However, under identical sunlight conditions the T_{90} for the FC suspended in freshwater was 114 min, and 8% of this population of bacteria survived the 3-h incubation period, whereas the FS were much more resistant to sunlight, and 45% of this population of bacteria survived the 3-h exposure to sunlight. The greater resistance of FC and FS to the bactericidal effect of sunlight when these bacteria were suspended in freshwater as opposed to seawater was observed when another source of freshwater (Manoa Stream) was used as the diluent.

DISCUSSION

The demonstration that populations of sewage-borne FC and FS bacteria suspended in seawater were drastically inactivated within a

few hours in the presence of sunlight, whereas they persisted for days in the absence of sunlight, points out the danger of extrapolating data obtained under laboratory conditions (absence of sunlight) to interpret events which occur under field conditions (presence of sunlight). The failure to recognize sunlight as a primary factor controlling the survival of indicator bacteria in marine waters may explain the apparent discrepancies in the reported survival times of coliform bacteria in marine waters. Strong evidence that sunlight is a major factor controlling the survival of enteric bacteria in marine waters was originally reported by Gameson and Saxon (7). However, the results of their study were not immediately appreciated, and only recently have their findings been supported by Bellair et al. (2). After reassessing the reported literature, Chamberlin and Mitchell (4) concluded that sunlight is a major factor controlling the stability of coliform bacteria under field conditions. The data accumulated in this study support the results of Gameson and Gould (6) and the model of coliform inactivation proposed by Chamberlin and Mitchell (4); it is the visible rather than the ultraviolet light spectrum of sunlight which is primarily responsible for the bactericidal effect, and this bactericidal effect can be expected to penetrate glass, linear polyethylene, and at least 3.3 m of clear seawater. Our results further show that the bactericidal effect of sunlight was effective even when the sewage-borne bacteria were diluted into distilled water or PBW, indicating that the high salt concentration of seawater is not required for the bactericidal activity of sunlight. However, the observation that the same bacterial samples diluted into fresh mountain stream waters were considerably more resistant to the bactericidal effect of sunlight indicates a major difference in the survival rates of indicator bacteria in fresh and marine waters which should be recognized in setting water quality criteria to assess fecal contamination of fresh versus marine waters. In this regard the rapid inactivation of FC as opposed to FS when sewage was diluted into seawater and exposed to sunlight resulted in the reduction of the high ratio of FC to FS (>10) naturally present in sewage to a ratio of less than 1 within 2 to 3 h, indicating that the significance of the FC/FS ratio established under freshwater conditions by Geldreich and Kenner (13) should not be extrapolated to include the marine environment. Furthermore, the greater resistance of FS as compared with FC to inactivation by bright, direct sunlight was accentuated under indirect sunlight and indicates that FS can be expected to predominate as the residual population of enteric bacteria in the marine environment. Significantly, human as well as animal sources of FC and FS suspended in seawater and exposed to sunlight were inactivated to approximately the same extent. This is taken as evidence that FC and FS of animal origin will not be the predominating strains in the residual populations recovered from natural marine waters, a situation which would prove to be most confusing in terms of evaluating the public health aspects of marine waters based on indicator bacteria density.

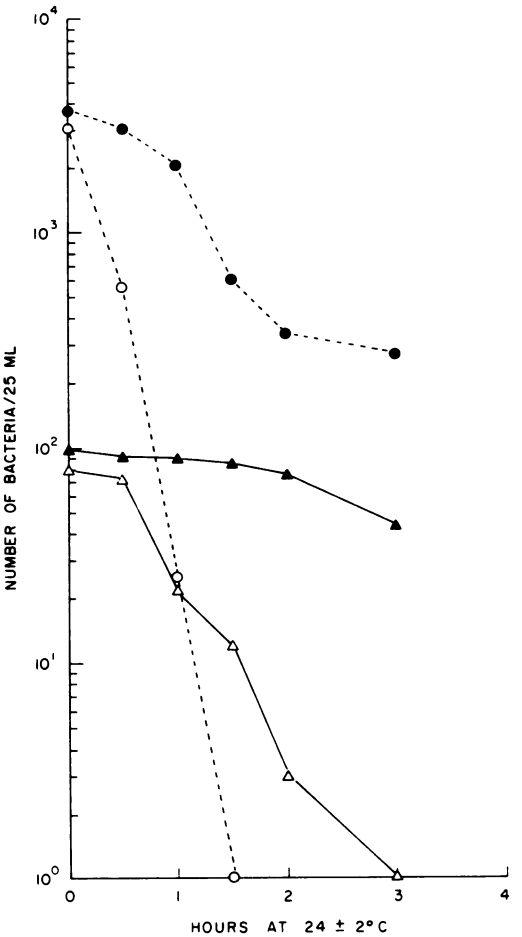

FIG. 3. *Comparative survival of FC suspended in freshwater (●) or seawater (○) versus FS suspended in freshwater (▲) or seawater (△) in the presence of sunlight.*

The identification of sunlight as a major bactericidal agent has many significant ramifications with regard to the expected survival of sewage-borne bacteria in the natural marine environment. For example, if fecal contamination of the marine environment occurred during the night, the enteric bacteria would not be expected

to be inactivated, and extensive dissemination of viable bacteria can be expected to occur until the following sunrise. On the other hand, if fecal contamination occurred during the day and especially in full sunlight, inactivation of bacteria would be rapid, and dissemination of viable bacteria would be drastically reduced. However, it must be recognized that turbidity, turbulence (foaming), or chemical composition of the water may interfere with the bactericidal effectiveness of sunlight. These factors should be considered in the selection of the sampling time and the sampling site as well as in the interpretation of the results. Furthermore, water samples collected under field conditions should be protected from sunlight during transportation, and the popular translucent linear polyethylene containers must be considered inadequate to block out the bactericidal effect of sunlight.

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