MAMALA BAY STUDY

INCIDENCE OF STAPHYLOCOCCUS AUREUS IN MAMALA BAY

PROJECT MB-SP3 / MB-7

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November 30, 1995

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PROJECT PERIOD: August 1, 1995 to December 31, 1995

I. INTRODUCTION AND DESCRIPTION OF STAPHYLOCOCCUS STUDY

A. Water-Contact Versus Water-Borne Diseases Associated with Swimming. Skin infections by staphylococcus bacteria following recreational use of water is classified as a water-contact disease defined as disease or microbial infection of eyes, ears, nose, throat, or skin of people obtained as a result of contact with water. The mode of transmission is contact with pathogenic microorganisms in water and the subsequent infection of some external part of man by one of more microorganisms. The normal habitats of microorganisms which cause water contact diseases are the water and/or some external sources on the human body such as eyes, ears, nose, throat, and skin. Since many of these microorganisms are found on the skin or mucus membranes of humans, they are readily washed off into the water environment when someone swims. Water-contact diseases are frequently reported following swimming. One explanation is that the water environment is not a normal environment for humans and swimming allows water and microorganisms to gain entrance to many parts of the body to establish conditions for microbial infections. Moreover, swimming often results in cuts and abrasions to swimmers which provide opportunities for microbial infections.

In contrast to water-contact diseases, water-borne diseases are caused by different groups of microorganisms and are transmitted by ingestion of the causative microorganism. The normal habitats of the microorganisms responsible for water-borne diseases are the intestinal tracts of man and animal. As a result the primary environmental source of these microorganisms is feces or sewage.

B. Staphylococcus Skin Infections. *Staphylococcus* is a genus of bacteria comprised of over 32 species which are characterized as gram-positive cocci which occurs in pairs, short chains or grape-like clusters (Kloos and Bannerman, 1995). The normal habitats of staphylococcus bacteria are the skin and mucous membranes of mammals and birds. Most of the staphylococcus bacteria are not pathogenic to man and can be found in man's environment, even in dust and soil. *Staphylococcus aureus* is the one species known to cause skin infections but even this pathogen is commonly found in the nose and skin of normal people who are not suffering from any skin infections. *S. aureus* is characterized by being coagulase positive or having enzymes capable of causing blood plasma to clot. Transmission of *S. aureus* skin infection is by contact but a healthy intact skin is generally resistant to even high concentrations of this bacteria (Marples and Kligman, 1985). However, when the skin is abraided, cut, and other defense mechanisms of man are lowered, much lower concentrations of *S. aureus* are required to establish a skin infection.

C. Staphylococcus Skin Infection Following Recreational Use of Water. Skin infection with *S. aureus* is commonly observed following recreational use of waters. Pien et al (1983) reported that activities related to recreational use of waters create conditions (cuts, skin abrasions, softening of skin, penetration of water) which are conducive to skin infections. There are two mechanisms by which one contracts staphylococcus skin infection resulting from recreational use of water. In the first mechanism, *S. aureus* is carried on the external skin of an individual and swimming provides the opportunity for this staphylococcus bacteria to gain entrance under the skin of that individual to cause a skin infection. This mechanism results in self-infection of an individual and therefore does not constitute a public health problem. Many physicians believe in this mechanism because repeated staphylococcus skin infections often occurs on the same individual. In the second mechanism, *S. aureus* is washed off the bodies of one swimmer into the water and water serves as the vehicle for the transmission of skin infection to another swimmer. This mechanism results in the transmission of disease from one individual to another and therefore creates a public health problem. Epidemiologist support this second theory and believe that some people are more susceptible to staphylococcus skin infections than others.

Evans (1977) was one of the first to evaluate the possibility that *S. aureus* could be transmitted by recreational use of water. He concluded that swimmers can be expected to become infected with *S. aureus* following recreational use of waters for two reasons. First, since the source of this bacteria is the skin and mucus membranes of people, *S. aureus* will be washed off the bodies of swimmers into the bathing waters. Second, since *S. aureus* is known to be much more resistant to environmental conditions such as drying, high salt concentrations and even disinfection than fecal coliform bacteria, this bacteria can be expected to persist in the water and to increase the potential time it can be infectious in the water environment. Evans (1977) also pointed out that *S. aureus* has very complex nutritional requirements and is therefore unlikely to multiply in most waters because the considerable array of organic nutrients it requires are not likely to be found in waters suitable for recreational use by humans.

To this day, a reliable method to selectively enumerate staphylococcus bacteria and especially *S. aureus* from all types of water has not been developed. It has been this lack of methodology that has prevented definitive studies on how *S. aureus* is being transmitted via recreational use of waters. Some studies monitor water for all species of staphylococcus (total staphylococcus), while others try to additionally determine the concentrations of the pathogenic *S. aureus* in the water sample. A review of the literature shows that studies conducted in Israel (Yoshpe-Purer and Golderman, 1987), in Spain (Alonso et al. 1989) in Canada (Seyfried et al. 1985) and Hawaii (Charoenca and Fujioka, 1993) have concluded that concentrations of total staphylococci bacteria or *S. aureus* in water can be correlated with swimmer density and can be used as an overall index of water quality. It should be noted that two major studies (Fujioka and Charoenca, 1991; Fujioka and Pennington, 1993) were completed in Hawaii which addressed the problem of staphylococcus bacteria in recreational waters. Some of the findings in these two studies are as follows: (1) A reliable but tedious and time consuming method was developed to enumerate the concentrations of total staphylococcus and *S. aureus* from marine waters in Hawaii. (2) Beaches in Hawaii could be categorized as high or low concentrations of total staphylococcus bacteria and this classification correlated with the number of people using the beach and the flushing capacity of the water at the beach sites. (3) Based on a 24 hour sampling study, the concentrations of staphylococcus in the water correlated with the numbers of swimmers during the day and persisted in the water after the swimmers left the water. (4) *S. aureus* recovered from south beaches (Waikiki, Ala Moana, Hanauma) of Oahu were similar in biotype (antibiotic sensitivity and phage typing) to *S. aureus* recovered from skin lesions of patients from Dr. Jeremy Lam's office in Honolulu. Dr. Lam had previously correlated staphylococcus skin infections of children and their recent experience in swimming in south beaches of Oahu. (5) Concentrations of *S. aureus* diluted in waters from the Ala Wai Canal and from Hanauma Bay remained stable and did not multiply in the absence of sunlight but was inactivated in the presence of sunlight.

In a recent study, Ahtiainen et al (1991) reported median concentration of staphylococcus of 1-2 CFU/100 ml in pristine water, 60 to 110 CFU/100 ml in waters impacted by agricultural run-off and 1000 to 1600 CFU/100 ml in treated wastewater. When just treated wastewater was assayed, they reported a median concentration of 500 CFU/100 ml for staphylococcus, 16,900 CFU/100 ml for fecal streptococci and 17,800 CFU/100 ml for fecal coliform. Thus, fecal indicator bacteria are present in much higher concentrations in sewage than staphylococcus bacteria. In this same report, the authors reported that only 2% of the staphylococcus in sewage was identified as *S. aureus* whereas 18% of the staphylococcus recovered from waters in uninhabited areas was identified as *S. aureus*. In environmental waters, total staphylococcus can always be expected to be present in higher concentrations than *S. aureus*. Moreover, the concentrations of *S. aureus* can be expected to vary considerably. For this reason, a reasonable limit for total staphylococcus for recreational water is not to exceed a geometric mean of 100/100 ml (Charoenca and Fujioka, 1993).

D. Decisions and Final Agreement on Staphylococcus Study. The MB-7 (Microbiological Assessment) Team of the Mamala Bay Study was aware of previous

studies which reported higher incidences of water-contact diseases (infections of eye, ear, nose, throat, and skin infections) of people following recreational use of waters than water-borne diseases (diarrheal diseases). Moreover, that in Hawaii, staphylococcus skin infections following recreational use (swimming, surfing, paddling) in the waters of Mamala Bay have been reported by a significant number of people. However, the MB-7 Team made a deliberate decision against the inclusion of staphylococcus study because the prevailing scientific evidence indicated that the source of staphylococcus in recreational waters is swimmers and the major goal of the Mamala Bay Study was to determine the impact of discharges of ocean sewage outfalls versus discharges of non-point sources (streams, storm drains, canals, harbors) on the quality of waters in Mamala Bay. In keeping with the primary goal of the Mamala Bay Study, the MB-7 Team used its resources to monitor waters in Mamala Bay for the presence of as many microbial indicators and pathogens known to be present in sewage and which represent water-borne diseases.

In June of 1995, the Mamala Bay Commissioners held a meeting in San Francisco to receive the preliminary final reports from all of the principal investigators. During this meeting, the Mamala Bay Commissioners reported that some citizens in Honolulu believed that the source of staphylococcus bacteria in Mamala Bay is sewage and requested the inclusion of a staphylococcus study as part of the Mamala Bay Study. As a result, the Commissioners requested that Russell Hill coordinate with Roger Fujioka to conduct a short, supplemental study to determine whether the concentrations of staphylococcus bacteria in Mamala Bay pose a threat to swimmers and to address the concerns raised by Honolulu citizens that sewage contamination of Mamala Bay is the cause of the increased level of staphylococcus skin infections among people using the waters in Mamala Bay. In response to the Commissioners request, Russell Hill and Roger Fujioka planned and implemented the completion of a short term supplemental staphylococcus study in two phases. Phase I Study was conducted by Roger Fujioka to assess the sources, the concentrations and fate of staphylococcus bacteria using cultural methods. Phase II Study was conducted by Russell Hill to assess the concentrations, the virulence and the fate of staphylococcus bacteria using molecular gene probe methods.

II. OBJECTIVES OF PHASE I STAPHYLOCOCCUS STUDY

This supplemental staphylococcus study was initiated after the completion of all the planned projects of the Mamala Bay Study because of continuing concerns expressed by citizens of Hawaii that recreational users (swimmers, surfers, paddlers, boaters) of Mamala Bay have had recurring staphylococcus skin infections. A spokesman for the citizens of Honolulu on the problem of staphylococcus skin infections is Mr. Robert Rodman, Vice President of the Waikiki Residential Association. Mr. Rodman scheduled a meeting with Mr. Felix Limtiaco, Director of the Department of Wastewater Management during the last week of May 1995 to explain his concerns that sewage being discharged into Mamala Bay was responsible for causing staphylococcus skin infections in people using the waters in Mamala Bay. Roger Fujioka was asked to attend this meeting because

of his experience in monitoring waters in Hawaii for staphylococcus bacteria. Having heard of Mr. Rodman's concerns, Roger Fujioka invited Mr. Rodman to the Water Resources Research Center, University of Hawaii on September 29, 1995 to meet with members of the Mamala Bay Team, to answer questions that Mr. Rodman had about staphylococcus, to clearly understand his concerns, and to explain how experiments could be designed to address his specific claims that sewage was entering Mamala Bay (Waikiki Beach) from underground discharges either from hotel basements or from broken sewer lines. Mr. Rodman stated his belief that staphylococcus bacteria was entering Mamala Bay directly from this source of sewage or the nutrients provided by these sewage discharges allowed staphylococcus bacteria to multiply in the waters of Mamala Bay.

The objectives of Phase I as outlined below were determined based on the general Mamala Bay Study approach, discussions with Russell Hill and the concerns of Mr. Rodman:

1. To determine the concentrations of total staphylococcus bacteria and the pathogenic species of staphylococcus (*Staphylococcus aureus)* at beach sites (Ala Moana, Waikiki, Queen's Surf, Sand Island park, Hanauma Bay, Ewa Beach) established earlier by the Mamala Bay Study on a weekly basis for two reasons. First to determine the concentrations of staphylococcus at these beaches. Second, to provide Dr. Hill with isolates of staphylococcus bacteria, especially *S. aureus* from these sites for his molecular probe tests.

2. To coordinate and to plan for a field experimental study with Dr. Hill to sample all sites routinely monitored during the quarterly sampling by the MB-7 Team for total staphylococcus and *S. aureus.* By monitoring the concentrations of staphylococcus bacteria at these sites, a conclusion can be made whether the ocean sewage outfall is a major source of staphylococcus bacteria which is then being transported to the shoreline waters of Mamala Bay.

3. To determine the stability of *S. aureus* in the marine waters of Mamala Bay and to determine the effect of nutrients (peptone and 50% sewage) on the ability of this bacteria to grow. This objective was included specifically to test Mr. Rodman's theory that *S. aureus* was multiplying in the waters of Mamala Bay.

4. To determine the stability of *E. coli* and *S. faecalis* in the marine waters of Mamala Bay and to determine the effect of nutrients (peptone and 50% sewage) on the ability of this bacteria to grow. This objective was included as a means to compare the response of *S. aureus* with that of *E. coli* and *S. faecalis* under the same set of conditions since most of the available monitoring data includes concentrations of these two fecal indicators.

5. To determine whether nutrients such as 50% sewage and peptone will have a stabililizing effect on the inactivation of *S. aureus* by sunlight. We previously determined that sunlight will inactivate *S. aureus* suspended in clean water. This objective was

included to determine the effects of nutrients (consequence of pollution) on the inactivating effect of sunlight for *S. aureus*.

III. RESULTS: Sources and Concentrations of Staphylococcus Bacteria.

A. Objective and Experimental Design. To address objectives 1 and 2 of this study, grab samples of water were collected from popular recreational beach sites, offshore and deep ocean sites and analyzed for total staphylococcus bacteria and *S. aureus* using the method as previously described by Charoenca and Fujioka (1993). Beach water samples were collected once a week over a 4-week period in September and October. During the second week of October, all of the sites established for quarterly sampling by the MB-7 team were sampled for staphylococcus bacteria over a three day period using a boat to collect the offshore sites. During this field sampling study, Dr. Russell Hill from the University of Maryland and his assistant, Victoria Boccuzi, collected their own samples while personnel from the Hawaii team collected water samples from each of the MB-7 sites. The Hawaii Team also provided the University of Maryland Team with laboratory facilities to process their samples and also collected, clarified effluent from Sand Island Wastewater Treatment Plant (SIWWTP).

Samples were assayed on VJ agar supplemented with 0.005% sodium azide using the membrane filtration technique and incubated at 35°C for 44 ± 4 hours using the method developed in our laboratory (Fujioka and Charoenca, 1991, Charoenca and Fujioka, 1993). It should be noted that this medium was optimized to selectively recover staphylococci bacteria from marine water samples as it inhibits the growth of marine bacteria. Black, shiny colonies were determined to be presumptive staphylococcus bacteria. A representative number of presumptive staphylococcus colonies were streaked on Tyrpticase soy agar and incubated at 35°C for 22 ± 2 hours. The presumptive isolates were then gram stained and tested for catalase. Gram positive cocci colonies which were positive on the catalase test were confirmed as total staphylococcus bacteria. The number of gram positive cocci that were also catalase positive divided by the number of isolates from each site was considered the percentage of total staphylococci from that site. This number was then multiplied by the presumptive staphylococci count (all black, shiny colonies on VJ agar) to obtain the number of total staphylococci. These staphylococcus isolates were further tested for characteristics of *S. aureus* based on the latex agglutination and coagulase tube tests. Positive results on both tests confirmed that the isolate was *S. aureus*. All positive isolates on the latex agglutination and coagulase tube tests were confirmed by a gene test for *S. aureus* using the Gen Probe Kit which detects specific ribosomal RNA sequences unique to *S. aureus*. Approximately 50 isolates, a representative number from each beach site, were screened using the Gen Probe. The results correlated well with the coagulase test results.

B. Discussion of Total Staphylococcus Counts. Results of total staphylococcus bacteria counts from the beach sites are summarized in Table 1. Waikiki, Queens Surf,

Hanauma Bay, and Iroquois beaches had geometric mean concentrations of total staphylococcus bacteria which were above 100 CFU/100 ml, a level which was previously used (Charoenca and Fujioka, 1993) to categorize beaches with high concentrations of staphylococcus. Waikiki beach had consistently high counts above this level for all four samples, with a range of 264 to 1,264 CFU/100 ml. It was also the beach with the highest numbers of swimmers. These results support previous observations that high concentrations of staphylococcus bacteria occur at beaches with high numbers of swimmers.

Only one sample of water was obtained from Hanauma Bay and from Black Point as these were our control sites. Hanauma Bay beach is a beach with high numbers of swimmers and poor flushing capacity. The concentration of total staphylococcus bacteria in water at this beach (148 CFU/100 ml) exceeded the 100 CFU/100 ml guideline while the concentration of total staphylococcus (88 CFU/100 ml) in water from Black Point beach characterized by fewer swimmers and better flushing capacity was below the guideline number. An attempt was made to determine the concentrations of total staphylococcus bacteria in the Sand Island sewage using the modified VJ medium, although this medium was known to be unsuitable for stream water and sewage samples. When this medium was used to analyze the sewage sample, a presumptive staphylococcus count of 8.2×10^5 CFU/100 ml was obtained. However, only 1 out of 8 presumptive isolates was a gram positive coccus and 6 of these isolates were catalase positive. None of these isolates were both gram positive or catalase positive Therefore, none of these isolates were confirmed as staphylococcus bacteria. These results confirm that non staphylococcus bacteria in sewage are present in much higher concentrations than staphylococcus and the VJ medium is unsuitable to analyze sewage water. Based on the report by Ahtiainen et al (1991), one can expect a total staphylococcus count of 1,000 to 1600 CFU/100 ml with very low percentage of *S. aureus* in sewage. Dr. Hill's gene probe method may be able to provide some information on the concentrations of total staphylococcus and *S. aureus* bacteria in the Sand Island sewage sample.

Total staphylococci counts at the offshore Waikiki (W2) and offshore Ala Wai (AW2) sites were very low (<1 to 4 CFU/100 ml) compared to the near-shore sites (Table 2). Similarly, at the deep ocean sites, including the Sand Island ocean outfall (D2), total staphylococci counts were very low. It should be noted that to increase the sensitivity of the method, a total of 1000 ml or ten times the volume of water sampled at beach sites were sampled at each of the deep ocean sites to recover staphylococcus bacteria. The geometric mean concentrations of total staphylococci bacteria at all of the offshore sites, including Sand Island ocean outfall site (D2), were less than 10 CFU/100 ml.The only slightly elevated site was at the mouth of Pearl Harbor where the total staphylococcus count from the surface sample was 39.4 CFU/100 ml and most probably reflect the contribution of discharges from the Fort Kamehameha WWTP and other land-based discharges in that area.

C. Results of *S. aureus* Counts. After the total staphylococcus counts are determined, the percent of the total staphylococcus bacteria recovered which are *S. aureus*

latex agglutination and coagulase tests. The isolates of *S. aureus* were then confirmed using the Gen Probe assay, a specific molecular detection test for *S. aureus*. When these tests were applied, the geometric mean concentrations of *S. aureus* was detected only at 3 CFU/100 ml at Waikiki Beach, and only 1 CFU/100 ml at Ala Moana and Ewa beaches (Table 3). The low geometric mean concentrations of *S. aureus* reflect the fact that *S. aureus* was detected in only one of four samples from these three sites. None of the total staphylococcus isolates collected at the offshore and deep ocean sites, including the Sand Island ocean outfall could be confirmed as *S. aureus*, using the latex agglutination and coagulase tests. It should be noted that these results reflect only 1 to 4 samples per site taken over a 1-2 month period. The brief period of this study is a limitation in the interpretation of data.

D. Conclusions. The data collected show that concentrations of staphylococcus bacteria were highest at shoreline beach sites and lowest from ocean sites located between the ocean outfall and the shoreline beach sites. The actual concentrations of total staphylococcus bacteria in sewage being discharged into Mamala Bay could not be determined because the culture method used was not suitable for sewage sample. However, based on the results of sampling the ocean water sites near and away from the sewage ocean outfall site, the concentrations of total staphylococcus at these sites were very low, even when ten times the volume of water sample were analyzed. These results provide evidence that sewage being discharged into the ocean via the Sand Island Outfall is not the source of staphylococcus bacteria being recovered from the waters from shoreline beach sites. The best explanation for these results is that sewage discharged via the Sand Island Ocean Outfall is effectively diluted with sea water and ocean currents primarily transport the sewage away the beaches. Moreover, high salt concentrations, sunlight, and predation by other organisms are factors known to inactivate most of the sewage-borne microorganisms.

The concentrations of staphylococcus bacteria were consistently higher at beach sites and were higher at beach sites (Waikiki, Hanauma Bay) with greater numbers of swimmers. Since the normal habitat of staphylococcus is skin and mucus membranes of people, the best explanation is that swimmers are the major source of staphylococcus bacteria recovered from beach water samples. Moreover, based on the results of this study, only a small percentage of the total staphylococcus bacteria recovered from beach water could actually be confirmed as being the pathogenic *S. aureus*. Clinical evidence show that staphylococcus skin infections is primarily caused by *S. aureus* and not by other species of the genus *Staphylococcus* which are measured as total staphylococcus. Thus, the data collected during this short-term study, indicate that the risk of skin infections by acquiring *S. aureus* from the waters in Mamala Bay was very low.

IV. RESULTS: Fate (stability, growth, death) of *S. aureus* in Environmental Waters

A. Objectives and Experimental Design. Objective 3 of this study was included to address the specific concerns of citizens of Honolulu that sewage is entering the beaches

near Waikiki and is the source of staphylococcus bacteria in these waters or the sewage is providing the nutrients to enable *S. aureus* to multiply in the marine water and thereby increase the risk of staphylococcus infections to people who use the waters for recreational uses. Low fecal indicator bacterial counts at Waikiki Beaches indicate that measurable amounts of sewage are not directly being discharged into these beaches. However, groundwater sources with or without sewage are probably entering the beaches at Waikiki and these may be sources of nutrients being discharged into Mamala Bay. To determine whether sewage could serve as a nutrient source for the multiplication of *S. aureus*, an experiment was conducted to determine whether *S. aureus* would multiply in ocean water containing 50% sewage.

For this experiment, water samples from Black Point beach, Manoa Stream and primary sewage effluent from the Sand Island Wastewater Treatment Plant (SIWWTP) were collected and filter-sterilized through a 0.22 μm filter into a sterile flask. Samples of sewage and stream waters were pre-filtered in order to remove suspended sediments and large particles. An estuary-type water was also prepared by preparing an equal mixture (50:50) of filter-sterilized ocean and stream water. The growth of *S. aureus* was then determined in these three types of waters (ocean, stream, stream/ocean mixture) in the absence of added nutrients and in the presence of nutrients in the form of 50% sewage and 0.05 to 0.5% peptone, a nutrient used to prepare commercial bacteriological medium to culture *S. aureus*. The filter-sterilized samples were held in sterile glass beakers lined with foil and kept in the lab at room temperature (24 +/- 2°C). These samples were then seeded with approximately 10^4 cells/ml of an 18-24 hr overnight culture of *S. aureus* (ATCC: 25912). Growth was monitored over 6 days and samples were taken at 0 and 6 hours each day. Samples were assayed on VJ agar supplemented with 0.005% sodium azide and incubated at 35°C for 44 ± 4 hours.

B. Results and Discussions. The results of this experiment are tabulated in Table 4 and the results for each of the water samples graphed. The results graphed in Figure 1 show that in filter-sterilized stream water, the concentrations of *S. aureus* slowly declined when the stream water was not supplemented with any nutrients or when supplemented with 50% sewage. However, when the stream water was supplemented with 0.05% peptone, substantial growth (5 logs) of *S. aureus* was observed. Similar response was observed when the suspending medium was filter sterilized marine water (Figure 2) and filter sterilized estaurine water (Figure 3). These results indicate that stream water, estaurine waters and marine waters do not have enough nutrients to support the growth of *S. aureus*. However, *S. aureus* can readily persist in these waters for at least 24 hours. The addition of 50% sewage to these waters did not result in sufficient nutrients to allow *S. aureus* to multiply but the presence of sewage allowed *S. aureus* to persist longer in these waters indicating that the bacteria was utilizing some nutrients from the sewage. The addition of 0.05% and 0.5% peptone to these three types of waters provided sufficient nutrients required for *S. aureus* to multiply. It should be noted that peptone is the source of nutrients used to prepare culture medium for the growth of a wide variety of bacteria.

C. Conclusions. The design of this experiment was to create conditions to specifically determine whether the nutrients available will enable *S. aureus* to multiply in

environmental waters. This was done by filter sterilizing the three types of environmental waters (stream, estuary, marine) to remove indigenous microorganisms but not the nutrients in these waters. Under natural conditions high concentrations of indigenous microorganisms will be present in the test water samples and can be expected to out-compete *S. aureus* in the uptake of nutrients and may even inhibit the growth of *S. aureus* using a number of known mechanisms. Moreover, the inactivating effect of sunlight was also excluded in these experiments. Since natural environmental conditions differ from the conditions of this experiment, caution must be applied in the interpretation of the results of this experiment.

Under the conditions of this experiment, the results showed that there is insufficient nutrients in natural marine, estuary or stream waters for *S. aureus* to multiply. However, *S. aureus* is able to persist for at least 24 hours indicating the resistance of this bacteria to high salt concentrations found in marine waters. In the absence of nutrients, the concentrations of *S. aureus* in these natural waters are reduced after 24 hours. A similar reaction was observed when 50% sewage was added to the three types of environmental waters. Thus, even 50% sewage does not contain all the essential nutrients required for *S. aureus* to multiply. The presence of sewage and nutrients in sewage did enable *S. aureus* to persist longer in environmental waters. However, when 0.05% and 0.5% peptone were added to the same three types of environmental waters, *S. aureus* was able to multiply and to persist even longer. These results support data in the literature that *S. aureus* is a hardy bacteria which can survive or persist under environmental conditions. However, due to its complex growth requirements (organic nutrients), growth of this bacteria under recreational water conditions is not likely, even when recreational waters is polluted with up to 50% with sewage.

V. RESULTS: Comparative Fate of *E. coli* and *S. faecalis* in Environmental Waters

A. Objectives and Experimental Design. The presence of sewage in environmental waters has traditionally been measured based on the concentrations of fecal bacteria such as *E. coli* and *S. faecalis*. Considerable more data is available on the concentrations of fecal bacteria in environmental waters than other kinds of bacteria such as *S. aureus*. Objective 4 of this study was to determine the fate of *E. coli* and *S. faecalis*, in the same three types of water under the same set of conditions as *S. aureus* so water quality data obtained based on concentrations of *E. coli* and *S. faecalis* can be applied to *S. aureus*.

To address objective 4 of this study, the same methodology and experimental design used for monitoring the growth of *S. aureus* was used. Filter-sterilized stream and ocean water samples were seeded with *E. coli* and *S. faecalis* (10^5 cells/ml for each bacterium) and monitored for growth over a period of 4 days. A 50:50 stream-ocean mixture was also prepared and seeded with the same bacteria to simulate an estuary-type water. The seeded samples were kept in the laboratory at room temperature (25°C) during the study period. The membrane filter technique was used to assay the samples. *E. coli* was recovered on *m*FC and incubated at 35°C for 24 ± 4 hours. This was done in order to

recover some of the stressed bacteria which would not have survived the standard temperature of 44.5°C. *S. faecalis* was recovered on *m*E and incubated at 41°C ± 4 hours 44 ± 4 hours.

B. Results and Discussions. The results of this experiment are tabulated in Table 5 and the results for the individual water samples graphed in Figures 4-9. The results for *E. coli* (Figure 4) and *S. faecalis* (Figure 7) in ocean waters show that in unsupplemented ocean water both *E. coli* and *S. faecalis* did not grow but remained stable over the 4 day period. These results show that ocean water does not contain sufficient nutrients to support the multiplication of *E. coli* and *S. faecalis* and the high salt concentrations of ocean water did not inactivate these two fecal indicator bacteria. However, when this ocean water was supplemented with 50% sewage or with 0.05% and 0.5% peptone, multiplication of both *E. coli* and *S. faecalis* was evident. These results indicate that 50% sewage and as well as 0.05% peptone provided enough nutrients for the growth of both *E. coli* and *S. faecalis* in ocean water.

The same experiment was conducted in stream water and a 50:50 mixture of stream water and ocean water. Stream water is considered a better environment than ocean water because its low salt concentrations will provide less stress to the bacteria and because stream water can be expected to contain more nutrients than ocean water. In unsupplemented stream water, some multiplication of *E. coli* (Figure 5) was observed while no multiplication of *S. faecalis* (Figure 8) was observed under these same conditions. These results indicate that stream water contains sufficient nutrients to allow *E. coli* but not *S. faecalis* to multiply in the absence of indigenous microorganisms. These results support previous reports that *E. coli* has simple nutritional requirements for growth while *S. faecalis* has more complex nutritional requirements for growth. When stream water was supplemented with 50% sewage or peptone, multiplication of both *E. coli* and *S. faecalis* was evident showing that nutrients in the form of 50% sewage and peptone will enhance the multiplication of both of these fecal indicators in stream water. Similar results were obtained when the water used was a 50:50 mixture of stream water and ocean water (Figures 6 and 9).

C. Conclusions. The conditions of this experiment were similar to the conditions of the previous *S. aureus* experiment and therefore the same precautions established for interpreting the results of the previous *S. aureus* experiment apply to this set of experiment. Although the results of this test do not reflect conditions under natural environment, the results measures the ability of *E. coli* and *S. faecalis* to utilize nutrients and multiply in environmental waters. Moreover, the results of this experiment should be comparable to the previous *S. aureus* experiment.

The following conclusions can be drawn from the results. Ocean water did not contain enough nutrients to allow *E. coli* and *S. faecalis* to grow whereas stream water contained enough nutrients to allow *E. coli* but not *S. faecalis* to grow. The addition of 50% sewage or 0.05% peptone to ocean water or stream water resulted in sufficient nutrients to allow both of the fecal indicator bacteria to grow. Since *S. aureus* did not

grow in these same waters even when 50% sewage was added, it can be concluded that the nutritional requirement for growth of *E. coli* is the simplest, followed by *S. faecalis* and that *S. aureus* has the most complex growth requirements. These results are consistent with previous reports which have documented the simple growth requirements of *E. coli*, the more complex growth requirement of *S. faecalis* and the very complex growth requirements of *S. aureus*. Based only on these data, one can conclude that the impact of nutrients in environmental waters will allow *E. coli* to multiply first, followed by *S. faecalis* and lastly *S. aureus*. However in natural waters, indigenous microorganisms will outcompete *S. aureus* and fecal bacteria for available nutrients. The natural stability of bacteria to environmental factors also determines the final concentrations of bacteria in water. In this regard, it has been reported that *E. coli* is highly susceptible to inactivation by environmental factors (high salt, sunlight, predation, disinfection) whereas *S. faecalis* and *S. aureus* are known to be more resistant to these environmental factors. The principle of microbial ecology states that microorganisms whose natural habitat is not environmental waters will generally not be able to multiply in environmental waters unless they can adapt to environmental conditions.

VI. RESULTS: Effects of Nutrients on Sunlight Inactivation of *S. aureus*.

A. Objective and Experimental Design. We previously determined that sunlight is a natural environmental agent which inactivated many bacteria, including *S. aureus*. However, in past experiments, the inactivating effect of sunlight was determined on *S. aureus* suspended in clean water samples. The results of this study showed that the presence of nutrients in environmental waters enabled *S. aureus* to persist longer (50% sewage) and to even multiply (0.05% peptone). The objective of this experiment was to determine whether the presence of these kinds of nutrients, which generally follow a pollution event, will prevent or retard the inactivating effect of sunlight.

In this experiment, ocean water from Black Point Beach was filter-sterilized and used to prepare the following three types of samples: a) unsupplemented ocean water, b) ocean water supplemented with 50% sewage, and c) ocean water supplemented with 0.05% peptone. All three water samples were clear to the naked eye. *S. aureus* was added to all three types of water. One set of these 1 liter samples were kept under laboratory conditions in the absence of sunlight and the concentrations of *S. aureus* determined at time 0 and after six hours in the absence of sunlight. The second set of these samples were placed into beakers with a magnetic stirrer and the samples continuously exposed to sunlight on the roof of Holmes Hall. As in previous sunlight experiments, the beakers were placed in a water bath to keep the water sample cool. Samples were obtained after the first 30 minutes exposure to sunlight and hourly over a four-hour period. All samples were measured for concentrations of *S. aureus* using the membrane filtration method and the modified VJ medium as previously described.

B. Results and Discussions. The results of this experiments are summarized in Table 6 and the data graphed in Figure 10. The results show that the concentrations of *S.*

aureus suspended in all three types of waters remained unchanged when held in the absence of sunlight indicating that ocean water itself did not inactivate this bacteria even after 6 hours. In contrast, the population of *S. aureus*, suspended in all three types of water was effectively inactivated after 1-2 hour exposure to sunlight. The inactivation of *S. aureus* was most rapid in clean ocean water indicating that nutrients such as 50% sewage and 0.05 peptone slightly reduces the rate of sunlight inactivation. However, since these nutrients only slightly reduced the rate of sunlight inactivation, it can be concluded that nutrients in the form of contamination are insufficient to protect against the inactivating effect of sunlight.

C. Conclusions. The results of this experiment confirm our previous findings that *S. aureus* is susceptible to sunlight inactivation and demonstrated that sunlight inactivation even occurred when the ocean water was supplemented with enough nutrients to maintain cell structure (50% sewage) and to allow for the multiplication of *S. aureus* (0.05% peptone). These results provide evidence that natural sunlight is an effective mechanism for the inactivation of many types of bacteria suspended in marine waters.

VII. FINAL ASSESSMENT AND RECOMMENDATIONS

In Phase I of this study, the data and conclusions were based on using standard cultural techniques to enumerate bacteria which are viable and able to be cultured on bacteriological medium. These are the kinds of methods used in clinical laboratory and in water monitoring programs to enumerate and identify bacteria such as *S. aureus*. These methods are used to determine whether water quality standards are met and used to assess the risks to human infection. However, it is well known that cultural techniques recover the more healthy bacteria and bacteria which are injured, starved or have converted to some survival form may not or will not be detected by cultural methods. The presence and significance of bacterial forms which are non-culturable will be addressed in Phase II of this study.

Recognizing that people do acquire staphylococcus skin infections and that this study did not determine how people are becoming infected, the following recommendations are made:

1. The development of isolation and enumeration methods for staphylococcus bacteria and for *S. aureus* which is reliable, feasible and which can be applied to all types of samples (sewage, feces, stream water, storm drain water, estuary water, ocean water).

2. The fate of total staphylococcus and *S. aureus* in sewage, stream, storm drain before and after it enters the natural ocean environment should be determined.

3. The concentrations of total and *S. aureus* in sand above the water line and under the water in swimming area should be determined as these sites provide opportunities for human infection.

4. The contribution of total staphylococcus and *S. aureus* by feces and bodies of pigeons who frequent the beach should be determined.

5. The contribution of total staphylococcus and *S. aureus* being washed off the bodies of humans entering the coastal water should be determined.

6. A study should focus on people who chronically obtain staphylococcus skin infections following recreational use of water for the purpose of determining the mechanisms by which these people acquire staphylococcus skin infections. Only after the mechanism(s) of acquiring *S. aureus* skin infection are known can effective preventive management decisions be made. At the present time, the best approach may be to provide showers so swimmers will shower before they enter coastal waters. Showering will wash off most of the bacteria on the bodies of people. This guideline is already instituted for swimming pools.

7. Characterization of the biotype as well as the virulence factors of *S. aureus* recovered from people's normal skin, from infected skin and from environmental sources (water, sand) should be determined and compared. Simpler methods should be developed for this characterization.

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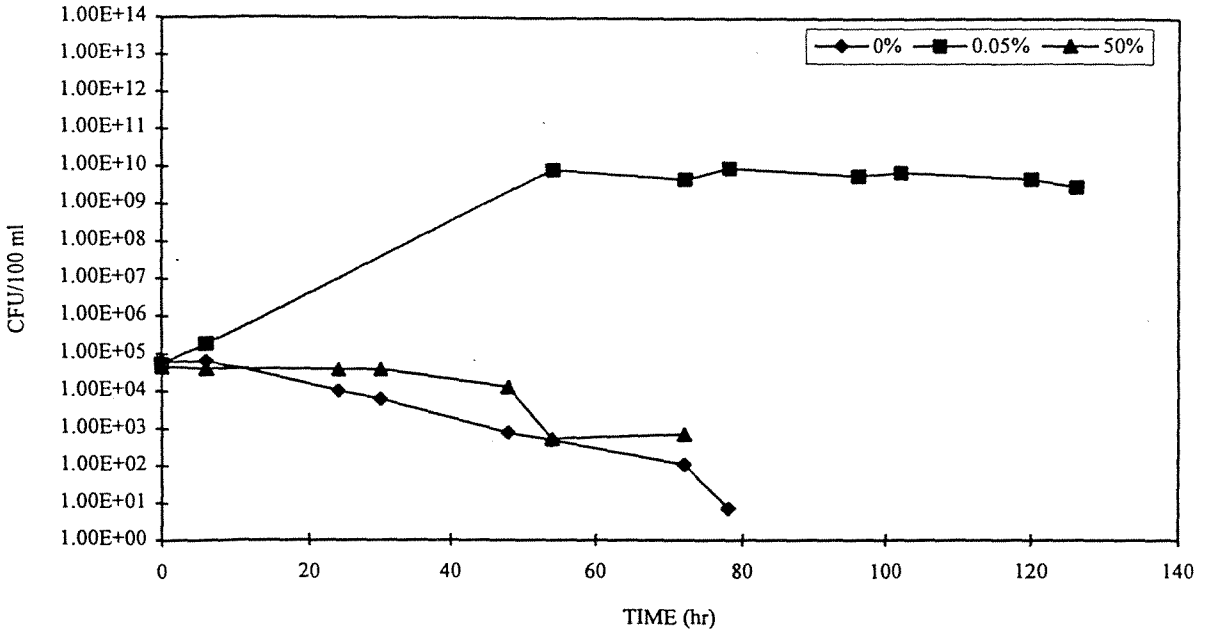

Figure 1. Growth of *S. aureus* in filter-sterilized stream water supplemented without nutrients (0%), with peptone (0.05%), and with filter-sterilized sewage (50%).

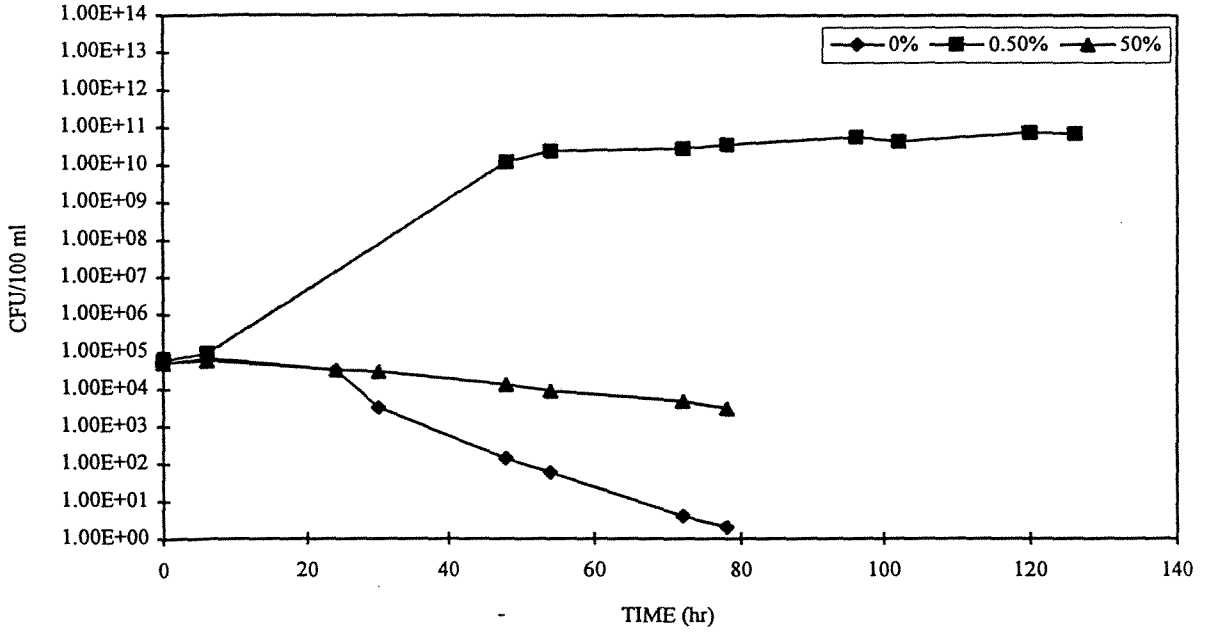

Figure 2. Growth of *S. aureus* in filter-sterilized marine water supplemented without nutrients (0%), with peptone (0.5%), and with filter-sterilized sewage (50%).

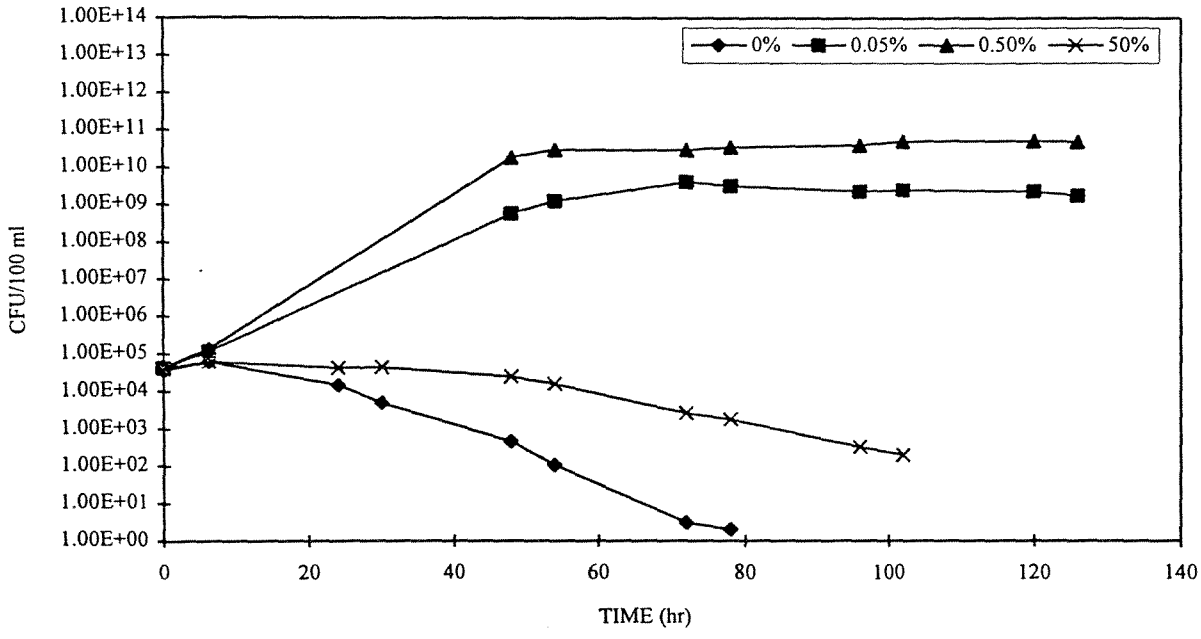

Figure 3. Growth of *S. aureus* in filter-sterilized stream-ocean water mixture (50:50) supplemented without nutrients (0%), with peptone (0.05% and 0.5%), and with filter-sterilized sewage (50%).

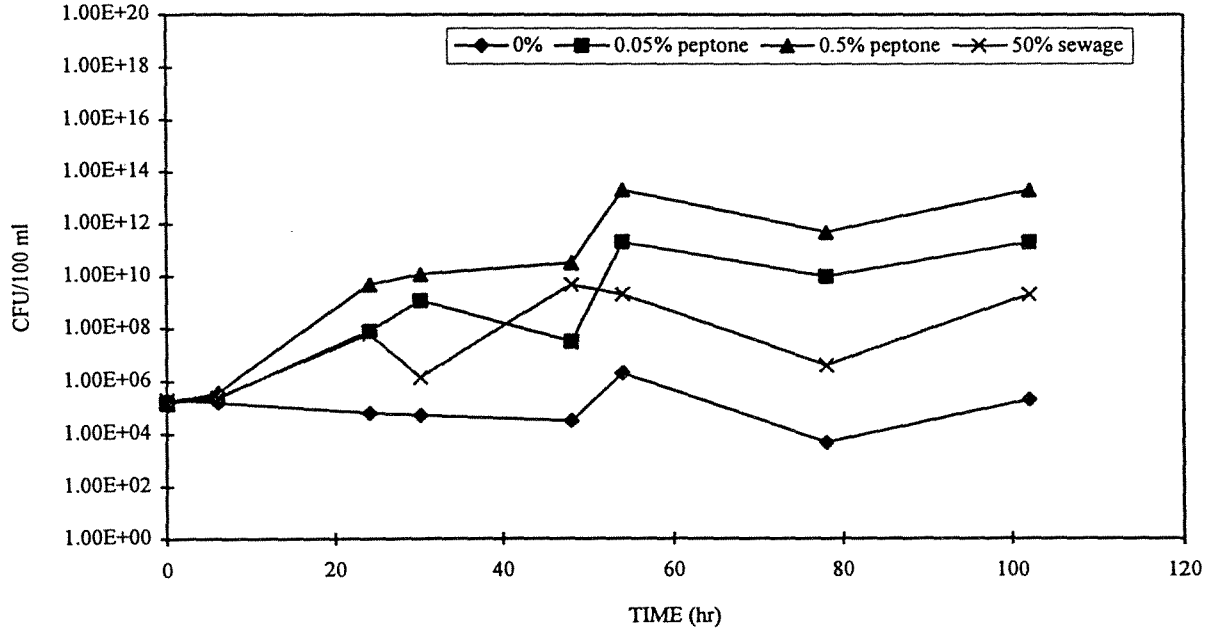

Figure 4. Growth of *E. coli* in filter-sterilized ocean water supplemented without nutrients (0%), with 0.05% and 0.5% peptone, and with 50% filter-sterilized sewage.

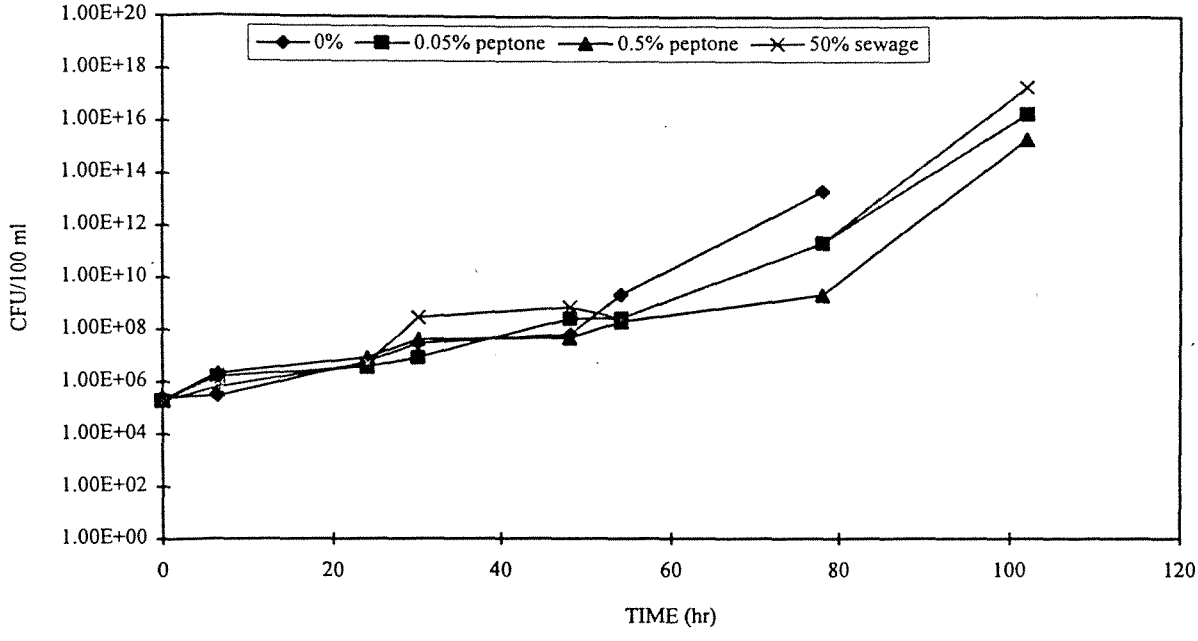

Figure 5. Growth of *E. coli* in stream water supplemented without nutrients (0%), with 0.05% and 0.5% peptone, and with 50% filter-sterilized sewage.

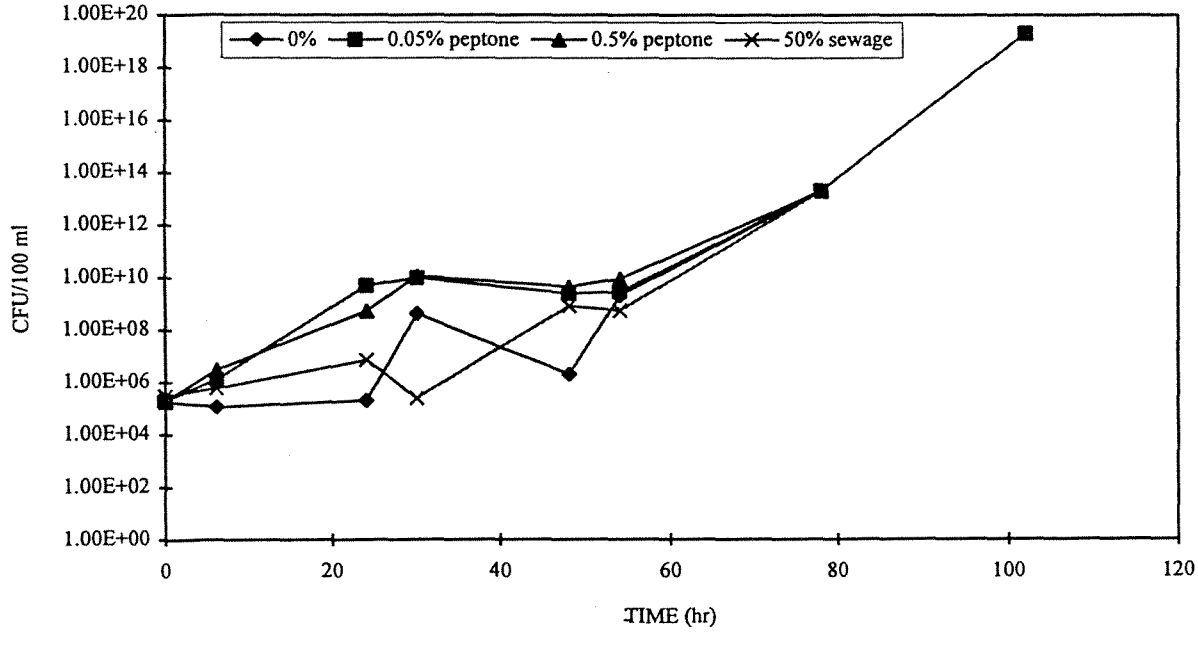

Figure 6. Growth of *E. coli* in stream-ocean water (50:50) supplemented without nutrients (0%), with 0.05% and 0.5% peptone, and with 50% filter-sterilized sewage.

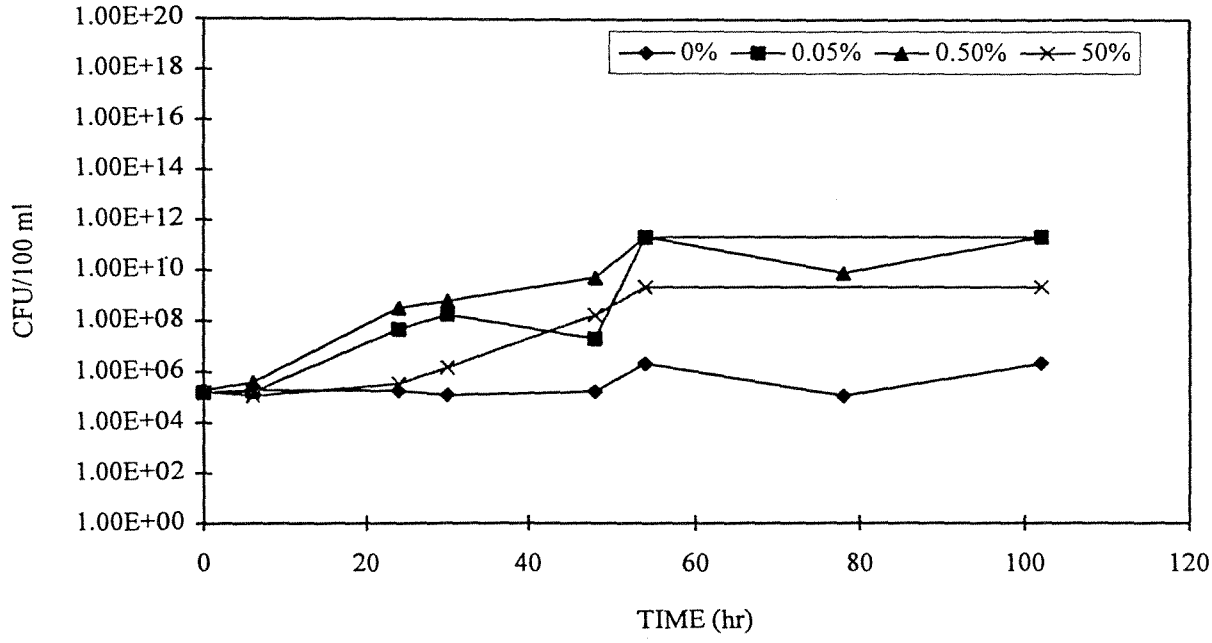

Figure 7. Growth of *S. faecalis* in ocean water supplemented without nutrients (0%), with 0.05% and 0.5% peptone, and with 50% filter-sterilized sewage.

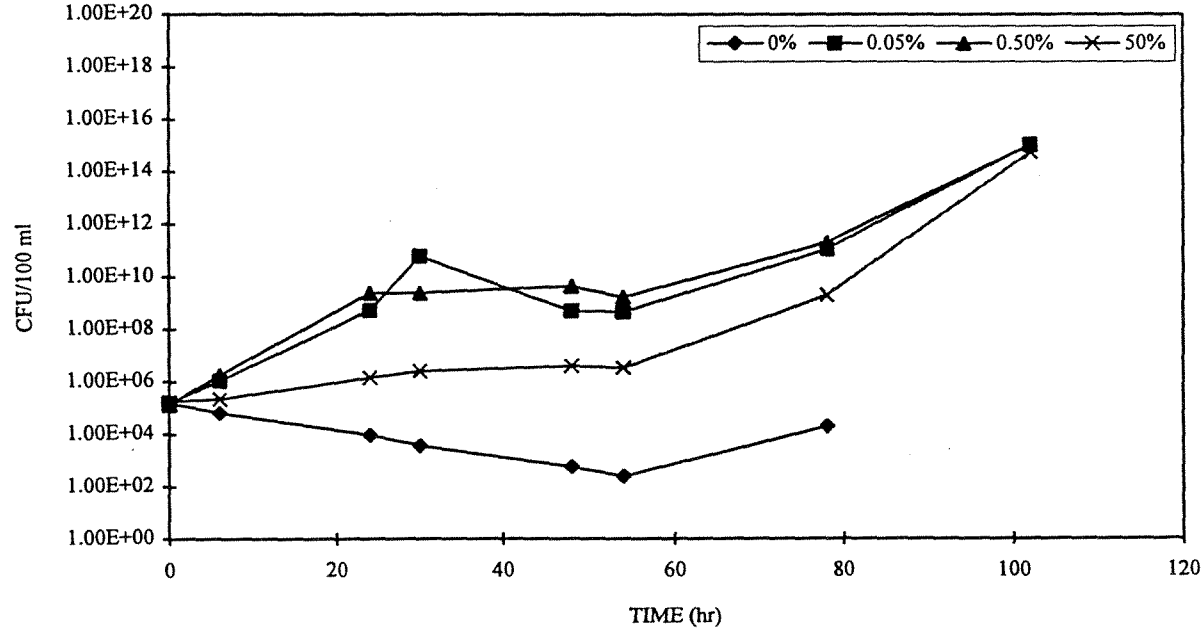

Figure 8. Growth of *S. faecalis* in stream water supplemented without nutrients (0%), with 0.05% and 0.5% peptone, and with 50% filter-sterilized sewage.

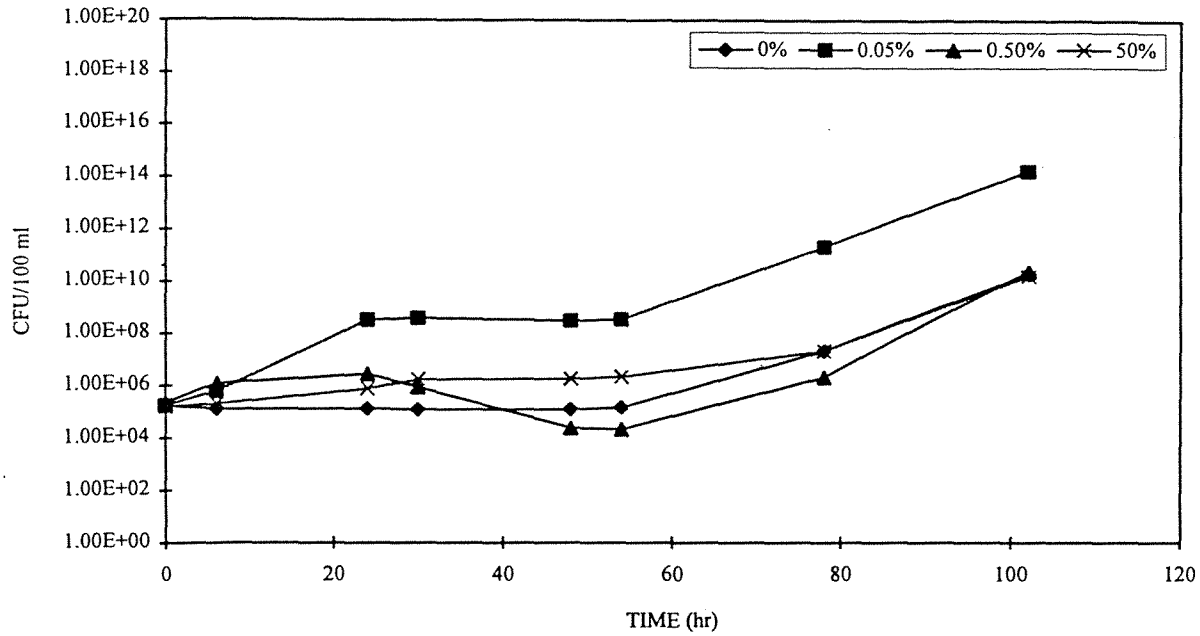

Figure 9. Growth of *S. faecalis* in stream-ocean water (50:50) supplemented without nutrients (0%), with 0.05% and 0.5% peptone, and with 50% filter-sterilized sewage.

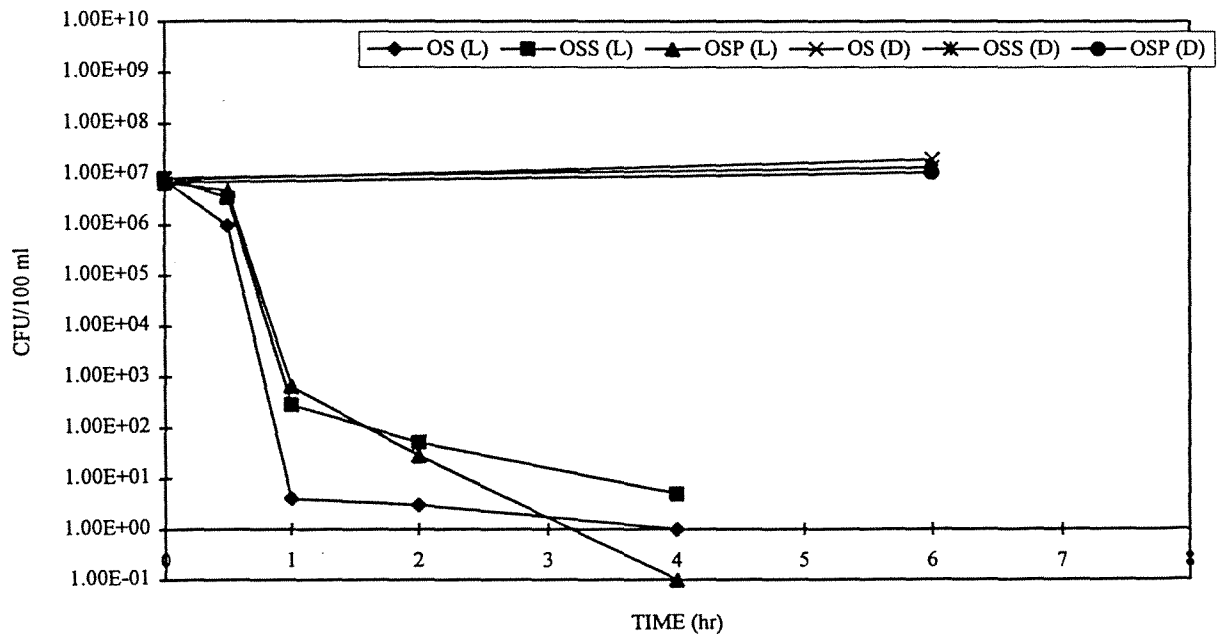

Figure 10. Inactivation of *S. aureus* in ocean water (OS-L), ocean water containing 50% filter sterilized sewage (OSS-L), and ocean water containing 0.05% peptone (OSP-L) when exposed to sunlight after 4 hours versus dark control (OS-D, OSS-D, and OSP-D).

Table 1.
Geometric mean and range of total staph bacteria, temperature, salinity, turbidity and pH in selected beach sites, Ala Wai Canal and primary effluent from Sand Island Wastewater Treatment Plant (SWWTP).

Site	Code	N	CFU/100 ml	Temperature (^{0}C)	Salinity (ppt)	Turbidity (NTU)	pH
Queens Surf Beach	QB1	4	123 (70-260)*	27.7 (27-29)	35.2 (35-36)	3.53 (2.2-4.9)	8.41 (8.24-8.58)
Waikiki Beach	W1	4	484 (264-1248)	28.4 (27-29.5)	34.7 (34-35)	3.62 (3.2-4.5)	8.46 (8.26-8.66)
Ala Wai Canal	AW1	4	21 (2-105)	27.9 (26-30)	26.6 (23-31)	2.19 (1.2-6.9)	8.22 (8.16-8.28)
Alamoana Beach	AM1	4	65 (53-108)	27.9 (26-29)	35 (35)	4.59 (3.4-6.2)	8.34 (8.31-8.37)
Sand Island Beach	SB1	4	39 (7-236)	27.4 (26-30)	34.7 (34-35)	3.82 (2.5-7.9)	8.33 (8.31-8.36)
Iroquios Beach	EW1	4	145 (16-500)	27.6 (26-29)	33.9 (32-35)	3.29 (1.5-15)	8.36 (8.34-8.39)
Ewa Beach	EW2	4	12 (2-908)	27.3 (26-29.5)	34.5 (34-35)	8.94 (4-18)	8.31 (8.29-8.34)
Hanauma Bay	HB1	1	148	27	35	2.00	8.26
Black Point	BP1	1	88	29	34	1.5	8.63
Primary Effluent (SWWTP)	S1	1	820000	ND	ND	ND	ND

NOTE: N = number of samples collected.
* = range of staph bacteria concentration and other water quality parameters.

Table 2.
Concentration of presumptive staph bacteria, salinity, turbidity, temperature and pH in selected offshore and deep ocean sites, including Sand Island ocean outfall.

Site	Code	N	CFU/100 ml	Temperature (^{0}C)	Salinity (ppt)	Turbidity (NTU)	pH
Diamond Head	E4S	1	.6	28	34	0.25	8.35
	E4M	1	0	28	35	0.30	8.39
	E4B	1	5.4	28	35	0.32	8.33
Waikiki - Offshore	W2S	1	3.9	29	35	0.65	8.30
	W2B	1	.1	29	35	0.70	8.32
Ala Wai - Offshore	AW2S	1	.1	31	34	0.77	8.28
	AW2M	1	.8	30	35	0.55	8.32
	AW2B	1	.2	31	35	0.51	8.29
Sand Island Outfall - inshore	D1S	1	1.4	30	35	0.71	8.28
	DIM	1	6.9	29	35	0.64	8.38
	D1B	1	6.7	29	35	0.52	8.43
Sand Island Outfall	D2S	1	4.7	29	36	0.59	8.46
	D2M	1	.3	28	36	0.49	8.45
	D2B	1	5.1	27	36	0.47	8.43
Sand Island Outfall - beyond	D3S	1	0	28	36	0.42	8.56
	D3M	1	.5	27	36	0.65	8.55
	D3B	1	.3	26	36	0.40	8.58
Upper - Pearl Harbor	C1S	1	.8	29	36	0.83	8.26
	C1M	1	.7	28.5	36	1.10	8.28
	C1B	1	8	28	35	4.90	8.26
Mid - Pearl Harbor	C2S	1	.9	29	35	0.95	8.24
	C2B	1	.4	28.5	35	0.89	8.24
Mouth of Pearl Harbor	C3S	1	39	29	35	1.00	8.22
	C3B	1	.6	28	36	0.84	8.18
Keehi Lagoon	D0S	1	0	30	35	2.20	8.21
	D0B	1	0	29	35	2.40	8.22

NOTE: S = surface
M = middle
B = bottom
N = number of samples collected.

Table 3.
Geometric mean concentration of presumptive staph†, total staph‡, and *S. aureus* in selected beach sites.

Site	Code	N	Presump. Staph	Total Staph	*S. aureus*
Queens Surf Beach	QB1	4	130 (78-260)*	123 (70-260)	0
Waikiki Beach	W1	4	484 (264-1248)	484 (264-1248)	3 (0-138)
Ala Wai Canal	AW1	4	34 (2-140)	21 (2-105)	0
Alamoana Beach	AM1	4	65 (53-108)	65 (53-108)	1 (0-11)
Sand Island Beach	SB1	4	39 (7-236)	39 (7-236)	0
Iroquios Beach	EW1	4	145 (16-500)	145 (16-500)	1 (0-6)
Ewa Beach	EW2	4	13 (2-908)	12 (2-908)	0
Hanauma Bay	HB1	1	148	148	0
Black Point	BP1	1	88	88	0
Primary Effluent (SWWTP)	S1	1	820000	0£	ND

NOTE: † = total counts of black, shiny colonies on VJ media.
‡ = total counts of positive isolates of black colonies which were gram positive cocci and catalase positive.
£ = 1 out of 8 isolates was a gram positive coccus but 6 out of 8 was catalase positive; none of the isolates were both gram positive cocci and catalase positive.
* = range of bacteria counts.
N = number of samples collected.
ND = not done.

Table 4.
Growth of *S. aureus* in filter-sterilized stream, ocean, and 50:50 mixture of stream and ocean water supplemented with no nutrients (0%), 0.05% and 0.5% peptone, and 50% filter-sterilized sewage.

	Stream			Ocean			Stream/Ocean (50:50)			
	Untreated	Peptone	Sewage	Untreated	Peptone	Sewage	Untreated	Peptone	Peptone	Sewage
TIME (hr)	0%	0.05%	50%	0%	0.5%	50%	0%	0.05%	0.5%	50%
0	5.92E+04	5.40E+04	4.52E+04	4.8E+04	6E+04	5.08E+04	3.52E+04	4.16E+04	4.32E+04	4.04E+04
6	6.32E+04	1.80E+05	4.76E+04	6.8E+04	9.2E+04	5.84E+04	6E+04	1.12E+05	1.32E+05	6E+04
24	1E+04	>2.00E+07	4.04E+04	3.2E+04	>2.00E+07	3.32E+04	1.44E+04	>2.00E+07	>2.00E+07	4.16E+04
30	6E+03	>2.00E+07	3.8E+04	3.2E+03	>2.00E+07	3E+04	4.8E+03	>2.00E+07	>2.00E+07	4.36E+04
48	7.6E+02	6E+09	1.24E+04	1.41E+02	1.2E+10	1.36E+04	4.38E+02	5.5E+08	1.8E+10	2.36E+04
54	4.73E+02	8E+09	6E+03	59	2.36E+10	9.2E+03	1.03E+02	1.2E+09	2.9E+10	1.48E+04
72	1.05E+02	4.52E+09	5.2E+02	4	2.79E+10	4.88E+03	3	4E+09	2.9E+10	2.48E+03
78	7	9.04E+09	6.8E+02	2	3.56E+10	3.04E+03	2	3.1E+09	3.4E+10	1.68E+03
96	ND	5.6E+09	ND	ND	5.7E+10	ND	ND	2.2E+09	3.9E+10	3.12E+02
102	ND	7E+09	ND	ND	4.3E+10	ND	ND	2.4E+09	4.9E+10	1.88E+02
120	ND	4.6E+09	ND	ND	7.5E+10	ND	ND	2.2E+09	5E+10	ND
126	ND	2.8E+09	ND	ND	6.9E+10	ND	ND	1.7E+09	4.8E+10	ND

NOTE: ND = not done.

Table 5.
Growth of *E. coli* and *S. faecalis* in stream, ocean, and stream-ocean mixture supplemented without nutrients, with 0.05% and 0.5% peptone, and with 50% filter-sterilized sewage.

TIME (hr)	CONC.	STREAM		OCEAN		STREAM-OCEAN	
		E. coli	*S. faecalis*	*E. coli*	*S. faecalis*	*E. coli*	*S. faecalis*
0	0%	2.48E+05	1.48E+05	1.92E+05	1.48E+05	1.76E+05	1.68E+05
	0.05%	1.96E+05	1.52E+05	1.52E+05	1.56E+05	1.92E+05	1.72E+05
	0.5%	2E+05	1.32E+05	1.48E+05	1.96E+05	1.88E+05	2.2E+05
	50%	1.8E+05	1.72E+05	2.04E+05	1.64E+05	3.04E+05	1.52E+05
6	0%	2.92E+05	6.4E+04	1.56E+05	1.92E+05	1.12E+05	1.28E+05
	0.05%	1.6E+06	1.04E+06	2.32E+05	1.64E+05	1.28E+06	6E+05
	0.5%	2.16E+06	1.72E+06	3.68E+05	3.84E+05	2.96E+06	1.2E+06
	50%	6.4E+05	2.16E+05	2.56E+05	1.12E+05	5.76E+05	2E+05
24	0%	5.68E+06	9.2E+03	6.2E+04	1.7E+05	2E+05	1.3E+05
	0.05%	3.6E+06	5.3E+08	8E+07	4.4E+07	4.8E+09	2.96E+08
	0.5%	8E+06	2.4E+09	4.8E+09	3.12E+08	5.28E+08	2.68E+06
	50%	5E+06	1.4E+06	6E+E07	3.2E+05	6.88E+06	7.2E+05
30	0%	2.8E+07	3.6E+03	5.08 E+04	1.2E+05	4.16E+08	1.2E+05
	0.05%	8E+06	6E+10	1.16 E+09	1.68E+08	9.6E+09	3.6E+08
	0.5%	4E+07	2.4E+09	1.2 E+10	5.72E+08	1.12E+10	8.4E+05
	50%	2.8E+08	2.48E+06	1.4 E+06	1.44E+06	2.4E+05	1.64E+06
48	0%	6E+07	5.56E+02	3.2 E+04	1.64E+05	2E+06	1.2E+05
	0.05%	2.4E+08	4.92E+08	3.2 E+07	1.87E+07	2.4E+09	2.8E+08
	0.5%	4.4E+07	4.4E+09	3.32 E+10	4.8E+09	4.4E+09	2.4E+04
	50%	6.56E+08	3.84E+06	4.8 E+09	1.6E+08	8.04E+08	1.68E+06
54	0%	2E+09	2.48E+02	2 E+06	> 2E+06	2E+09	1.4E+05
	0.05%	2.44E+08	4.52E+08	2E+11	> 2E+11	2.8E+09	3.16E+08
	0.5%	1.88E+08	1.68E+09	2E+13	> 2E+11	8.8E+09	2.16E+04
	50%	2.48E+08	3.36E+05	2E+09	> 2E+09	5.44E+08	2.04E+06
78	0%	2E+13	> 2E+04	4.8E+03	1.06E+05	> 2E+13	> 2E+07
	0.05%	2E+11	1.12E+11	9.6E+09	ND	> 2E+13	> 2E+11
	0.5%	2E+09	> 2E+11	4.8E+11	7.2E+09	> 2E+13	> 2E+06
	50%	2E+11	> 2E+09	4E+06	ND	> 2E+13	> 2E+07
102	0%	> 2E+05	ND	> 2E+05	> 2E+06	> 2E+19	1.76E+10
	0.05%	1.84E+16	1.07E+15	> 2E+11	> 2E+11	> 2E+19	1.68E+14
	0.5%	> 2E+15	1.05E+15	> 2E+13	> 2E+11	> 2E+19	2.28E+10
	50%	> 2E+17	5.52E+14	> 2E+09	> 2E+09	> 2E+19	1.48E+10

NOTE: CONC. = concentration of treatments.
ND = not done.

Table 6.
Inactivation of *S. aureus* in ocean water (OS), ocean water containing 50% sewage (OSS) and ocean water containing 0.5% peptone (OSP) when exposed to sunlight compared to the dark control..

	CFU/100 ml					
	SUNLIGHT			DARK CONTROL		
TIME (hr)	OS	OSS	OSP	OS	OSS	OSP
0	7.6E+6	8.4E+6	6.8E+6	7.6E+6	8.4E+6	6.8E+6
0.5	9.6E+5	3.48E+6	4.8E+6	ND	ND	ND
1	4	280	640	ND	ND	ND
2	3	51	28	ND	ND	ND
4	1	5	0	ND	ND	ND
6	ND	ND	ND	1.96E+7	1.36E+7	1.08E+7

NOTE: OS = ocean + staph
OSP = ocean + staph + 0.05% peptone
OSS = ocean + staph + 50% sewage
ND = not done

PHASE II REPORT

TITLE: Molecular Detection of *Staphylococcus aureus* in Waters of Mamala Bay, Hawaii

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PROJECT PERIOD: August 1, 1995 to December 31, 1995

I. INTRODUCTION AND DESCRIPTION OF MOLECULAR ASPECTS OF STAPHYLOCOCCUS STUDY

It has previously been established that skin infections caused by *S. aureus* are strongly associated with exposure to marine waters and there is good evidence that marine recreational waters serve as the transmission medium for *S. aureus* on Hawaiian beaches (Charoenca and Fujioka, 1993; 1994). In view of the importance of *S. aureus* on bathing beaches in Hawaii and because this organism has not been studied by any investigators in the MB-7 section of the Mamala Bay Study, we proposed to investigate the presence of this pathogen in water samples from Mamala Bay by using molecular approaches. In addition, we characterized *Staphylococcus* isolates obtained by Dr. Fujioka to confirm identification as *S. aureus* and to determine their pathogenic potential. A comprehensive general introduction to *Staphylococcus* skin infections and the particular concerns to be addressed

by this Supplemental *Staphylococcus* Study is given by Dr. Fujioka in his report on Phase I of this study.

Two molecular approaches were used in this investigation for detection *of S. aureus*. In the first approach, *S. aureus* was detected by polymerase chain reaction (PCR) amplification of the *nuc* gene, as described by Brakstad et al. (1992). The *nuc* gene encodes an extracellular thermostable nuclease and this gene has been cloned, sequenced (Kovacevic et al. 1985) and found to have species-specific sequences suitable for identification of *S. aureus* (Liebl et al., 1987). *S. aureus* strains produce the extracellular thermostable nuclease encoded by the *nuc* gene with a frequency similar to that at which they produce coagulase (Madison and Baselski, 1983). This PCR-based approach was selected to ensure detection of all *S. aureus* cells, including those that may be present in a viable but nonculturable state (Roszak and Colwell, 1987) (Section IV).

The second approach was designed to detect culturable *S. aureus* by plating on non-selective medium. This approach was quantitative and was undertaken to ensure maximal recovery of culturable *S. aureus* cells since selective media, which may inhibit stressed cells, were avoided. Colonies growing on nonselective medium were transferred to filters and probed with a putative *S. aureus*-specific gene probe derived from the *nuc* gene (Section V).

Specific PCR amplification of the *S. aureus nuc* gene, used for detection of *S. aureus* in environmental samples, was also used to confirm the identity of putative *S. aureus* isolates obtained by Dr. Fujioka's group (Section VI). Conventional confirmation is based on latex agglutination and coagulase tube tests, which can be somewhat subjective and difficult to interpret. A molecular technique such as detection of the *S. aureus*-specific *nuc* gene, was expected to give very accurate identification of isolates.

In addition to causing skin infections, *S. aureus* is an important cause of food poisoning, has been associated with toxic shock syndrome, and can cause other serious infections including endocarditis and osteomyelitis. Some *S. aureus* strains produce one or more staphylococcal enterotoxins and toxic shock syndrome toxin 1 (TSST-1) (Neill et al., 1990). We investigated the pathogenic potential of selected *S. aureus* isolates obtained from Hawaiian waters by PCR analysis for detection and differentiation of *S. aureus* strains containing genes for enterotoxins A, B, and TSST-1 (Johnson et al., 1991) (Section VII).

An important consideration in transmission of *S. aureus* in marine waters is survival of the organism and its entry into the viable but nonculturable state. We investigated the survival of *S. aureus* and monitored its entry into the viable but nonculturable state under optimal conditions i.e. in the absence of sunlight and other bacteria and zooplankton that may inhibit or consume the *S. aureus* cells (Section VIII).

II. OBJECTIVES OF PHASE II STAPHYLOCOCCUS STUDY

The general objectives of this Supplemental Staphylococcus Study are outlined in the Phase I Report (Dr. Fujioka). The specific objectives of the Phase II study were:

1. To coordinate and plan a field experimental study with Dr. Fujioka to sample all sites routinely monitored by the MB-7 Project group during the quarterly sampling periods.

2. To use PCR, the most sensitive technique currently available for detection of pathogens in the environment, to determine the presence or absence of *S. aureus* in water samples from Mamala Bay. The distribution pattern of this organism was expected to be useful in concluding whether *S. aureus* was present in Mamala Bay as a result of inputs of *S. aureus* in sewage discharged at the Sand Island outfall.

3. To use the most sensitive culture-based technique currently available (detection of *S. aureus* colonies on nonselective medium by using gene probes) for accurate quantification of culturable *S. aureus*. A positive PCR result and a negative culture-based result in a particular sample would indicate the presence of *S. aureus* but an inability to culture the *S. aureus* cells, suggesting that the cells are present in a viable but nonculturable state. Therefore, comparison of culturable *S. aureus* counts obtained by this culture-based approach (and by Dr. Fujioka's enumeration on selective medium) with PCR detection of *S. aureus* would indicate whether this organism was present in the environment in the viable but nonculturable state

4. To analyze putative *S. aureus* isolates obtained by Dr. Fujioka's group by using PCR amplification of *S. aureus*-specific *nuc* gene sequences.

5. To analyze all confirmed *S. aureus* isolates obtained from Dr. Fujioka for the presence of enterotoxin genes, thereby elucidating the pathogenic potential of these environmental *S. aureus* isolates.

6. To investigate the long term survival and entry into the viable but nonculturable state of *S. aureus* in seawater.

III. SAMPLING.

Sampling was conducted at stations in Mamala Bay from 11 September to 15 September, 1995. Stations sampled were those routinely monitored during the quarterly samplings of the MB-7 Research Team. The sampling procedure followed was identical to that described in Hill, 1995. Sampling was done in close collaboration with Dr. Roger Fujioka. Total bacterial counts in water samples were determined by the DAPI direct counting procedure (Coleman, 1980) and counts are given in Table 1. Bacterial counts are generally similar to those obtained during dry sampling periods in the main MB-7 study.

IV. PCR DETECTION OF *S. aureus*

A. Objective and Experimental Design. PCR detection of *S. aureus*-specific *nuc* gene sequences was done on DNA extracted from Mamala Bay water samples. The distribution pattern of this organism revealed by this PCR analysis was expected to be useful in concluding whether *S. aureus* cells were entering Mamala by the ocean sewage outfall.

Essential first steps in PCR detection of bacteria in environmental samples are obtaining representative DNA samples from the total bacterial community and purifying DNA samples to remove compounds that inhibit PCR. The DNA extraction procedure of Somerville et al. (1989) was used in this study and, in general, gave good yields of DNA. This DNA was purified by the procedure described by Boccuzzi et al. (1995), prior to PCR. Suitable PCR primers and an internal probe were constructed in our molecular core facility and used for PCR detection of the *nuc* gene. PCR was performed as described by Brakstad et al. (1992) and PCR products were resolved by agarose gel electrophoresis. PCR products were transferred to membranes by the Southern hybridization procedure and *S. aureus nuc* gene products were confirmed by hybridization with an internal probe specific for the *S. aureus nuc* gene, as described by Brakstad et al. (1992). Samples for which PCR with *nuc*-specific primers gave a ca. 270-bp-amplification product, which hybridized with the specific internal probe on Southern analysis, were concluded to be positive for *S. aureus*.

B. Results and Discussion. All samples analyzed, expect one from a station in Pearl Harbor (C2), were positive for *S. aureus* (Fig. 1). There was no amplification product of ca. 270 bp that hybridized with the *nuc* internal probe in control samples containing DNA from *Staphylococcus epidermidis* and *Micrococcus luteus*, bacteria closely related to *S. aureus*. *S. aureus* control strains gave appropriate amplification products. These controls and the extensive verification of this method by Brakstad et al. (1992) indicated the specificity of this technique for *S. aureus*.

C. Conclusion PCR detection of *S. aureus*-specific *nuc* sequences indicated that *S. aureus* was ubiquitous in waters of Mamala Bay in the period during which sampling was done. This result contrasts sharply with results of Dr. Fujioka's group, who isolated *S. aureus* on selective medium only very rarely. The PCR technique will detect very low numbers of cells (<20 cells) (Brakstad et al., 1992) and these cells need not be culturable. Three possible explanations for the discrepancy between the PCR- and culture-based detection of *S. aureus* are that 1) cell numbers of *S. aureus* are present at below the detection limit of the culture technique but above the limit of the PCR-based technique 2) poor recovery is being obtained on selective medium and therefore some culturable *S. aureus* cells are not being enumerated by the culture-based technique or 2) *S. aureus* is commonly present in the viable but nonculturable state in Mamala Bay waters.

There are several possible sources of the *S. aureus* cells found in Mamala Bay. Bathers on beaches along the coast are likely to shed *S. aureus* cells that may become dispersed throughout the Bay. *S. aureus* may enter Mamala Bay in sewage discharged from the Sand Island outfall. Non point sources such as the Ala Wai canal may be a source of *S. aureus*. Finally, *S. aureus* may be growing and multiplying in marine waters. The last possibility is highly unlikely, since in this case one would expect to isolate *S. aureus* readily on selective media and, in addition, Dr. Fujioka has shown that *S. aureus* is incapable on growth in Mamala Bay water even when this water is amended with 50% sewage. If *S. aureus* had been present in only a limited number of samples from a single area, it may have been possible to deduce which of the first three possibilities is the most likely. However, since *S. aureus* was present in almost all samples tested, it is not possible to determine the relative contributions of bathers, the sewage outfall, and non-point sources.

V. MOLECULAR DETECTION OF *S. AUREUS* ON NONSELECTIVE MEDIUM

A. Objective and Experimental Design. The objective was to enumerate all culturable *S. aureus* cells present in samples. Samples were plated on nonselective medium in order to obtain optimal growth of all culturable *S. aureus* cells present. Water and sediment samples were plated on suitable nonselective medium within 4 h of collection. Resultant colonies were enumerated and transferred to solid support membranes for probing. Over 300 colony lifts were prepared following this procedure. The probe used was a radiolabeled 33-mer designed to be specific for the *S. aureus nuc* gene (Brakstad et al., 1992).

B. Results and Discussion. The *S. aureus nuc* gene probe was used to probe control colony lifts of "known" bacterial isolates, including several *S. aureus* isolates, to test the specificity and sensitivity of this probe. Hybridizations were performed under two sets of conditions. Results of one of these experiments are shown in Fig. 1. This probe has proven to be unsatisfactory since it hybridized, under both sets of conditions tested, to some but not all of the *S. aureus* control strains. In addition, a hybridization signal was detected for enterotoxic *Escherichia coli* (Fig. 1) indicating that the probe is not specific for *S. aureus* This probe was originally designed for use as an "internal probe" for confirmation of *S. aureus nuc* gene amplification products and has sufficient specificity for this application but proved not to be suitable as a *S. aureus*-specific probe for probing colony blots. Time constraints did not allow development of a *S. aureus*-specific probe that would work in this application.

C. Conclusion. A *S. aureus*-specific gene probe that is suitable for probing colonies grown on nonselective medium would enable optimal enumeration of culturable *S. aureus* cells and should be developed for future studies of this type.

VI. CONFIRMATION OF PUTATIVE *S. aureus* ISOLATES BY *nuc* GENE ANALYSIS

A. Objective and Experimental Design. The objective was to confirm putative *S. aureus* strains isolated by Dr. Fujioka's group on selective medium and which were tested by the latex agglutination and coagulase tube tests. Cells of each isolate were boiled for 5

min and cell debris was pelleted by centrifugation. PCR analysis for *S. aureus*-specific *nuc* gene sequences was performed on supernatants. PCR was performed and PCR products analyzed and confirmed by Southern hybridization as described in Section IV A.

B. Results and Discussion. Thirty-seven isolates obtained from Dr. Fujioka were tested. Nine of these isolates (24.3%) were confirmed as *S. aureus* by presence of the appropriate *nuc* gene sequences. Results for individual isolates are given in Table 2 and electrophoretic analysis of PCR products from *nuc* primers is shown in Fig. 3.

Dr. Fujioka's group tested this set of isolates using the Gen Probe Kit which detects specific ribosomal RNA sequences unique to *S. aureus* and obtained three positive results. All three of these isolates were also found positive by the *nuc* method. However, six isolates identified as *S. aureus* by the *nuc* method were negative by the Gen Probe method. This discrepancy should be addressed by additional research, possibly including 16S RNA gene sequencing of these anomalous strains. For the purposes of Dr. Fujioka's analysis on the incidence of *S. aureus* at selected beach sites, a conservative approach was adopted and only isolates identified as *S. aureus* by both molecular approaches were considered to be *S. aureus* strains.

C. Conclusion. PCR analysis of the *nuc* gene was useful in confirming the identity of putative *S. aureus* isolates. Less than 25% of isolates obtained on Staphylococcal selective medium were found to be *S. aureus* by *nuc* gene analysis indicating that these conventional tests are unreliable for identification of *S. aureus* from environmental samples. We recommend that both the *nuc* PCR method and the Gen Probe method be used and that strains identified as *S. aureus* by both methods be reported as confirmed *S. aureus* in future studies of this type, until discrepancies between these two methods are resolved.

VII. DETECTION OF STAPHYLOCOCCAL ENTEROTOXIN GENES

A. Objective and Experimental Design. Strains of *S. aureus* isolated from Mamala Bay-water samples by Dr. Fujioka's group that were positive for *S. aureus*-specific *nuc* gene sequences were analyzed for the presence of enterotoxin genes to determine their pathogenic potential. PCR analysis was used for detection of TSST-1 and staphylococcal enterotoxin genes A and B. Primer sets were as described by Johnson et al. (1991) and PCR conditions were as follows: 94°C at 5 min, 30 cycles of denaturation at 94°C for 1

min, primer annealing at 55°C for 2 min, and extension at 72°C for 1 min, followed by extension at 72°C for 5 min. Strains for each enterotoxin-producing type of *S. aureus* were a gift from Dr. Peter Gemski and were used as positive controls in these studies. PCR products were analyzed by agarose gel electrophoresis and products of appropriate sizes, as described by Johnson et al. (1991), were taken to indicate presence of toxin genes.

B. Results and Discussion. One strain of seven *S. aureus* strains tested was found to possess genes encoding staphylococcal enterotoxins A and B. No strains were positive for the TSST-1 gene (Table 3).

We have demonstrated the presence of enterotoxin genes in a *S. aureus* strain isolated from Waikiki Beach [strain W1-2 (8/10)]. The staphylococcal enterotoxins are agents of staphylococcal food poisoning and may also be implicated in other staphylococcal infections (Bergdoll, 1983) and this strain can therefore be considered to have a confirmed pathogenic potential. Although the incidence of *S. aureus* strains carrying genes encoding was fairly low (one in seven tested), demonstration of toxin genes in environmental isolates of *S. aureus* raises the possibility that toxin gene profiles may be useful "markers" *of S. aureus* environmental isolates. These profiles could be considered in future studies as a method for tracking the origin of particular isolates.

C. Conclusion. A culturable *S. aureus* strain that contained genes encoding staphylococcal toxins which may be implicated in the pathogenicity of these bacteria was isolated from a Waikiki Beach water sample. It is difficult to assess the implications for public health of this finding, since *S. aureus* appears to be present at very low concentrations. However, *S. aureus* strains carrying genes encoding toxins may be potentially more pathogenic that toxin-negative strains.

VIII. SURVIVAL STUDIES

A. Objective and Experimental Design. Survival studies on *S. aureus* were done to determine the ability of this bacterium to survive for prolonged periods in seawater and to investigate its entry into the viable but nonculturable state. Experiments were performed in microcosms containing water collected from station D2B, in the vicinity of the sewage outfall, since this water was expected to contain high concentrations of nutrients which may enhance survival. Total cell counts were determined by microscopic enumeration of DAPI fluorochrome-stained cells (Coleman, 1980) and culturable cell counts were obtained by

plating on both nonselective and selective medium. The selective medium used was modified VJ agar, identical to that used by Dr. Fujioka for enumeration of *S. aureus*. The CTC procedure (Rodriguez et al. 1992) was used to determine viable cell counts. Microcosms were inoculated with ca. 10^8 cells/ml of *S. aureus*, and held at room temperature in the dark. Microcosms were monitored for 17 days.

B. Results and Discussion. Results obtained from microcosms containing water collected at station D2B and filtered though a 0.1 µm filter to remove all bacteria are shown in Figs 4-6. Survival at room temperature (ca. 26°C) of two strains of *S. aureus*, ATCC12600 and W12 are shown in Figs. 4 and 5 respectively. Survival of one of these strains (W12) was also determined at 4°C (Fig. 6). In all cases, total cell numbers decreased by ca. ten-fold over 17 d, and numbers of viable cells remained at or above 10^6 cell/ml. Culturable cell counts decreased more markedly and were less than 10^3 cells/ml after 17 d, when determined on nonselective medium. Culturable cell counts determined on selective medium were generally less than ten-fold lower than on nonselective medium during the first three days, but this difference was very pronounced after 17 d, when *S. aureus* cells were undetectable on selective medium and were between 10^2 and 10^3 cells/ml on nonselective medium.

These findings indicate that *S. aureus* can survive for prolonged periods in seawater. It is important to note that conditions in these experiments were optimal for survival. Experiments were conducted in the dark and possible inactivation by zooplankton and other bacteria was eliminated since water was prefiltered though 0.1 µm filters. However, these data do indicate the potential for long-term survival of *S. aureus* in the marine environment.

Viable counts of *S. aureus* were generally several orders of magnitude greater than culturable counts, indicating that *S. aureus* enters into a viable but nonculturable state in Mamala Bay water. Previous work has shown that viable but nonculturable cells may retain the potential to be pathogenic and may be an unrecognized reservoir of disease (Colwell et al., 1985; Colwell et al., 1990; Rahman et al., 1994). Although the pathogenicity of viable but nonculturable *S. aureus* has not been established, the possibility cannot be discounted that viable but nonculturable *S. aureus* cells in marine waters present a public health risk.

C. Conclusion. *S. aureus* can survive for long periods in the marine environment, remaining culturable for several days and persisting in a viable but nonculturable state for weeks or months.

VII. FINAL ASSESSMENT AND RECOMMENDATIONS

Molecular studies on detection and characterization of *S. aureus* proved to be useful in confirming the presence of *S. aureus* at some beach sites and showing that one isolate contained genes encoding an enterotoxin. The most important finding resulting from the use of a molecular approach was the ubiquitous presence of *S. aureus* in water samples from Mamala Bay Hawaii, even though *S. aureus* could be cultured from only very few of these samples. Survival studies demonstrated the ability of *S. aureus* to survive for long periods in the marine environment and to enter into the viable but nonculturable state in which bacteria potentially remain pathogenic. A comparison of recovery of *S. aureus* on selective and nonselective medium indicated that cell counts on selective medium are likely to underestimate numbers of culturable *S. aureus* cells, particularly when cells are starved. It is reasonable to conclude that culturable *S. aureus* cells may be underestimated by present methods and that *S. aureus* is likely to be present in Mamala Bay waters in the viable but nonculturable state

Several recommendations are made, in addition to the recommendations of Dr. Fujioka in Phase I of this study:

1. *S. aureus* was shown to be ubiquitous in Mamala Bay waters, although it was not recoverable on selective medium except occasionally from beach sites. This implies that high numbers of *S. aureus* cells are entering Mamala Bay and reinforces the need for determining the relative contributions of bathers, sewage and nonpoint sources to *S. aureus* inputs into Mamala Bay.

2. Enumeration of culturable *S. aureus* remains a problem since selective media may underestimate numbers of starved cells. The most promising approach is plating on nonselective medium, followed by identification of *S. aureus* isolates by using a gene probe. This requires development of a *S. aureus*-specific probe suitable for use in this application.

3. *S. aureus* strains carrying genes encoding specific staphylococcal toxins can be identified in *S. aureus* isolates from environmental samples and this approach may be useful in tracing particular populations of *S. aureus* cells.

4. A molecular approach was essential in determining that *S. aureus* is ubiquitous in Mamala Bay water samples and all future studies should include molecular and microbiological components that are well-coordinated and complementary.

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Table 1. Total bacterial counts in water samples obtained from Mamala Bay in September 1995.

Station Description	Station Code	Cell Count ($x10^5$/ml)
Manoa Stream	MS	8.41
Ala Wai Canal	AW1	12.70
Ala Wai Offshore	AW2	4.91
Waikiki Beach	W1	7.14
Waikiki Offshore	W2	4.66
Sewage Outfall 0	DO	5.27
Sewage Outfall 1 Surface	D1S	6.83
Sewage Outfall 1 Bottom	D1B	3.06
Sewage Outfall 2 Surface	D2S	3.31
Sewage Outfall 2 Middle	D2M	4.85
Sewage Outfall 2 Bottom	D2B	1.74
Sewage Outfall 3 Surface	D3S	3.18
Sewage Outfall 3 Bottom	D3B	3.51
Pearl Harbor 1	C1	7.46
Pearl Habor 2	C2	9.10
Pearl Harbor 3	C3	11.35
Diamond Head Offshore Surface	E4S	3.32
Diamond Head Offshore Middle	E4M	5.38
Diamond Head Offshore Bottom	E4B	6.63
Hanauma Bay Beach Park	HB	2.51
Ala Moana Beach	AM1	5.37

Table 2. Screening of putative *S. aureus* isolates obtained from R. Fujioka for the presence of the *S. aureus*-specific nuc gene.

Strain Designation	Presence (+) or absence (-) of *nuc* gene
AW1-1 (8/16)	-
AW1-3 (8/16)	-
AW1-4 (8/16)	-
AW1-9 (8/16)	-
AM1-1 (8/10)	+
AM1-2 (8/10)	-
AM1-2 (8/16)	-
AM1-4 (8/16)	-
AM1-5 (8/16)	-
WNAT-1 (8/10)	-
WNAT-2 (8/10)	+
EW1-2 (8/16)	-
EW1-3 (8/10)	-
EW1-3 (8/16)	-
EW1-4 (8/16)	-
EW1-5 (8/10)	-
EW1-5 (8/16)	-
EW2-2 (8/16)	-
EW2-9 (8/16)	-
EW2-10 (8/16)	-
SB1-1 (8/10)	-
SB1-1 (8/16)	-
SB1-3 (8/10)	+
SB-3 (8/16)	
SB1-4	-
SB1-5	-
SB1-6 (8/10)	-
SB1-6 (8/16)	+
QB1-6	-
W1-1 (8/10)	+
W1-1 (8/16)	-
W1-2 (8/10)	+
W1-3 (8/16)	-
W1-4	-
W1-6 (8/16)	+
WKB-1 (8/10)	+
WKB-5 (8/10)	+

Table 3. Screening of *S. aureus* strains isolated from Mamala Bay water samples for the presence of genes encoding TSST-1 and enterotoxins A and B.

S. aureus Strain (UH Identification)	TSST-1	Staphylococcal enterotoxin A	Staphylococcal enterotoxin B
AM1-1 (8/10)	-	-	-
SB1-3 (8/10)	-	-	-
W1-1 (8/10)	-	-	-
W1-2 (8/10)	-	+	+
W1-6 (8/16)	-	-	-
WKB-1 (8/10)	-	-	-
WNAT-2 (8/10)	-	-	-

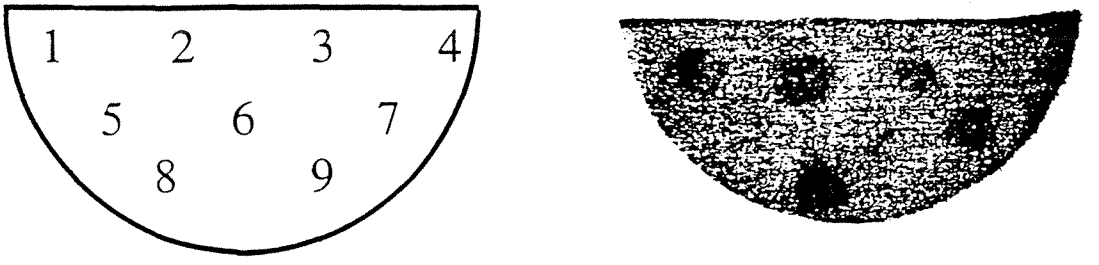

Fig. 1. Probe detection of *S. aureus* colonies on nonselective medium. A radioactively labeled probe derived from the *nuc* gene was hybridised with colonies of control strains of *S. aureus* and other bacterial species. Colony 1, *S. aureus* ATCC 25923; Colony 2, enterotoxigenic *Escherichia coli*; Colony 3, *S. aureus* UH W1C1; Colony 4 *S. aureus* UHSB1-C6; Colony 5, *S. epidermidis* ATCC 12228; Colony 6, *S. aureus* methicillin resistant strain 7509-3R81103; Colony 7, *S. aureus* methicillin resistant strain 75421; Colony 8, *S. aureus* ATCC 12600; Colony 9, *Micrococcus luteus* ATCC 27523.

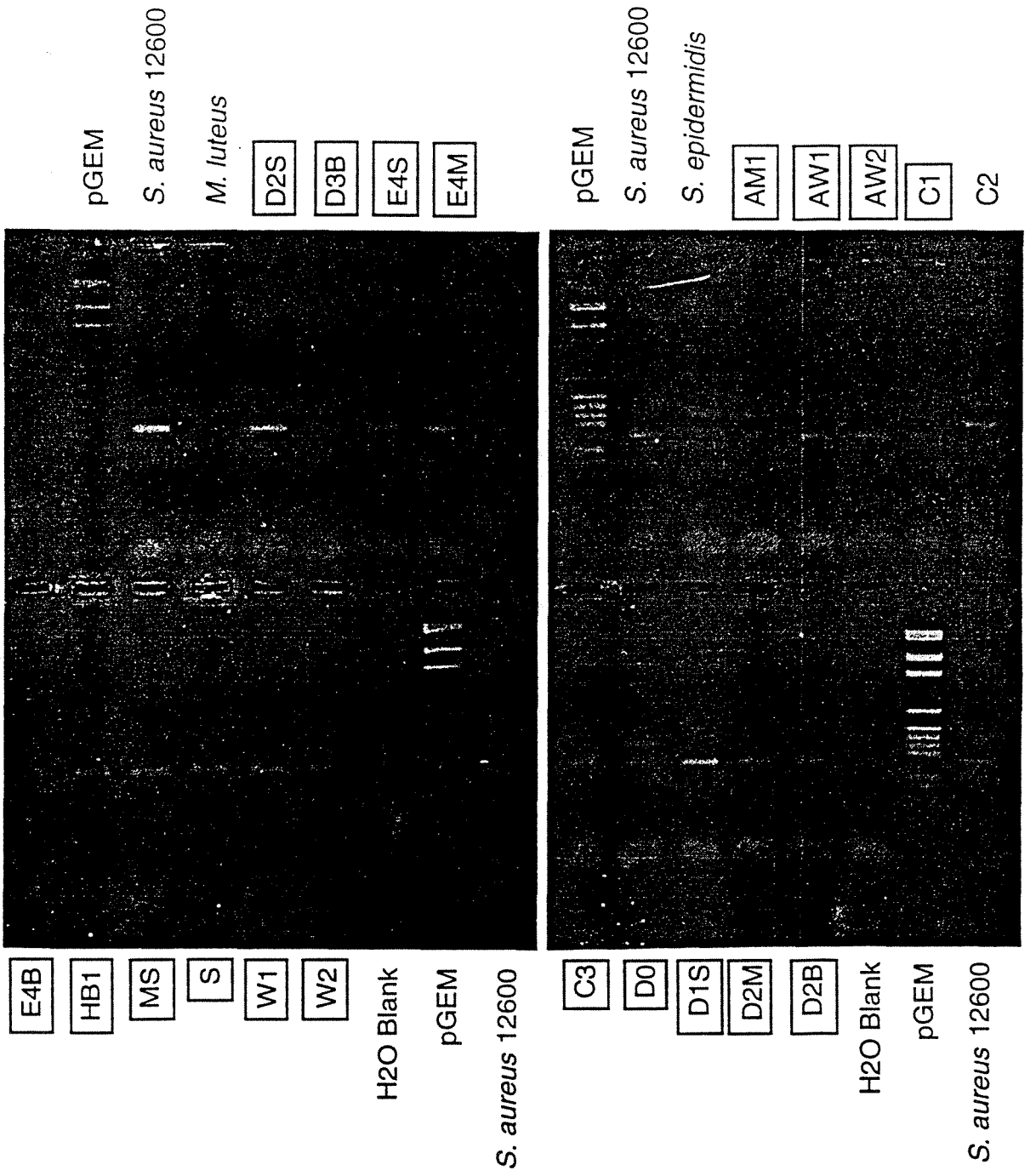

Fig. 2. PCR detection of the *S. aureus*-specific *nuc* gene in DNA extracted from Mamala Bay water samples. Sites designations are as given in Table 1. Putative *nuc* gene-positive sites, where an amplification product of ca. 270 bp was detected, are boxed. pGEM is a molecular weight marker, *S. aureus* ATCC12600 is a positive control, *M. luteus* and *S. epidermidis* are negative controls and H_2O blank is a control without added template DNA.

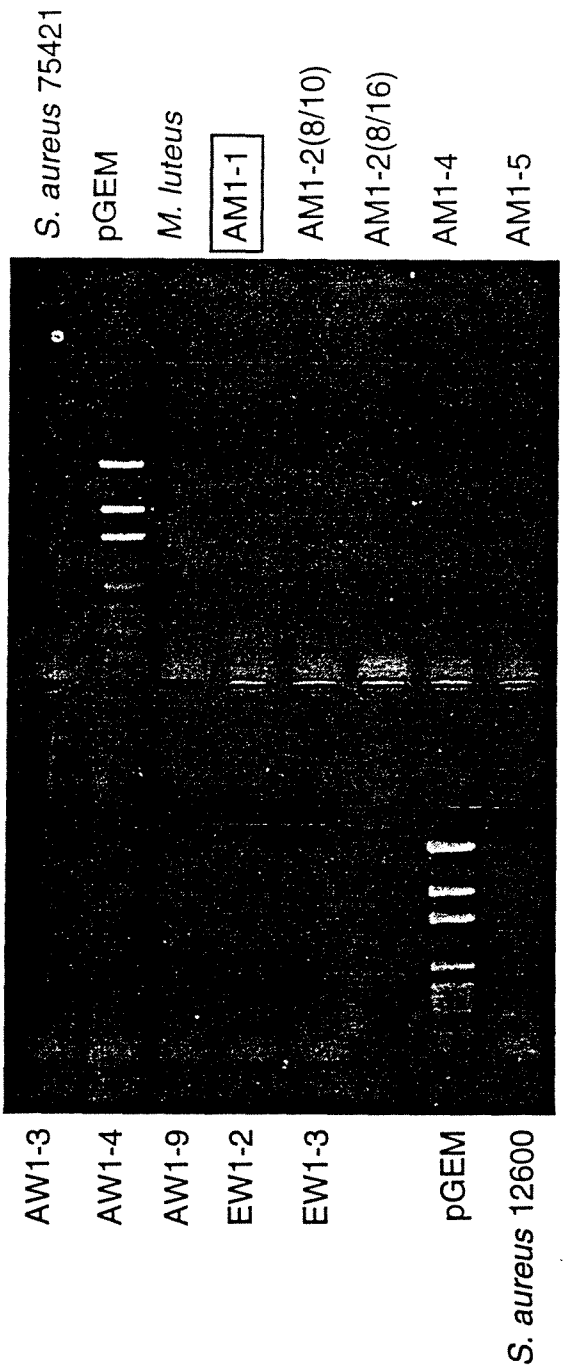

Fig. 3. Screening of putative *S. aureus* isolates obtained from R. Fujioka for the presence of the *S. aureus* specific *nuc* gene. Isolates are designated with a station number (stations are described in Table 1.) and an identification number. Putative *nuc* gene-positive isolates, where an amplification product of 270 bp was obtained are boxed. *S. aureus* ATCC 75421 and ATCC 12600 are positive controls, and *M. luteus* is a negative control.

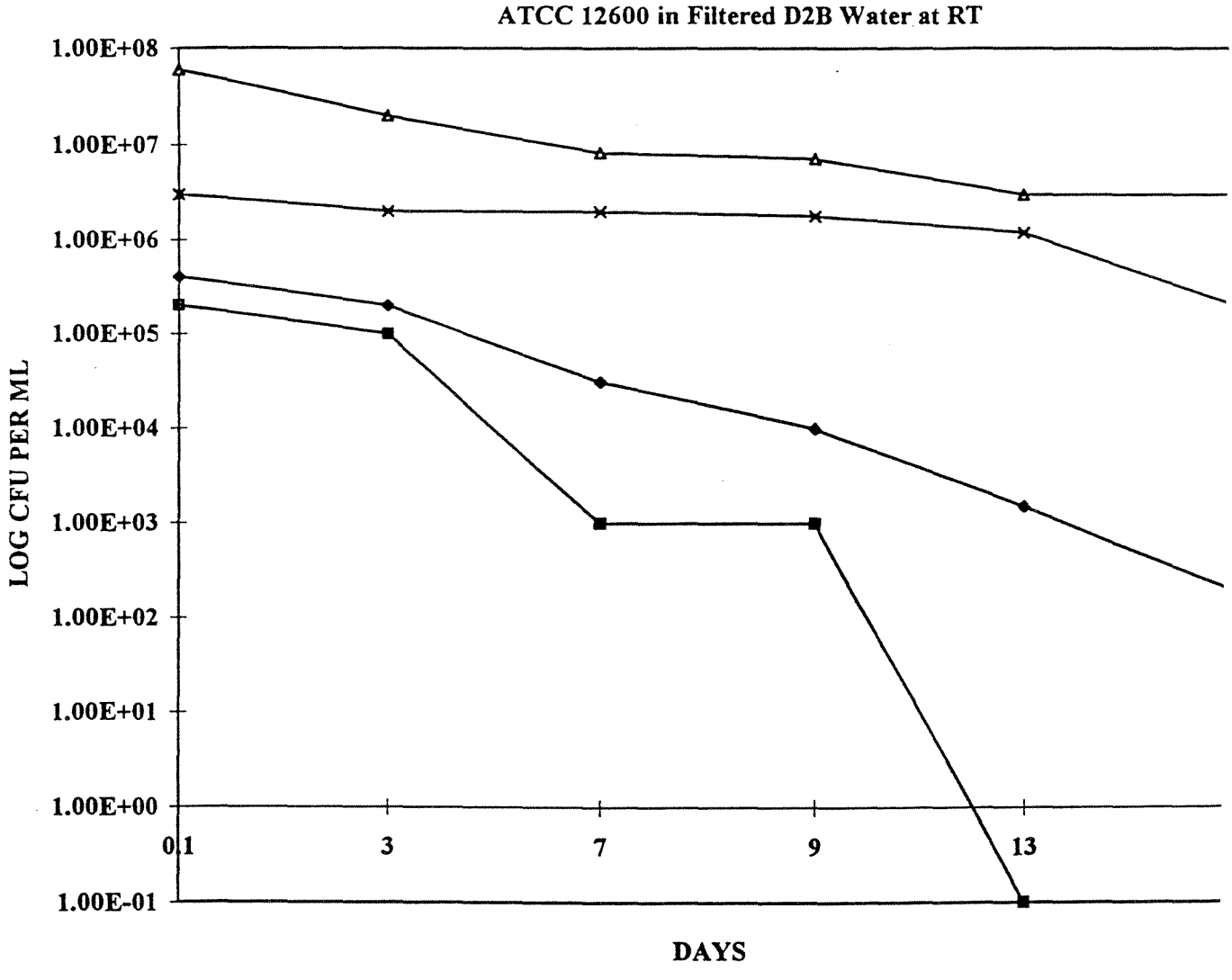

Fig. 4. Culturable, viable and total counts of *S. aureus* ATCC12600 in microcosms containing filtered water from station D2B and held at room temperature (ca. 26°C). Culturable counts were determined by plating on nonselective medium (diamonds) and selective medium (squares), viable counts (x) by the CTC method of Rodriguez et al. (1992), and total counts (triangles) by microscopic enumeration of DAPI-stained cells.

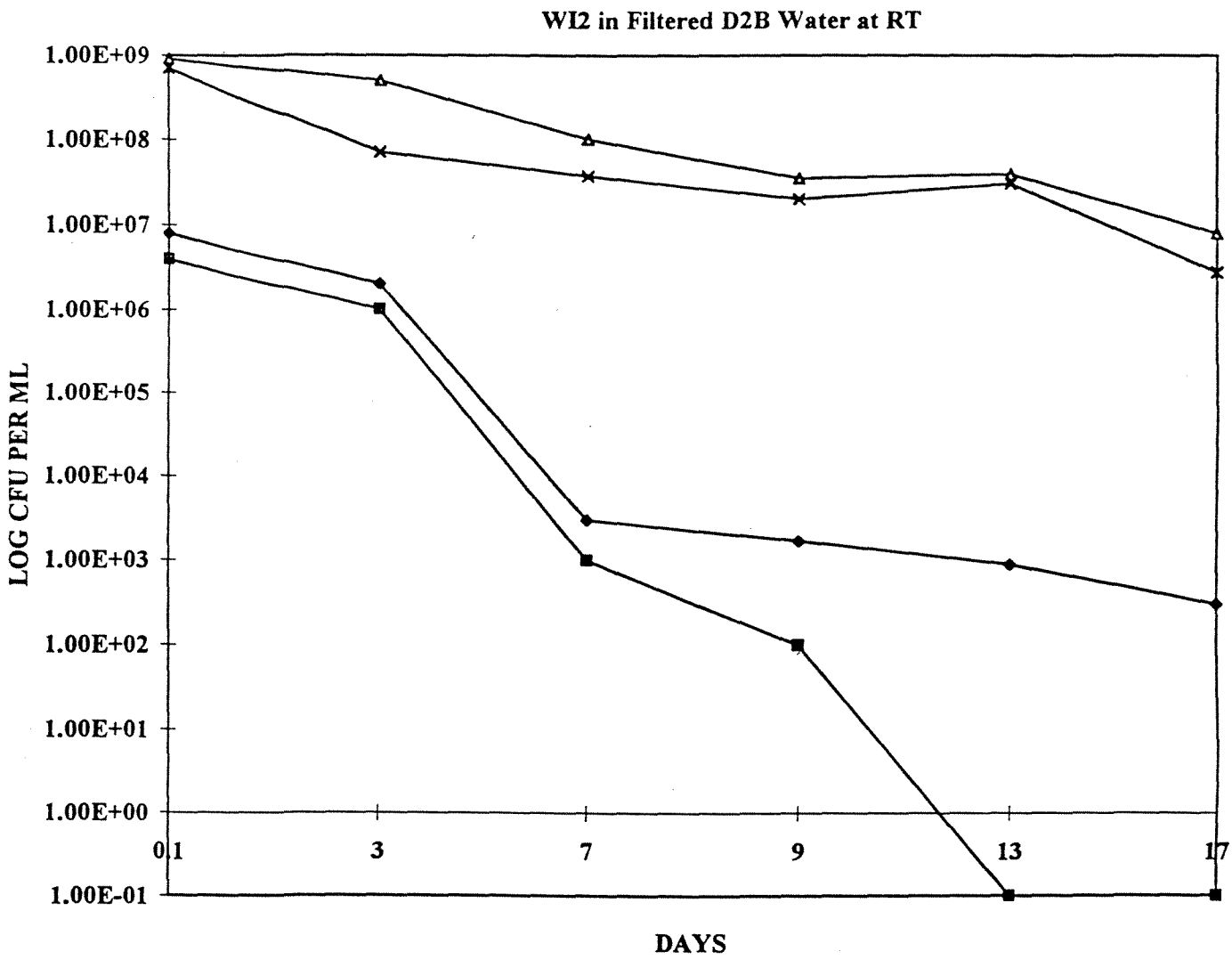

Fig. 5. Culturable, viable and total counts of *S. aureus* W2 in microcosms containing filtered water from station D2B and held at room temperature (ca. 26°C). Culturable counts were determined by plating on nonselective medium (diamonds) and selective medium (squares), viable counts (x) by the CTC method of Rodriguez et al. (1992), and total counts (triangles) by microscopic enumeration of DAPI-stained cells.

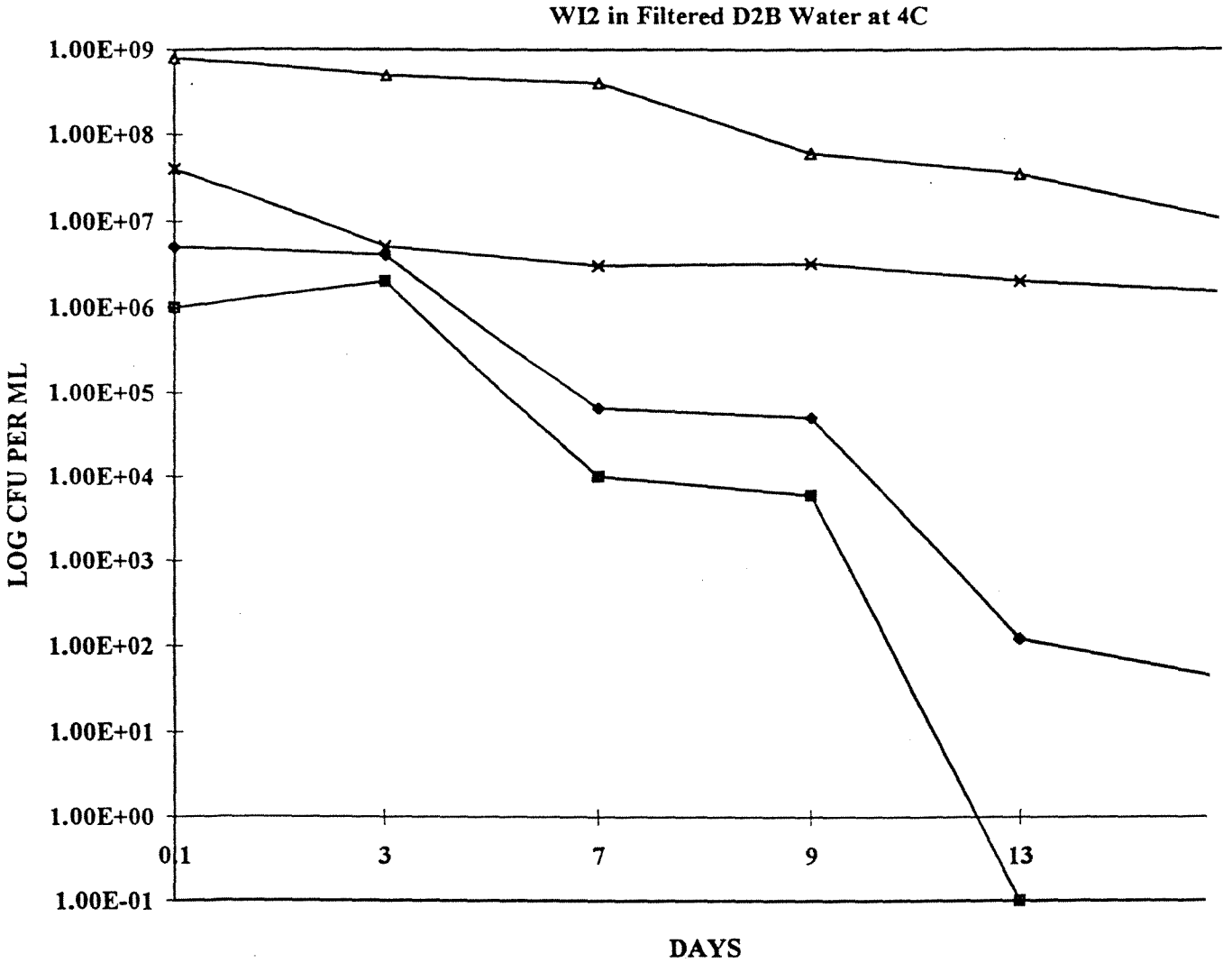

Fig. 6. Culturable, viable and total counts of *S. aureus* W2 in microcosms containing filtered water from station D2B and held at 4°C. Culturable counts were determined by plating on nonselective medium (diamonds) and selective medium (squares), viable counts (x) by the CTC method of Rodriguez et al. (1992), and total counts (triangles) by microscopic enumeration of DAPI-stained cells.