MAMALA BAY STUDY

MOLECULAR INVESTIGATION OF THE EFFECT OF POLLUTION ON PATHOGENIC AND INDIGENOUS BACTERIA IN MAMALA BAY

PROJECT MB-7

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1 EXECUTIVE SUMMARY

Two molecular methods were used to detect the pathogens *Campylobacter jejuni*, *Shigella* species, enterotoxigenic *E. coli* (ETEC), and Cholera Toxin (CT)-positive vibrios in samples from Mamala Bay. The first method used specific gene probes to detect and enumerate pathogens cultured on non-selective media. The second approach detected pathogens whether or not they were culturable and used the polymerase chain reaction (PCR) to amplify fragments of genes involved in pathogenicity in each of the organisms studied. The identity of PCR amplification products was confirmed by using gene probes designed to detect these amplification products.

The four bacterial pathogens of interest, *Shigella* spp., enterotoxigenic *E. coli*, *C. jejuni* and Cholera Toxin-positive vibrios, were detectable in waters of Mamala Bay by both the molecular techniques employed in this study. *Shigella* spp. and enterotoxigenic *E. coli* were present in multiple samples from the vicinity of the Sand Island sewage outfall and the mouth of the Ala Wai canal, and were detectable in primary sewage samples. Concentrations of these pathogens in water samples from the vicinity of the outfall were several orders of magnitude lower than in primary sewage, indicating rapid dilution of primary sewage at the outfall.

Culturable *Shigella* spp. were detected by a gene probe in samples from Waikiki Beach and Hanauma Bay. This constitutes a potential public health risk. The origin of *Shigella* found in these samples could not be determined by the techniques used in this study. *C. jejuni* was detected by PCR amplification in one sample from Ala Moana Beach and in two primary sewage samples. Absence of *C. jejuni* at all other stations and the very rapid loss of culturability found in survival studies with this organism suggest that the *C. jejuni* detected at a beach site is likely to have been shed by bathers.

Cholera Toxin-positive bacteria were detected by probe and PCR studies and were present in samples from many stations. Cholera Toxin is generally associated with *Vibrio*

cholerae O1, which has the potential to cause epidemics of cholera. However, the Cholera Toxin gene has also been detected in other vibrios and related organisms and in non-O1 strains of *V. cholerae* . There is considerable evidence that *V. cholerae* O1 and non-O1 strains are indigenous to the marine environment. It is therefore possible that the CT-positive bacteria detected in this study are part of the natural bacterial community of Mamala Bay. Molecular techniques should be used to determine the identity and pathogenic potential of these Cholera Toxin-positive bacteria.

Survival experiments showed that V. cholerae, Shigella spp. and enterotoxigenic E. coli remained culturable for extended periods under optimal conditions. However, our data indicate that, under typical conditions present in Mamala Bay, Shigella spp. and enterotoxigenic E. coli are likely to become nonculturable in less than one day and V. cholerae may remain viable and culturable for longer periods. In all cases, V. cholerae, Shigella spp. and enterotoxigenic E. coli retained viability considerably longer than culturability, indicating the importance of detection of viable as well as culturable cells. C. jejuni became nonculturable very rapidly in all experiments and culturable forms are unlikely to be detectable in environmental samples.

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2 INTRODUCTION

The discharge of sewage effluents into coastal waters is a controversial method of sewage disposal which may have adverse environmental consequences. Mamala Bay receives sewage from two major outfalls, the Sand Island Outfall and the Honouliuli (Barbers Point) Outfall. In addition, there are numerous non-point sources of pollution which drain into the bay. Major concerns of the Mamala Bay Study Plan are to study these point and non-point sources of pollution and analyze the effects of these sources on public health and the environment.

A standard approach for the detection of fecal contamination of aquatic ecosystems is enumeration of marker or indicator microorganisms such as fecal coliforms, enterococci, coliphage and *Clostridium perfringens*. *C. perfringens* has been found to be a particularly useful marker in the marine environment because it forms highly resistant endospores which persist for long periods in marine waters. *C. perfringens* has been used as a marker in several aquatic systems (Cabelli and Pedersen, 1982; Fujioka and Shizumura, 1985; Hill et al., 1993; Sorensen et al., 1989) including a study in Mamala Bay (Fujioka, et al., 1992) . Fecal coliforms are not useful markers in Hawaiian waters since these organisms grow in tropical freshwater and terrestrial environments (Hardina and Fujioka, 1991) and rapidly lose culturability in seawater (Fujioka et al., 1981) . Enterococci and coliphage have proven to be useful indicators of fecal pollution in the marine environment during this and previous studies.

While these marker bacteria are useful in environmental studies, they are indeed just markers. These marker organisms do not in general cause disease, nor does their isolation differentiate between human and animal sources of fecal contamination (Institute of Medicine, 1991) . In assessing the impact of sewage contamination, the best approach is clearly to be able to directly identify and enumerate specific important human pathogens in water and other environmental samples. This approach has previously not

been practical, due to limitations inherent in conventional microbiological techniques. Use of these techniques requires laborious (and expensive) identification of hundreds of bacterial colonies, each requiring a battery of biochemical tests for confirmation. The use of selective media may also affect the counts obtained, probably by inhibiting growth of stressed organisms (Wright et al., 1992) . Standard approaches also have a severe limitation in that they fail to detect viable but nonculturable organisms which will not grow on any media, selective or non-selective. This is particularly important in aquatic environments where many bacteria, including important pathogens, enter a dormant state in which they are not culturable, but remain viable (Roszak and Colwell, 1987) and retain pathogenicity (Colwell et al., 1985; Colwell et al., 1990) .

Molecular genetic and fluorescent antibody techniques provide a means of overcoming these difficulties. Genetic probes derived from critical, species-specific (and usually pathogenicity-related) genes provide a highly sensitive and specific means of identifying particular pathogens, e.g. Hoge et al. (1990); Miliotis et al. (1989); Morris et al. (1987); Wright et al. (1992); Wright et al. (1993). In the first approach used in this study, samples were plated on non-selective media and resultant colonies were transferred to hybridization membranes. Genetic probes, labeled radioactively or with alkaline phosphatase, were used to detect and enumerate specific bacterial colonies. This approach still requires that the organism of interest is culturable on nonselective media and will therefore not detect organisms that are viable but nonculturable.

Our second approach was to use the polymerase chain reaction (PCR) technique for detection of specific genes involved in pathogenicity of the organisms of interest. This approach ensures detection of viable but nonculturable bacteria. This approach was supplemented where possible by direct detection of cells of these pathogens by labeling with fluorescent antibodies and microscopic observation. All environmental samples were screened using PCR primers specifically designed for detection of the pathogens

Campylobacter jejuni, *Shigella* species, enterotoxigenic *E. coli* (ETEC), and Cholera Toxin-positive vibrios.

The impact of sewage disposal on the natural bacterial assemblage in Mamala Bay was studied by determining total and culturable bacterial numbers in water and sediment samples from impacted and relatively pristine stations and by determining the prevalence of *Vibrio alginolyticus*, a naturally occurring marine bacterium which was used as a surrogate of the natural bacterial community.

A supplementary aspect of this study was to determine inactivation rates of the four pathogenic bacteria on which the project was focused. Survival studies were performed on microcosms of water obtained from Mamala Bay. These studies provide important information on the long-term survival of pathogens in Mamala Bay.

2.1 Scope of Work

This study formed part of the MB-7 Project that investigated microbiological aspects for the Mamala Bay Study Commission. The scope of work was coordinated with other investigators in MB-7, under the leadership of Dr. Charles Gerba. Samples for this project were obtained during the quarterly sampling periods. In general, samples were collected at one time point from each station sampled during quarterly sampling periods. Limited additional samples were collected from selected stations (beach sites) at an additional time point, ca. 6 h after first sample collection in an attempt to address tidal effects. Water samples were collected from all stations and sediment samples were collected from stations where this was possible within the limitations of our sampling equipment.

Total and culturable bacterial counts were determined for all water samples. Culturable bacterial counts were determined for all sediment samples. All water and sediment samples were processed to detect culturable *Shigella* spp., enterotoxigenic *E. coli*, *C. jejuni* and *V. cholerae* by using gene probes. Water samples were filtered to

concentrate the total bacterial community present in samples and DNA was extracted from these concentrates. Presence of the same four pathogens in these samples was determined by using PCR amplification of specific genes.

Survival of the pathogens *Shigella* spp., enterotoxigenic *E. coli*, *C. jejuni* and *V. cholerae* was investigated in water collected from Mamala Bay. Long-term survival was studied in microcosms incubated at 28°C and 22°C. Culturable cell counts, direct viable counts and total cell counts were used to assess survival.

2.2 Objectives

2.2.1 Molecular Detection of Pathogens

Our primary objective was to use molecular techniques to directly detect the important bacterial pathogens *Shigella* spp., enterotoxigenic *E. coli*, *C. jejuni* and Cholera Toxin-positive vibrios in samples from Mamala Bay and to determine the influence of point and non-point sources of pollution on the incidence of these pathogens. Culturable and nonculturable bacterial pathogens were quantified. This objective included providing quantitative data on bacterial pathogens for use in a quantitative risk assessment model for Mamala Bay and in calibration of physical mixing and transport models.

2.2.2 Assessment of Changes in Indigenous Bacterial Communities

A secondary objective of our study was to determine the effect of pollution on autochthonous bacterial populations. This was studied by determining total and culturable bacterial numbers in water and sediment samples from impacted and relatively pristine stations and by determining the prevalence of *Vibrio alginolyticus*, a naturally occurring marine bacterium which was used as a "model" organism representative of the natural bacterial community

2.2.3 Bacterial Survival Studies

An additional objective established during the course of this project was to investigate survival of the pathogens *Shigella* spp., enterotoxigenic *E. coli*, *C. jejuni* and *V. cholerae* under conditions that replicated, as closely as possible, the natural conditions in Mamala Bay waters.

2.3 Project Organization

The project formed a part of the MB-7 Project under the overall leadership of Dr. Charles Gerba. Other investigators in the MB-7 section were Dr. Ian Pepper from the University of Arizona, Dr. Roger Fujioka from the University of Hawaii, Drs. John Paul and Joan Rose from the University of South Florida, and Drs. Fred Dobbs and Michael Landry from the University of Hawaii. All investigators other than Drs. Dobbs and Landry participated in the tightly coordinated quarterly sampling periods, during which all samples processed in this study were collected. Sampling was coordinated by Kimberly Roll of the University of Hawaii. Drs. John Paul and Russell Hill supervised ship-board sample collection. An essential contribution to our part of the study was made by Dr. Roger Fujioka, who arranged facilities in Hawaii which enabled us to perform one week of intensive laboratory work in Hawaii, immediately after sample collection. Stabilized and processed samples were transported back to our laboratories at the Center of Marine Biotechnology, the Center of Vaccine development and the Microbiology Department, University of Maryland at College Park, for further processing.

The following people made contributions to this research project:

Russell T. Hill. Research Assistant Professor, Center of Marine Biotechnology. Principal Investigator with primary responsibility for design and implementation of sampling strategy, PCR analysis of bacterial community DNA, bacterial counts, and bacterial survival studies.

J. Glenn Morris. Professor, Veterans Administration. Co-principal Investigator. Supervision of enumeration of culturable bacteria by using specific genetic probes. Lead investigator on *Campylobacter* studies. Advisor on probe and PCR primer design.

Anwar Huq. Research Assistant Professor, Microbiology Dept. UMCP. Co-principal investigator. Supervision of fluorescent antibody studies.

Anita Wright. Senior Scientist. Center of Vaccine Development. Lead researcher on probe detection of culturable bacteria. Participated in sample collection.

Victoria Boccuzzi. Graduate Student. Center of Marine Biotechnology. Participated in sample collection. Performed PCR studies on all water samples. Performed direct fluorescent microscopy for detection of *V. cholerae*. Developed technique for removal of inhibitory substances from samples prior to PCR.

K. Eric Wommack. Graduate Student. Center of Marine Biotechnology. Performed survival experiments. Assisted in counting of total bacteria present in Mamala Bay water samples.

Jacques Ravel. Graduate Student. Center of Marine Biotechnology. Participated in sample collection. Contributed to verification of PCR methods and development of technique for removal of inhibitory substances from samples prior to PCR.

Rome Voulhoux. Visiting Graduate Student. Center of Marine Biotechnology. Contributed to verification of PCR methods.

3 METHODS

3.1 Task Summary

3.1.1 Bacterial Counts

Total bacterial counts were determined for all water samples. Culturable bacterial counts were determined for all water and sediment samples. Stations at which water samples were collected are shown in Fig. 3.1 and descriptions of stations and standard abbreviations used throughout this study are given in Table 3.1.

3.1.2 Probe detection of culturable bacteria

All water and sediment samples were processed to detect culturable *Shigella* spp., enterotoxigenic *E. coli*, *C. jejuni* and Cholera Toxin-positive vibrios by using gene probes.

3.1.3 PCR detection of pathogens

Water samples were filtered to concentrate the total bacterial community present in samples and DNA was extracted from these concentrates. Presence of the same four pathogens in these samples was determined by using PCR amplification of specific genes.

3.1.. Direct fluorescent antibody detection of *V. cholerae*

Selected water samples were processed for direct detection of *V. cholerae* by using fluorescently-tagged antibodies specific for the *V. cholerae* O1 and O139 antigens.

3.1.5 Survival studies

Survival of the pathogens *Shigella* spp., enterotoxigenic *E. coli*, *C. jejuni* and *V. cholerae* was investigated in water collected from Mamala Bay. Long-term survival was

studied in microcosms incubated at 28°C and 22°C. Culturable cell counts, direct viable counts and total cell counts were used to assess survival.

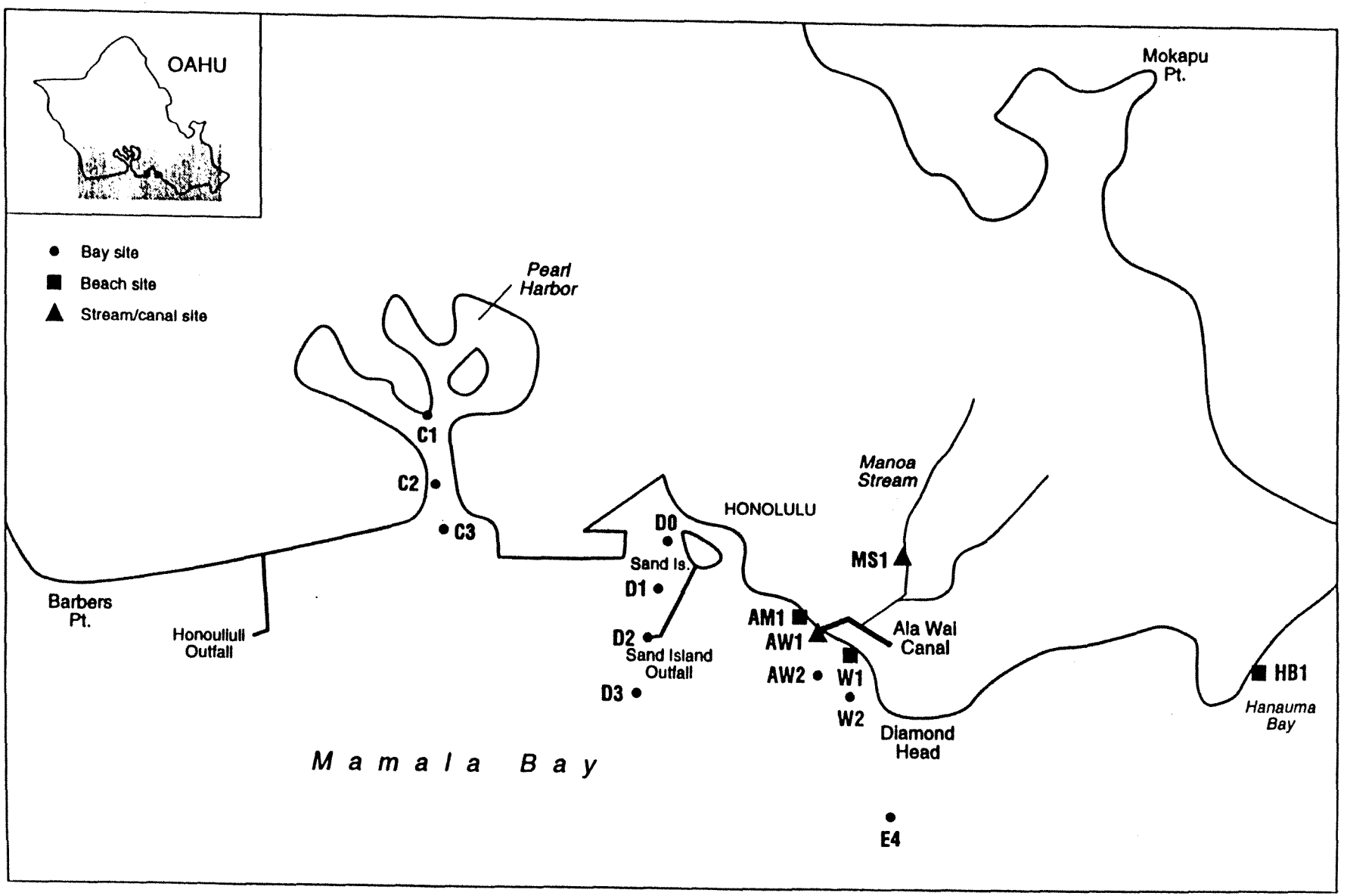

Fig. 3.1. Mamala Bay microbiological studies (MB-7) indicator/pathogen sampling sites.

Table 3.1. Abbreviations used to describe sampling stations.
Locations of stations are shown in Fig. 3.1. Sediment samples obtained from these stations are designated by the suffix sed and additional samples obtained in afternoons following normal sample collection are designated p.m.

Station Description	Station Code
Manoa Stream	MS
Ala Wai Canal	AW1
Ala Wai Offshore	AW2
Waikiki Beach	W1
Waikiki Offshore	W2
Sewage Outfall 0	D0
Sewage Outfall 1 Surface	D1S
Sewage Outfall 1 Bottom	D1B
Sewage Outfall 2 Surface	D2S
Sewage Outfall 2 Middle	D2M
Sewage Outfall 2 Bottom	D2B
Sewage Outfall 3 Surface	D3S
Sewage Outfall 3 Bottom	D3B
Pearl Harbor 1	C1
Pearl Harbor 2	C2
Pearl Harbor 3	C3
Diamond Head Offshore Surface	E4S
Diamond Head Offshore Middle	E4M
Diamond Head Offshore Bottom	E4B
Hanauma Bay Beach Park	HB
Primary Sewage	S
Ala Moana Beach	AM1

3.2 Task Methodology

3.2.1 Bacterial Counts

Total bacterial counts were done by direct microscopic counting of acridine orange-stained preparations i.e. the acridine orange direct count (AODC) procedure, as described by Hobbie et al. (1977) . Counts of viable cells in microcosm survival experiments were done based on the direct viable count procedure (Kogure et al., 1979) and by use of a fluorescent redox probe for direct visualization of actively respiring cells (Rodriguez et al., 1992) . Culturable cell counts were obtained by plating of appropriate dilutions of water and sediment samples on the nonselective medium LB agar (Maniatis et al., 1982) . Plates were incubated at 35°C for 24 h prior to enumeration of bacterial colonies.

3.2.2 Probe detection of culturable bacteria

Samples were plated on appropriate non-selective media and incubated under suitable conditions. LB agar (Difco Laboratories, Detroit, MI) and an incubation temperature of 35°C was used for culture of *Shigella*, enterotoxigenic *E. coli*, *V. vulnificus* and *V. cholerae*. *Campylobacter* were cultured on Trytic Soy Agar (Difco) under microaerophilic conditions at 42°C. Appropriate dilutions of samples were plated when necessary. Total numbers of viable heterotrophic bacteria were enumerated after an 18 to 24 h period of growth and plates containing between 20 and 1,000 colonies were overlaid with filter paper. Colonies were transferred to filter paper and directly identified and counted by using genetic probes. The probes that we used were oligonucleotides, attached to an alkaline phosphatase indicator. In some cases ^{32}P-radiolabeled probes were used. Details of the procedure are given in detail in Wright et al. (1993) .

Details of the genetic probes used are as follows:

chromosome and in multiple copies on a plasmid implicated in pathogenicity of *Shigella* spp. The primers were designated ipaIII (5'-GTTCCTTGACCGCCTTTCCGATACC GTC-3') and ipaIV (5'-GCCGGTCAGCCACCCTCTGAGAGTAC-3') and the sequence of the internal probe was 5'-AATCTCCGGAAAACCCTCCTGGTC-3' (Sethabutr et al., 1993).

Enterotoxigenic *E. coli*: PCR primers used to detect enterotoxigenic E. coli were those described by Lang et al. (1994) .and are designated LT1 (5'-TGTTTCCACTTCTCTTAG-3') and LT2 (5'-TATTCCCTGTTACGATGT-3'). The sequence of the internal probe is 5'-TACAGCCCTCACCCATATGAACA-3'.

***Campylobacter jejuni*:** PCR primers were developed and tested by Blaser and colleagues at Vanderbilt University. Primers amplify a fragment of the *C. jejuni peb1A* gene. The primers were designated CAMP1 and CAMP2 and their sequences are 5'-GCAGAAGGTAAACTTGAGTCTATT-3' and 5'-TTATAAACCCCATTTTTTCGCTA A-3', respectively. The internal probe used to confirm the identity of amplification products is the same probe used in detection of *C. jejuni* colonies, described in 3.2.2. above.

PCR conditions were based on previously described conditions for each set of PCR primers. No attempt will be made to obtain quantitative data from PCR detection in view of the difficulties in quantitative interpretation of PCR results. Samples screened by PCR amplification were reported as "positive" or "negative" for each pathogen, based on detection of an amplification product of the correct size by probing with each internal probe.

3.2.4 Direct fluorescent antibody detection of *V. cholerae*

Formaldehyde-preserved water samples were processed in a similar manner to that described by Islam et al. (1993) . Bacteria in water samples (50 ml) were precipitated by centrifugation and resuspended in 25 µl of 0.2 µm-filtered water from the

same sample. A 5-µl volume of this suspension was placed on a well glass slide, dried, fixed and challenged by addition of monoclonal antiserum to the well. After incubation, rinsing, and addition of appropriate fluorescein isothiocyanate-conjugated serum, cells were enumerated under an epifluorescence microscope. Suitable positive and negative control organisms were also processed. This procedure was followed using monoclonal antibodies for *V. cholerae* 01 and *V. cholerae* O139 available in our laboratory.

3.2.5 *V. alginolyticus* as a marker of the indigenous bacterial community

An alkaline phosphatase labeled probe was used to detect culturable *V. alginolyticus* cells in selected water samples from Mamala Bay. This probe was designed to detect the sucrase gene (Scholle et al., 1989) and was specific for *V. alginolyticus* (J. Johnson, pers. comm.). The probe sequence is 5'-CAAGACTACGATTCTCATGGTGTC-3'. The procedure used was as described in section 3.2.2. above.

3.2.6 Survival studies

Microcosm studies were performed with *Shigella* spp., enterotoxigenic *E. coli*, *C. jejuni* and *V. cholerae* in microcosms containing water collected from Station D2B. In all cases, microcosms were set up according to the method routinely employed in our laboratory, described in Ravel et al. (1995). Conditions were chosen to replicate the most favorable conditions likely to be present in Mamala Bay waters. For this reason, low levels of light were used (in a 12 h/12 h light dark cycle) and microcosms were performed in glass flasks which exclude UV light. All microcosm experiments were performed in triplicate.

4 RESULTS

4.1 Bacterial Counts

Total bacterial counts (AODC's) are given in Table 4.1. Culturable bacterial counts in water and sediment samples are given in Tables 4.2 and 4.3, respectively. Counts in sediment samples ranged between 1.0 x 10^2 and 4.6 x 10^4, but no clear trends of elevated counts at nearshore stations or stations where higher bacterial loads were expected (AW1, D2B) were observed. The following trends were apparent in total and culturable counts from water samples:

Ala Wai Canal: Water samples collected at AW1 had higher concentrations of total and culturable bacteria than at the offshore Ala Wai station (AW2). The mean total bacterial count was 3.4 x 106 cells/ml at AW1 and 3.3 x 105 cells/ml at AW2. The mean culturable bacterial count for all quarterly samples was 4.1 x 104 CFU/ml at AW1 and 2.1 x 101 CFU/ml at AW2. These counts suggest that a large bacterial load is entering Mamala Bay from the Ala Wai canal.

Sand Island Outfall: During June 1994 and November 1994, total bacterial counts in samples from D2B were more than three-fold greater than corresponding counts in samples from D2M. Culturable bacterial counts were more than 10-fold greater in the sample from D2B than in the sample from D2M in June 1994. However, differences of similar magnitudes were observed between samples collected from E4M and E4B off Diamond Head, indicating that bacterial counts may differ by these amounts in waters without a sewage input. The most striking result from bacterial enumeration at D2B is that the very high bacterial counts in primary sewage (1.8-6.5 x 10^7 cells/ml total counts, 1.5-42 x 10^5 CFU/ml culturable counts) are rapidly diluted by several orders of magnitude to give the counts observed in samples from D2B.

Table 4.1. Total bacterial counts in water samples obtained from Mamala Bay. Counts were obtained by the acridine orange direct counting procedure.

Station Code	Cell Count (x 10^5/ml) ± S. E.			
	October 93	February 94	June 94	November 94
MS	26 ± 3.0	54.0 ± 6.6	35.0 ± 5	20.0 ± 9
AW1	48 ± 5.0	59.8 ± 1.1	19.0 ± 3.6	8.8 ± 1.0
AW2	2.2 ± 0.2	6.8 ± 0.6	1.7 ± 0.2	2.6 ± 0.5
W1	8.1 ± 1.5	6.4 ± 1.1	13 ± 3	6.8 ± 1.5
W2	3.6 ± 0.4	9.8 ± 0.8	6.7 ± 0.5	3.2 ± 0.6
D0	8.1 ± 1.2	28.4 ± 3.6	28 ± 1.6	14 ± 2.3
D1S	3.6 ± 1.1	N.S.[1]	2.5 ± 0.3	0.5 ± 0.1
D1B	6.0 ± 1.5	6.2 ± 0.3	3.7 ± 0.5	N.S.
D2S	1.8 ± 0.2	7.4 ± 1.3	8.5 ± 2.4	8.0 ± 2.0
D2M	N.S.[1]	8.5 ± 1.3	7.5 ± 1.1	4.3 ± 1.0
D2B	4.2 ± 0.5	8.5 ± 1.4	22.0 ± 1.9	18 ± 11
D3S	3.2 ± 0.4	1.7 ± 0.6	5.9 ± 0.9	1.4 ± 0.2
D3B	N.S.	3.6 ± 0.8	11.0 ± 2.2	5.1 ± 2.6
C1	6.5 ± 2.0	22.8 ± 2.6	35.0 ± 6	3.0 ± 0.3
C2	8.5 ± 0.5	3.0 ± 0.5	12.2 ± 1.5	5.5 ± 1.2
C3	3.8 ± 0.4	9.1 ± 0.9	8.6 ± 0.7	7.3 ± 0.7
E4S	2.2 ± 0.5	2.1 ± 0.9	2.3 ± 0.6	8.5 ± 2.0
E4M	1.7 ± 0.3	3.9 ± 0.5	1.9 ± 0.3	1.7 ± 0.4
E4B	3.5 ± 0.4	1.9 ± 0.6	18.0 ± 2.3	3.6 ± 2.7
HB	2.6 ± 0.6	3.5 ± 0.5	3.4 ± 0.4	1.5 ± 0.6
AM1	N.S.	14.6 ± 1.1	24.0 ± 4.6	7.0 ± 1.3
S	N.S.	650 ± 1.5	350.0 ± 18	183 ± 27

N.S.[1] No sample

4.2 Probe detection of culturable bacteria

Results of DNA probe detection of specific pathogenic bacteria are given in Table 4.4 (Cholera Toxin-containing bacteria), Table 4.5 (*Shigella* spp.) and Table 4.6 (enterotoxigenic *E. coli*). Results for *Campylobacter jejuni* have were not obtained due to problems with the specificity of the *C. jejuni* probe, which appeared to be hybridizing with DNA from bacteria other than *C. jejuni*.

Detection limits varied for samples processed by this method, depending on which dilution gave plates most suitable for colony lifts (i.e. with a suitable number of colonies for lifting). For this reason, negative results are given as less than the specific detection limit, in each case.

This methodology was successful in detecting pathogenic vibrios carrying the Cholera Toxin gene, pathogenic *Shigella* spp. harboring the *ipaH* pathogenicity-related gene and enterotoxigenic *E. coli* in water and sediment samples from Mamala Bay. This is the first report, to our knowledge, of direct detection of these pathogens in Hawaiian waters. Detection of these pathogens is discussed by site:

Ala Wai Canal: *Shigella* spp. and enterotoxigenic *E. coli* were isolated from three samples and one sample respectively, from AW1. The Ala Wai Canal is therefore a potential source of these pathogenic bacteria. Cholera Toxin-positive bacteria were not isolated from water or sediment samples from AW1, although they were detected in two water samples from AW2.

Sand Island Outfall: Enterotoxigenic *E. coli* were found in five of eleven samples from D2 and was present in three of four samples of primary sewage. Pathogenic *Shigella* spp. were present in three of 11 samples from D2 and two of four samples of primary sewage. This suggests that the outfall is a source of both these pathogens. Cholera Toxin-positive bacteria were present in three of 11 samples from D2 and one of three samples of primary

sewage, suggesting that the outfall may be a source of these bacteria. However, Cholera Toxin-positive bacteria may also be indigenous to Mamala Bay waters (see below).

Pearl Harbor: Enterotoxigenic *E. coli* and *Shigella* spp. were each found in one of nine samples from Pearl Harbor. This suggests that the harbor may be a low-level source of these pathogens.

Beaches: Culturable *Shigella* spp. were detected in samples from beaches, at W1 and HB, whereas enterotoxigenic *E. coli* was not detected in samples from beaches.

Table 4.4. Counts of culturable bacteria containing the Cholera Toxin gene.

Station	*CT*+(CFU/100ml)			
	November 1993	February 1994	June 1994	November 1994
MS	-[a]	$<10^3$	**2.0×10^2**	**2.5×10^0**
MS sediment	-	-	$<10^2$	-
AW1	$<10^5$	$< 10^5$	$<10^2$	$<10^2$
AW1 sediment	-	-	-	$<10^2$
AW2	$<10^3$	**2.5×10^0**	$<10^1$	**4.0×10^2**
AW2 sediment	$<10^3$	**1.3×10^1**	$<10^2$	$<10^2$
W1	-	$<10^2$	$<10^1$	-
W1 sediment	-	$<10^3$	-	$<10^2$
W2	$<10^1$	$<10^1$	$<10^1$	**1.2×10^1**
W2 sediment	$<10^3$	-	$<10^2$	**5.0×10^3**
D0	$<10^3$	$<10^2$	**4.0×10^3**	-
D0 sediment	-	$<10^3$	-	-
D1S	-	**2.5×10^0**	$<10^1$	$<10^1$
D1B	-	-	$<10^1$	-
D1 sediment	-	$<10^3$	-	$<10^2$
D2S	-	$<10^2$	$<10^1$	**1.6×10^1**
D2M	-	$<10^2$	$<10^1$	$<10^1$
D2B	**1.3×10^2**	$<10^3$	$<10^2$	$<10^1$
D2 sediment	-	**8.0×10^2**	-	-
D3S	-	-	$<10^1$	-
D3B	-	-	$<10^1$	-
D3 sediment	-	-	-	-
C1	-	$<10^1$	$<10^1$	$<10^1$
C1 sediment	-	-	$<10^2$	-
C2	-	-	$<10^1$	-
C2 sediment	-	-	-	-
C3	-	-	$<10^1$	**8.0×10^2**
C3 sediment	-	$<10^1$	$<10^1$	**8.6×10^4**
E4S	$<10^3$	$<10^2$	$<10^2$	$<10^1$
E4M	$<10^3$	-	$<10^2$	$<10^1$
E4B	$<10^3$	$<10^3$	**9.0×10^2**	**4.0×10^0**
HB	$<10^3$	$<10^2$	$<10^1$	**1.2×10^1**
HB sediment	-	$<10^3$	$<10^2$	**1.0×10^1**
S	$<10^6$	**2.8×10^5**	$<10^6$	-
AM1	-	-	$<10^2$	$<10^1$
AM1 sediment	-	-	$<10^2$	$<10^2$

[a]Signifies no sample available.

Table 4.5. Counts of *Shigella* spp. detected by the presence of the *ipaH* gene.

Station	*Ipa*$^+$*Shigella* (CFU/100 ml)			
	October 1993	February 1994	June 1994	November 1994
MS	-[a]	$<10^3$	$<10^3$	$<10^1$
MS sediment	-	-	$<10^2$	$<10^2$
AW1	$<10^5$	**5.0×10^4**	**2×10^2**	$<10^2$
AW1 sediment	-	$<10^3$	$<10^2$	**2.0×10^2**
AW2	$<10^3$	$<10^1$	$<10^1$	$<10^1$
AW2 sediment	-	**2.0×10^3**	$<10^2$	$<10^2$
W1	-	$<10^2$	**4.0×10^2**	**8×10^0**
W1 sediment	-	$<10^3$	-	$<10^2$
W2	$<10^1$	$<10^1$	$<10^1$	$<10^1$
W2 sediment	-	$<10^3$	-	$<10^2$
D0	$<10^3$	$<10^2$	$<10^2$	**1.2×10^3**
D0 sediment	-	$<10^3$	-	$<10^2$
D1S	-	$<10^1$	$<10^1$	$<10^1$
D1B	-	$<10^1$	$<10^1$	-
D1 sediment	-	$<10^3$	-	**4×10^3**
D2S	-	**3.3×10^1**	$<10^1$	$<10^1$
D2M	-	**3.3×10^1**	$<10^1$	$<10^1$
D2B	$<10^1$	$<10^3$	$<10^2$	$<10^1$
D2 sediment	-	**2.5×10^3**	-	-
D3S	-	$<10^2$	$<10^1$	$<10^1$
D3B	-	$<10^3$	$<10^1$	**4.0×10^2**
D3 sediment	-	-	-	-
C1	-	$<10^1$	$<10^1$	$<10^1$
C1 sediment	-	-	-	-
C2	-	$<10^1$	$<10^1$	-
C2 sediment	-	-	-	-
C3	-	**1.6×10^0**	$<10^1$	$<10^1$
C3 sediment	-	-	-	$<10^2$
E4S	$<10^3$	$<10^2$	$<10^2$	$<10^1$
E4M	$<10^3$	$<10^3$	$<10^2$	$<10^1$
E4B	$<10^3$	$<10^3$	$<10^2$	$<10^1$
HB	$<10^3$	$<10^2$	$<10^1$	**1.6×10^3**
HB sediment	-	$<10^3$	$<10^2$	$<10^2$
S	**1.3×10^5**	$<10^6$	$<10^6$	**1.6×10^6**
AM1	-	-	$<10^2$	$<10^2$
AM1 sediment	-	-	$<10^2$	$<10^2$

[a]Signifies no sample available.

Table 4.6. Counts of enterotoxigenic *E. coli* detected by the presence of the LT gene.

Station	*LTH*$^+$(CFU/100 ml)			
	October 1993	February 1994	June 1994	November 1994
MS	-[a]	$<10^3$	$<10^3$	**5.0×10^2**
MS sediment	-	-	$<10^2$	**2.7×10^4**
AW1	$<10^5$	$< 10^5$	$<10^2$	**1.3×10^3**
AW1 sediment	-	$<10^3$	$<10^2$	$<10^2$
AW2	$<10^3$	$<10^1$	$<10^1$	**1.6×10^1**
AW2 sediment	-	$<10^3$	$<10^2$	**1.0×10^3**
W1	-	$<10^2$	$<10^1$	-
W1 sediment	-	$<10^3$	-	$<10^2$
W2	$<10^1$	$<10^1$	$<10^1$	$<10^2$
W2 sediment	-	$<10^3$	$<10^2$	**1.0×10^3**
D0	$<10^3$	$<10^2$	$<10^2$	$<10^1$
D0 sediment	-	$<10^3$	-	$<10^2$
D1S	-	$<10^1$	$<10^1$	$<10^1$
D1B	-	**2×10^0**	$<10^1$	-
D1 sediment	-	$<10^3$	-	$<10^2$
D2S	-	$<10^2$	$<10^1$	**2.0×10^3**
D2M	-	$<10^2$	$<10^1$	$<10^1$
D2B	**3.3×10^2**	**1.7×10^2**	$<10^2$	**6.8×10^3**
D2 sediment	-	**8.3×10^2**	-	-
D3S	-	$<10^2$	$<10^1$	**1.7×10^4**
D3B	-	$<10^3$	$<10^1$	$<10^1$
D3 sediment	-	-	-	-
C1	-	$<10^1$	$<10^1$	$<10^1$
C1 sediment	-	-	-	-
C2	-	$<10^1$	$<10^1$	-
C2 sediment	-	-	-	-
C3	-	$<10^1$	$<10^1$	$<10^1$
C3 sediment	-	-	-	**8.6×10^4**
E4S	$<10^3$	**3.2×10^2**	$<10^2$	**4.0×10^2**
E4M	$<10^3$	$<10^3$	$<10^2$	$<10^1$
E4B	$<10^3$	$<10^3$	-	$<10^1$
HB	$<10^3$	$<10^2$	$<10^1$	$<10^1$
HB sediment	-	$<10^3$	$<10^2$	$<10^2$
S	$<10^6$	**6.4×10^5**	**2.0×10^5**	**3.5×10^6**
AM1	-	$<10^2$	$<10^2$	$<10^1$
AM1 sediment	-	-	$<10^2$	$<10^2$

[a]Signifies no sample available.

4.3 PCR detection of pathogens

Detection limits of this method for *V. cholerae* and *S. flexneri* were determined by processing "spiked" water samples and were ca. 10 cells/100 ml (results not shown). The specificity of PCR primers and internal probes for *V. cholerae*, enterotoxigenic *E. coli* and *Shigella flexneri* was tested to ensure that each methodology did not result in detection of the other two bacteria. The LT gene amplification product in enterotoxigenic *E. coli* gave a very small hybridization signal with the Cholera Toxin internal probe (not apparent in Fig. 8.1) but this cross-reaction was judged to be insufficient to give incorrect results from our environmental samples. Additional testing of specificity of primers and probes was not necessary since probes and primers were all previously verified (Fields et al., 1992; Koch et al. 1993; Lang et al., 1994; Sethabutr et al., 1993) .

Results from PCR detection of pathogens are shown in Table 4.7 (Cholera Toxin-positive bacteria), Table 4.8 (Shigella spp.), Table 4.9 (enterotoxigenic *E. coli*), and Table 4.10 (*Campylobacter jejuni*). Detection of these pathogens is discussed by site:

Ala Wai: Cholera Toxin-positive bacteria were detected in two of four samples from AW1 but these bacteria were found in many other samples, including samples from stations off Diamond Head, indicating that they may be ubiquitous in Mamala Bay waters. AW1 samples were negative for PCR detection of *Shigella* spp. One of three samples from AW1 was positive for enterotoxigenic *E. coli*. The only other sample in which enterotoxigenic *E. coli* was detected by PCR was a sewage sample.

Sand Island Outfall: Enterotoxigenic *E. coli* was not detected at the D2 station by PCR, whereas *Shigella* spp. were detected in three of nine samples. Cholera Toxin-positive bacteria were present in seven of nine samples from D2 analyzed by PCR.

Pearl Harbor: *Shigella* spp. were present in three of ten samples and enterotoxigenic *E. coli* was not detected in samples from Pearl Harbor. Cholera Toxin-positive bacteria were detected in six of nine Pearl Harbor samples analyzed by PCR.

Beaches: Cholera Toxin-positive bacteria were detected in samples from Ala Moana and Hanauma Bay. *Shigella* spp. were detected in samples from Ala Moana, Hanauma Bay, and Waikiki Beach but enterotoxigenic *E. coli* was not detected in samples from beaches.

4.4 Comparison of results obtained by probe detection of culturable bacteria and by PCR detection

Positive results obtained by both molecular detection methods are summarized in Table 4.11. In 46 samples analyzed by both approaches, PCR detection gave positive results whereas no corresponding culturable pathogenic bacteria were detected by using gene probes. This suggests that pathogens are frequently present in the viable but nonculturable state. Another possibility is that detection by PCR is more sensitive. In trials to determine the sensitivity of PCR detection, concentrations of 10 cells/100 ml were readily detected by our methodology (results not shown). Detection limits for probing of culturable cells were frequently $>10^3$ cell/ml.

Of greater concern are cases in which pathogens were detected by probing of culturable cells but were not detectable by PCR in the same samples. One possible explanation is that PCR was inhibited by contaminating substances present in DNA preparations, in spite of precautions taken to avoid this problem. Experiments are underway where DNA samples that were negative for a particular pathogen are "spiked" with very small concentrations of purified DNA from that pathogen. Failure to detect PCR amplification products on these "spiked" samples will indicate that inhibitory substances are indeed present.

Table 4.7. Qualitative results of polymerase chain reaction (PCR) detection of CT-positive bacteria in water samples from Mamala Bay, Hawaii.
Positive results are indicated by Y and negative results by N.

Station Code	Oct. '93	Feb. '94	June '94	Nov. '94
AM1	-[a]	-	Y	N
AM1 p.m.	-	-	Y	N
AW1	N	N	Y	Y
AW1 p.m.	-	-	-	-
AW2	-	-	N	N
C1	-	N	Y	Y
C2	Y	N	Y	N
C3	-	Y	Y	-
D0	-	N	Y	Y
D1S	Y	-	Y	Y
D1B	-	-	Y	-
D2S	-	N	-	Y
D2M	-	Y	Y	Y
D2B	Y	N	Y	Y
D3S	-	Y	Y	Y
D3M	-	-	Y	Y
D3B	-	Y	Y	Y
E4S	Y	Y	Y	Y
E4M	-	N	Y	Y
E4B	-	-	Y	Y
HB1	-	N	Y	-
MS1	N	N	N	-
S	-	N	-	Y
W1	-	-	-	N
W2	-	-	Y	Y

[a] - Signifies no sample available

Table 4.8. Qualitative results of polymerase chain reaction (PCR) detection of pathogenic *Shigella* spp. in water samples from Mamala Bay, Hawaii. Positive results are indicated by Y and negative results by N.

Station Code	Oct. '93	Feb. '94	June '94	Nov. '94
AM1	-[a]	Y	Y	-
AM1 p.m.	-	N	N	N
AW1	-	N	N	N
AW1 p.m.	-	N	-	-
AW2	-	N	N	N
C1	-	Y	N	N
C2	N	N	Y	N
C3	-	N	N	N
D0	-	N	Y	-
D1S	N	N	N	Y
D1B	-	N	Y	-
D2S	N	N	-	N
D2M	-	Y	-	N
D2B	Y	Y	N	N
D3S	-	N	N	N
D3M	-	-	N	N
D3B	-	N	Y	N
E4S	N	N	Y	N
E4M	N	N	N	N
E4B	-	N	N	N
HB1	N	N	Y	N
MS1	N	N	N	N
S	-	Y	Y	Y
W1	N	N	N	N
W1 p.m.	-	-	Y	-
W2	-	N	N	N

[a] - Signifies no sample available

Table 4.9. Qualitative results of polymerase chain reaction (PCR) detection of enterotoxigenic *E. coli* in water samples from Mamala Bay, Hawaii. Positive results are indicated by Y and negative results by N.

Station Code	Oct. '93	Feb. '94	June '94	Nov. '94
AM1	-[a]	N	N	N
AM1 p.m.	-	-	N	N
AW1	N	N	Y	N
AW1 p.m.	-	-	-	-
AW2	N	N	N	N
C0	-	-	N	-
C1	N	N	N	N
C2	N	N	N	N
C3	N	N	N	N
D0	N	N	N	-
D1S	N	N	N	N
D1B	N	N	N	-
D2S	N	N	-	N
D2M	-	N	-	N
D2B	N	N	N	N
D3S	N	N	N	N
D3M	-	-	N	N
D3B	N	N	N	N
E4S	N	N	N	N
E4M	N	N	N	N
E4B	N	N	N	N
HB1	N	N	N	N
MS1	N	N	N	N
S	-	N	Y	N
W1	N	N	N	N
W1 p.m.	-	-	N	-
W2	N	N	N	N

[a] - Signifies no sample available

Table 4.10. Qualitative results of polymerase chain reaction (PCR) detection of *Campylobater jejuni* in water samples from Mamala Bay, Hawaii. Positive results are indicated by Y and negative results by N.

Station Code	Oct. '93	Feb. '94	June '94	Nov. '94
AM1	-[a]	N	N	N
AM1 p.m.	-	-	Y	N
AW1	N	N	N	N
AW1 p.m.	-	-	-	-
AW2	N	N	N	N
C0	-	-	N	-
C1	N	N	N	N
C2	N	N	N	N
C3	N	N	N	N
D0	N	N	N	N
D1S	N	N	N	N
D1B	N	N	N	-
D2S	N	N	-	N
D2M	-	N	-	N
D2B	N	N	N	N
D3S	N	N	N	-
D3M	-	-	N	N
D3B	N	N	N	N
E4S	N	N	N	N
E4M	N	-	N	N
E4B	-	N	N	N
HB1	N	-	N	N
MS1	N	N	N	N
S	-	N	Y	Y
W1	N	N	N	N
W1 p.m.	-	-	N	-
W2	N	N	N	N

[a] - Signifies no sample available

4.5. Significance of Cholera Toxin-positive results

The Cholera Toxin is generally associated with *Vibrio cholerae* O1, which has the potential to cause epidemics of cholera. However, the Cholera Toxin gene has also been detected in other vibrios and related organisms, including *Vibrio mimicus* and *V. cholerae* O139 (Chowdhury et al., 1994) , a non-O1 strain which recently caused a pandemic of cholera in southern Asia (Ramamurthy et al., 1993) . The Cholera Toxin gene may also be present in other non-O1 strains of *V. cholerae* (Tamplin et al., 1990) . There is considerable evidence that *V.* cholerae O1 and non-O1 strains are indigenous to the marine environment (Colwell et al., 1990; Xu et al., 1982) . It is therefore likely that the Cholera Toxin-positive bacteria detected in this study are part of the natural bacterial community of Mamala Bay and are not necessarily present as a result of sewage discharge into the Bay. This is supported by detection of Cholera Toxin-positive bacteria in two samples collected at the Diamond Head station (E4). However, Cholera Toxin-positive bacteria were detected at concentrations of 2.8 x 10^5 CFU/ml and 3.5 x 10^6 CFU/ml in two primary sewage samples, suggesting that the Sand Island outfall is a possible source of these organisms.

4.6 Direct fluorescent antibody detection of *V. cholerae*:

A limited number of samples have been processed for direct fluorescent detection of V. cholerae O1 and V. cholerae O139. These results are presented in Table 4.12. Detection of both O1 and non-O1 strains of V. cholerae by this technique provides additional evidence that these organisms are ubiquitous in Mamala Bay waters.

Table 4.11. Molecular detection of pathogens: Summary of positive results from probing and PCR experiments.

Bacteria detected are designated as follows: Cholera Toxin-positive bacteria (V); *Shigella* spp. (S); enterotoxigenic *E. coli* (E); *Campylobacter jejuni* (C). Bold type indicates pathogens detected by PCR and plain type indicates culturable bacteria detected by using specific gene probes.

Station	Date of Sample Collection			
	October 1993	February 1994	June 1994	November 1994
MS			V	V E
AW1		S	**V S E**	**V S E**
AW2		V		V E
W1			**S S**	S
W2			**V**	**V V**
D0			**V V S**	**V S**
D1S	**V**	V	**V**	**V S**
D1B		E	**V S**	
D2S		S		V **V** E
D2M		**V S S**	**V**	**V**
D2B	V **V** E **S**	E **S**	**V**	**V** E
D3S		**V**	**V**	**V** E
D3B			**V S**	**V S**
C1		**S S**	**V**	**V**
C2	**V**		**V S**	
C3		**V**	**V**	V
E4S	**V**	V E	**V S**	**V** E
E4M			**V**	**V**
E4B			**V V**	**V V**
HB			**V S**	S V
S	S	V **E S**	V **S E E C**	**V S S E C**
AM1		**S**	**V S**	

4.7 V. *alginolyticus* as a marker of the indigenous bacterial community

A limited number of samples were processed for detection of *V. alginolyticus* by using a *V. alginolyticus*-specific probe to screen colonies that grew on nonselective medium. Results from these samples are presented in Table 4.13. No clear trends were present in distribution patterns of *V. alginolyticus*.

4.8 Survival studies

Results of survival studies are shown in Figs. 8.1-8.10 (Appendix). In some cases, results of direct viable counts and total counts are not included since these samples are still being processed. All culturable counts are shown in these figures. Loss of culturability measured in all microcosm experiments are summarized in Table 4.14. It is important to note that rates of loss of culturability and viability are conservative, since natural intensities of UV radiation were not replicated in microcosm experiments. Cells in microcosms were exposed to negligible UV intensities and survival rates are likely to be greater for this reason. Most microcosm experiments were performed with 0.1 μm-filtered water, in which all bacteria protozoal grazers would be absent, although one set of experiments were performed in "whole" (unfiltered) water. Microcosm experiments were performed at 28°C and 22°C. These temperatures were selected as representative high and low temperatures in Mamala Bay waters. Water from Station D2B was used in microcosms. This water is obtained in the vicinity of the Sand Island outfall and is likely to be nutrient-enriched and should therefore enhance survival. Thus, inactivation rates obtained in these experiments are likely to approach the minimum

Table 4.12. Detection of *V. cholerae* O1 and O139 by direct fluorescent antibody microscopy.

Station	February 1994		November 1994	
	V. cholerae O1 (cells/ml)	*V. cholerae* O139 (cells/ml)	*V. cholerae* O1 (cells/ml)	*V. cholerae* O139 (cells/ml)
AM1 a.m.	0	7	7*	2*
AM1 p.m.	-[a]	-	4*	6*
AW1 a.m.	0	7	0	0
AW1 p.m.	-	-	0	0
AW2	3	7	2	0
C1S	0	0	0	0
C2S	0	0	0	0
C3S	0	0	0	0
D0	0	0	1	0
D1S	0	0	3	0
D1B	0	0	-	-
D2S	18	10	2	0
D2M	1	-	8	0
D2B	0	0	2	0
D3S	0	0	5	0
D3B	0	0	0	0
E4S	0	-	3	0
E4M	0	-	0	0
E4B	0	-	0	0
HB1	0	0	0	0
MS1	1	-	2	0
S	17	17	0	0
W1 a.m.	0	0	0	0
W1 p.m.	-	-	0	0
W2	0	-	1	0

[a]Signifies no sample available.

Table 4.13. Counts of culturable *V. alginolyticus* determined by probing colony lifts with a *V. alginolyticus*-specific probe.

Station	*V. alginolyticus*$^+$(CFU/100ml)			
	November 1993	February 1994	June 1994	November 1994
MS	-[a]	**5.0×10^0**	**2.0×10^1**	**5.0×10^0**
MS sediment	-	-	$<10^2$	$<10^2$
AW1	$<10^4$	**8.0×10^5**	**2.5×10^4**	**2.8×10^2**
AW1 sediment	-	-	-	**1.5×10^3**
AW2	**5.0×10^2**	-	**6.8×10^1**	**2.4×10^2**
AW2 sediment	-	-	-	**6.3×10^4**
W1	$<10^3$	**3.3×10^1**	**5.0×10^1**	**2.1×10^2**
W1 sediment	-	-	-	**1.3×10^4**
W2	**2.0×10^1**	**4.0×10^1**	**1.7×10^1**	**6.0×10^1**
W2 sediment	-	**8.0×10^3**	**2.0×10^3**	**7.5×10^3**
D0	-	**6.0×10^1**	**2.0×10^2**	**1.1×10^2**
D0 sediment	-	**2.5×10^2**	-	$<10^2$
D1S	-	$<10^1$	**2.0×10^1**	-
D1B	-	**5.0×10^1**	**2.3×10^1**	-
D1 sediment	-	**2.0×10^3**	-	**4.5×10^4**
D2S	-	**4.3×10^2**	**8.0×10^0**	**1.0×10^1**
D2M	-	-	**1.8×10^1**	$<10^1$
D2B	**5.0×10^2**	$<10^3$	$<10^2$	$<10^1$
D2 sediment	-	**7.0×10^3**	-	-
D3S	-	$<10^2$	$<10^1$	-
D3B	-	$<10^3$	$<10^1$	-
D3 sediment	-	-	-	-
C1	-	$<10^1$	**1.0×10^2**	**6.0×10^2**
C1 sediment	-	-	**2.0×10^3**	**6.5×10^4**
C2	-	**1.0×10^1**	**1.8×10^1**	-
C2 sediment	-	-	-	-
C3	-	**3.3×10^0**	$<10^1$	**7.0×10^2**
C3 sediment	-	-	-	-
E4S	$<10^3$	$<10^2$	$<10^2$	$<10^1$
E4M	$<10^3$	-	**6.6×10^1**	$<10^1$
E4B	$<10^3$	-	$<10^2$	$<10^1$
HB	**1.3×10^2**	**1.7×10^2**	**9.3×10^1**	**1.6×10^2**
HB sediment	-	$<10^3$	**5.5×10^1**	**3.3×10^4**
S	$<10^5$	**2.5×10^3**	$<10^6$	$<10^6$
AM1	-	**2.0×10^2**	**3.3×10^1**	**6.0×10^1**
AM1 sediment	-	-	**7.2×10^3**	**3.0×10^2**

[a]Signifies no sample available.

Table 4.14. Rates of loss of culturability and viability in microcosm experiments.
Microcosms contained water from Mamala Bay (Station D2B) and were incubated at low light intensity (12 h light/12 h dark cycle).

Organism	Type of count	Temperature (_C)	Water treatment[c]	Rate (h^{-1})[d] x 10^{-2} ±S.D.
Vibrio cholerae	Culturable[a]	22	Filtered	-1.10 ± 0.10
	Viable[b]	22	Filtered	-0.70 ± 0.13
Escherichia coli	Culturable	22	Filtered	-18.0 ± 2.50
	Viable	22	Filtered	-0.95 ± 0.14
Shigella flexneri	Culturable	22	Filtered	-14.0 ± 6.75
	Viable	22	Filtered	-8.02 ±0.54
Vibrio cholerae	Culturable	28	Filtered	-0.67 ± 0.06
	Viable	28	Filtered	-0.31 ± 0.03
Escherichia coli	Culturable	28	Filtered	-0.79 ± 0.07
	Viable	28	Filtered	-0.24 ± 0.09
Shigella flexneri	Culturable	28	Filtered	-20.7 ± 21.0
	Viable	28	Filtered	-6.38 ± 4.0
Vibrio cholerae	Culturable	28	Unfiltered	-14.6 ± 1.4
Escherichia coli	Culturable	28	Unfiltered	-11.5± 0.67
Shigella flexneri	Culturable	28	Unfiltered	-17.8 ± 0.92

[a]Culturable counts were determined from counts of colony forming units on Luria agar.

[b]Viable counts were determined using the direct viable count technique (Kogure et al., 1979).

[c]Water treatments were: Filtered-microcosms contained water from station D2B which was filtered through a 0.1 μm filter. Unfiltered-microcosms contained "whole" untreated water from station D2B.

[d]Loss rates were determined according to the following formula: $\ln C_t - \ln C_0/t$, where C_t equals cell concentration at time t; C_0 equals cell concentration at time 0 and t equals number of hours elapsed between C_t and C_0.

5 CONCLUSIONS

1. The four bacterial pathogens of interest, *Shigella* spp., enterotoxigenic *E. coli*, *C. jejuni* and Cholera Toxin-positive vibrios, were detectable in waters of Mamala Bay by both the molecular techniques employed in this study.

2. The pathogens *Shigella* spp. and enterotoxigenic *E. coli* were present in multiple samples from the vicinity of the Sand Island sewage outfall and the mouth of the Ala Wai Canal, and were detectable in primary sewage samples. Concentrations of these pathogens in water samples from the vicinity of the outfall were several orders of magnitude lower that in primary sewage, indicating rapid dilution of primary sewage at the outfall.

3. Culturable *Shigella* spp. were detected by a gene probe in samples from Waikiki Beach and Hanauma Bay. This constitutes a potential public health risk. The origin of *Shigella* found on Waikiki Beach could have been the Sand Island outfall, the Ala Wai Canal, shedding from bathers, or some other source. We could not distinguish between these possible origins by the techniques used in this study. The presence of *Shigella* spp. in the enclosed space of Hanauma Bay does, however, suggest that shedding from bathers is a likely source. *Shigella* spp. were also detected by PCR amplification in samples from Ala Moana Beach and Hanauma Bay.

4. *C. jejuni* was detected by PCR amplification in one sample from Ala Moana Beach and in two primary sewage samples. This is the first report, to our knowledge, of detection of *C. jejuni* in environmental samples. Absence of *C. jejuni* at all other stations and the very rapid loss of culturability found in survival studies with this organism suggest that the *C. jejuni* detected at a beach site is likely to have been shed by bathers.

5. Cholera Toxin-positive bacteria were detected by probe and PCR studies and were present in samples from many stations. Cholera Toxin is generally associated with

Vibrio cholerae O1, which has the potential to cause epidemics of cholera. However, the Cholera Toxin gene has also been detected in other vibrios and related organisms and in non-O1 strains of *V. cholerae* . There is considerable evidence that *V. cholerae* O1 and non-O1 strains are indigenous to the marine environment. It is therefore likely that the Cholera Toxin-positive bacteria detected in this study are part of the natural bacterial community of Mamala Bay and are not necessarily present as a result of sewage discharge into the Bay.

6. In some samples analyzed by both molecular methods used in this study, PCR detection gave positive results whereas no corresponding pathogenic bacteria were detected by using gene probes. This suggests that pathogens might be present in the viable but nonculturable state or that detection by PCR is more sensitive under some conditions. In some cases, culturable bacteria were detected by gene probing techniques but PCR amplification of the same samples gave negative results. PCR may have been inhibited by contaminating substances present in some samples, in spite of precautions taken to avoid this problem. We conclude that use of both detection methods is necessary for optimal detection of pathogens.

7. No changes were detected in the indigenous bacterial community in the vicinity of the Sand Island outfall by using *V. alginolyticus* as a "marker" of the indigenous bacterial community. No clear trends in distribution patterns of *V. alginolyticus* were apparent and this organism was not ubiquitous in marine waters as we had expected. *V. alginolyticus* was therefore found not to be a useful marker of the indigenous marine bacterial community. We were unable to draw any conclusions on the possible effects of sewage disposal on the indigenous microbial community in Mamala Bay.

8. Survival experiments showed that *V. cholerae*, *Shigella* spp. and enterotoxigenic *E. coli* remained culturable for extended periods under optimal conditions, but that under typical conditions present in Mamala Bay, *Shigella* spp. and

enterotoxigenic *E. coli* were likely become nonculturable in less than one day, with *V. cholerae* remaining culturable for longer periods. In all cases, *V. cholerae*, *Shigella* spp. and enterotoxigenic *E. coli* retained viability considerably longer than culturability, indicating the importance of detection of viable as well as culturable cells. C. *jejuni* became nonculturable very rapidly in all experiments and culturable forms are unlikely to be detectable in environmental samples.

6 RECOMMENDATIONS

6.1 Recommended Actions

This study indicated that pathogenic bacteria were present in some water samples from beaches and this is a concern in terms of public health. Our data do not exclude the possibility that these pathogens are shed by bathers. However, we have shown that there is an input of pathogens into Mamala Bay from the Sand Island outfall and the Ala Wai Canal. Any measures that reduce these inputs may have public health benefits.

6.2 Future Studies

1. There are no standards in recreational waters for concentrations of pathogenic bacteria such as those investigated in this study. Direct detection of pathogens has been shown to be feasible. Additional direct molecular monitoring of pathogens will be useful in establishing "background data" which is necessary before standards can be set.

2. The presence of Cholera Toxin-positive bacteria in Mamala Bay is intriguing and may have important public health implications. However, further molecular studies are required to determine potential risks of these bacteria. Are these Cholera Toxin-positive bacteria *V. cholerae* O1 strains or other strains of vibrios? Do these strains have the potential to cause epidemics? Research on the molecular biology of these strains is required to address these questions.

3. Further research is needed in order to develop techniques that can be used to track specific clonal populations of bacteria. This would enable us to determine the origin of pathogenic strains isolated from samples collected on beaches.

4. The pathogen *Staphylococcus aureus* has not been investigated in this study. Molecular techniques have been established to detect *S. aureus* [e.g. Neill et al. (1990)].

The presence of this pathogen in waters of Mamala Bay should be investigated by using a combination of conventional and molecular approaches.

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8 APPENDICES

Appendix 1.

Boccuzzi, V. M., W. L. Straube, J. Ravel, R. R. Colwell and R. T. Hill. 1995. Presented at the 95th General Meeting of the American Society for Microbiology, Washington, D. C.

N-168 Removal of Contaminating Substances from Environmental Samples Prior to PCR by Using Sephadex G-200 Spun Columns. V.M. BOCCUZZI*, W.L. STRAUBE, J. RAVEL, R.R. COLWELL, and R.T. Hill. Ctr. of Marine Biotechnology, Univ. of Maryland Biotechnology Inst., Baltimore, MD.

PCR is an important technique that is used to analyze nucleic acids recovered from environmental samples. PCR of DNA recovered from environmental samples can be inhibited by contaminating components, including humic substances. A number of purification techniques, including size exclusion chromatography, have been reported to remove these contaminating components. Sephadex G-200 spun columns, designed to purify proteins, have been used for DNA purification prior to PCR; however, optimization and efficiency of DNA recovery of this procedure have not been reported. We determined the effects of several parameters, including spin speed, ion concentration, frit composition, and number of spins, on recovery efficiency. Our method includes using a high salt TE buffer (pH 8.0)., and four low spin speeds (100 x g) in a swing-out centrifuge. The effectiveness of this procedure in removal of substances inhibiting PCR was estimated by PCR detection of *Vibrio cholerae* and *Shigella flexneri* -specific DNA sequences in "spiked" water samples from Mamala Bay, Hawaii. detection limits of these pathogens were compared, prior to and after clean-up of DNA by the optimized Sephadex G-200 spun column procedure. Optimization of DNA recovery was achieved and recovery of nucleic acids was calculated to be approximately 71%.

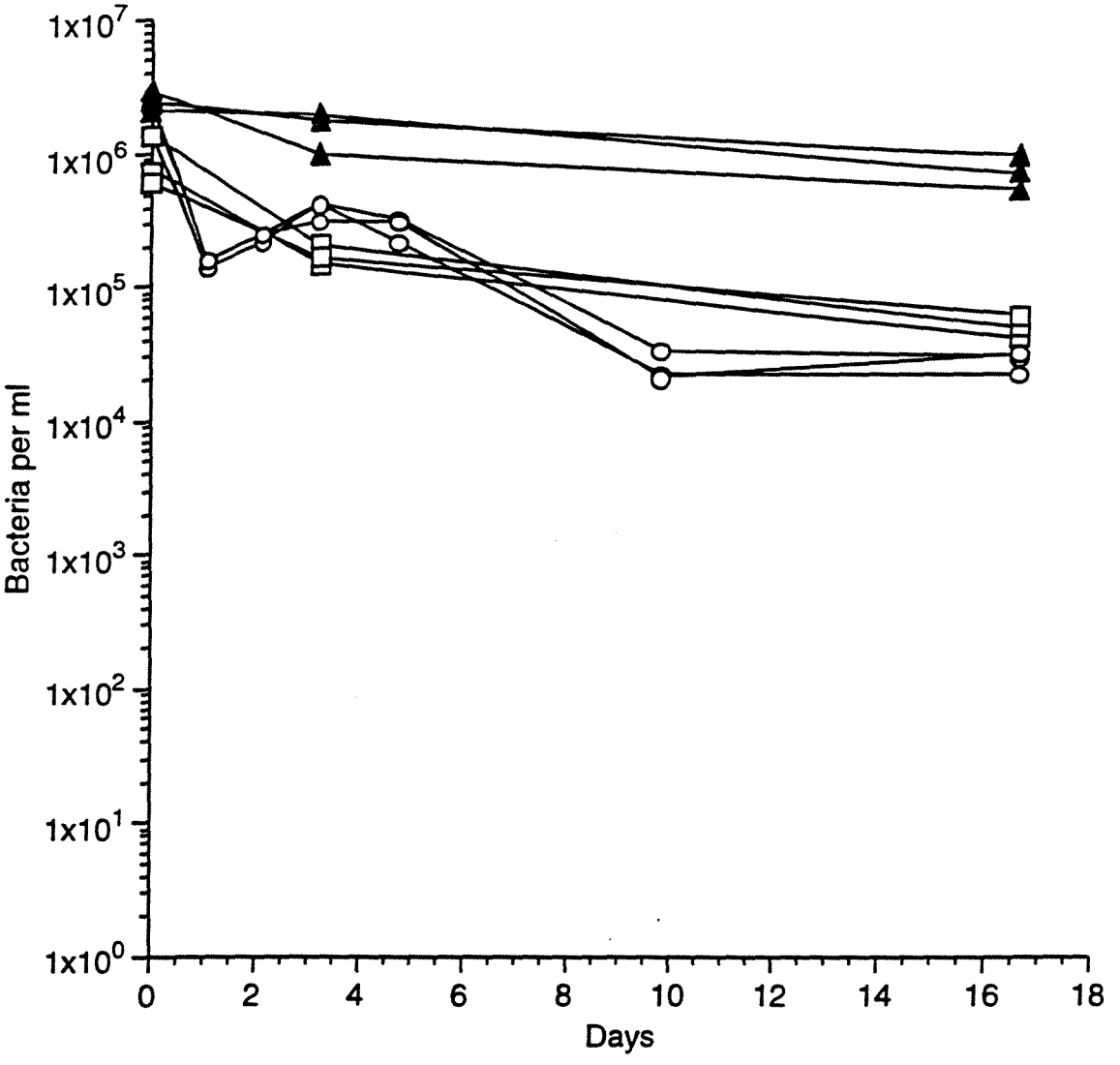

Fig. 8.1 Culturable, viable and total counts of *V. cholerae* in triplicate microcosms containing filtered water (22°C). Culturable counts (open circles) were determined by plating on nonselective medium, viable counts (open squares) by the method of Kogure et al. (1979), and total counts (closed triangles) by microscopic enumeration of acridine orange-stained cells.

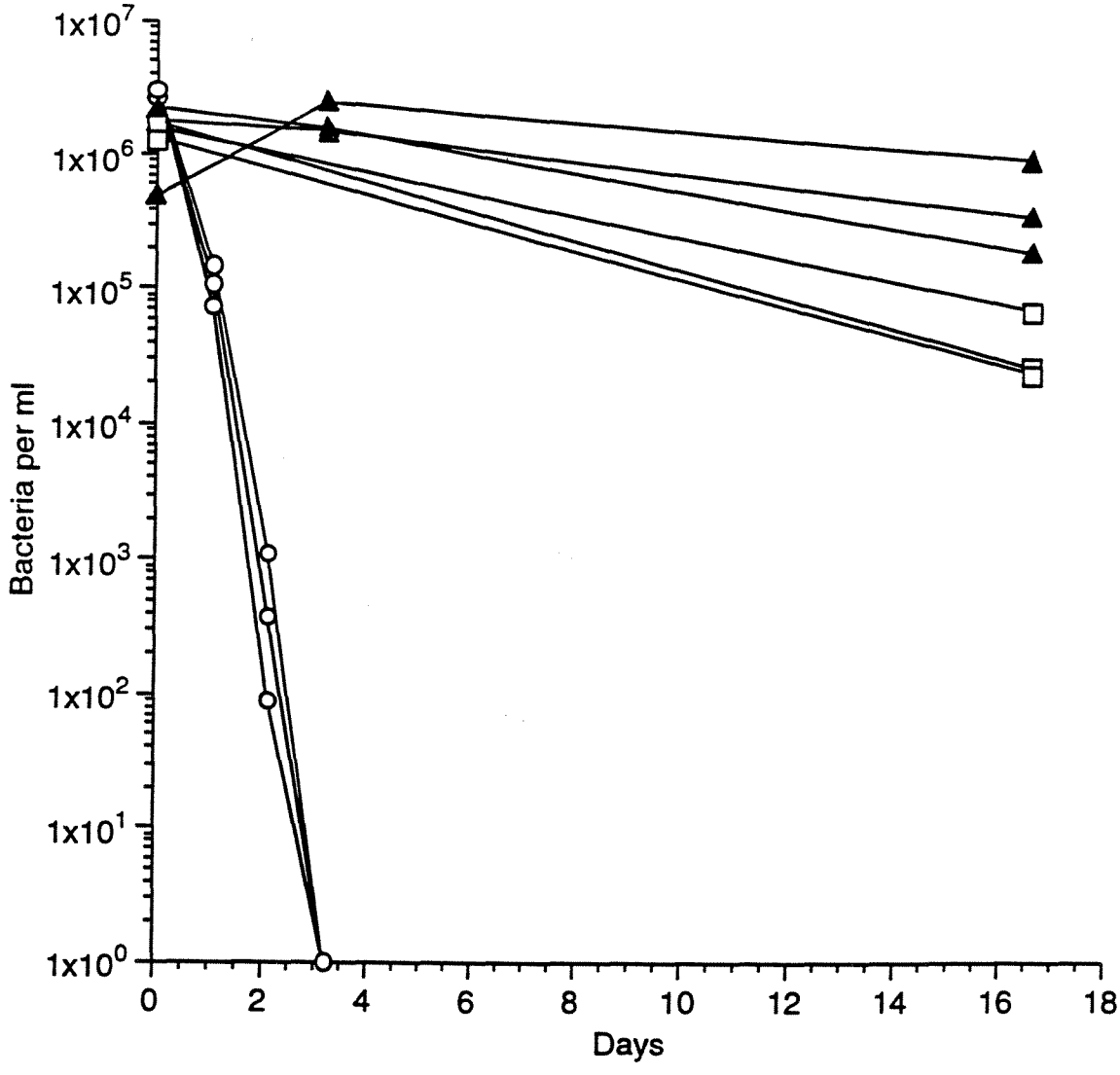

Fig. 8.2 Culturable, viable and total counts of enterotoxigenic *E. coli* in triplicate microcosms containing filtered water (22°C). Culturable counts (open circles) were determined by plating on nonselective medium, viable counts (open squares) by the method of Kogure et al. (1979), and total counts (closed triangles) by microscopic enumeration of acridine orange-stained cells.

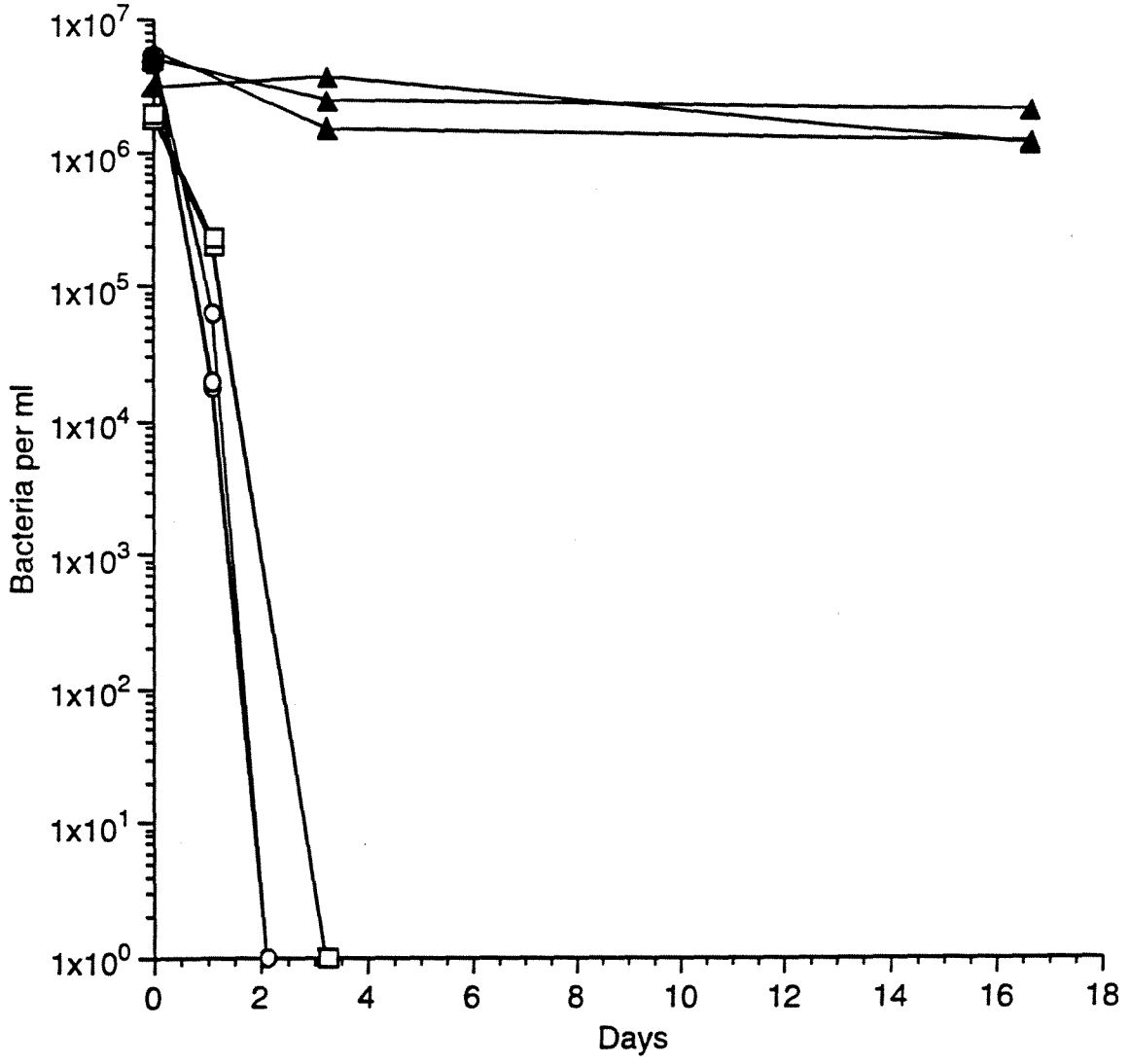

Fig. 8.3 Culturable, viable and total counts of *S. flexneri* in triplicate microcosms containing filtered water (22°C). Culturable counts (open circles) were determined by plating on nonselective medium, viable counts (open squares) by the method of Kogure et al. (1979), and total counts (closed triangles) by microscopic enumeration of acridine orange-stained cells.

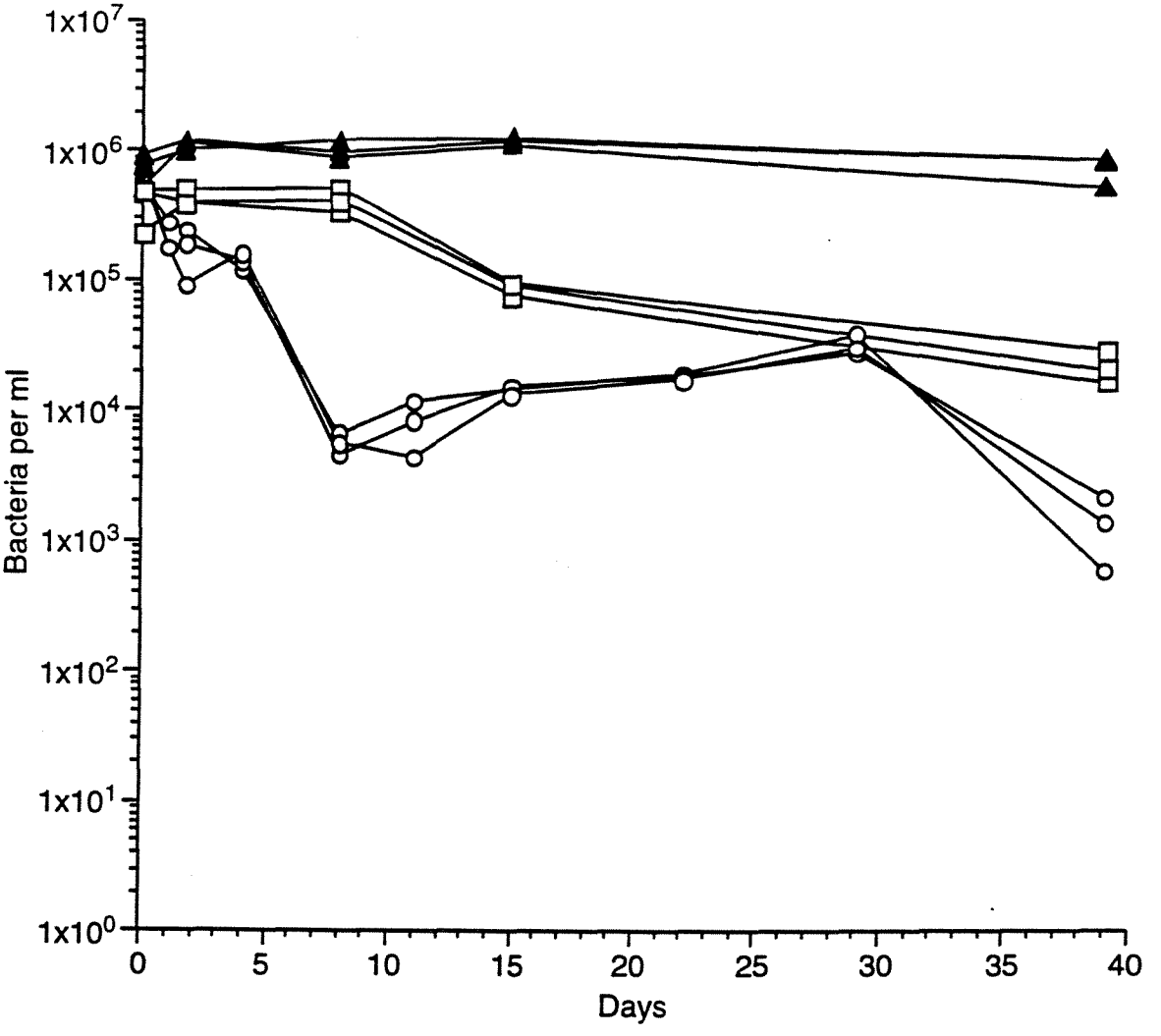

Fig. 8.4 Culturable, viable and total counts of *V. cholerae* in triplicate microcosms containing filtered water (28°C). Culturable counts (open circles) were determined by plating on nonselective medium, viable counts (open squares) by the method of Kogure et al. (1979), and total counts (closed triangles) by microscopic enumeration of acridine orange-stained cells.

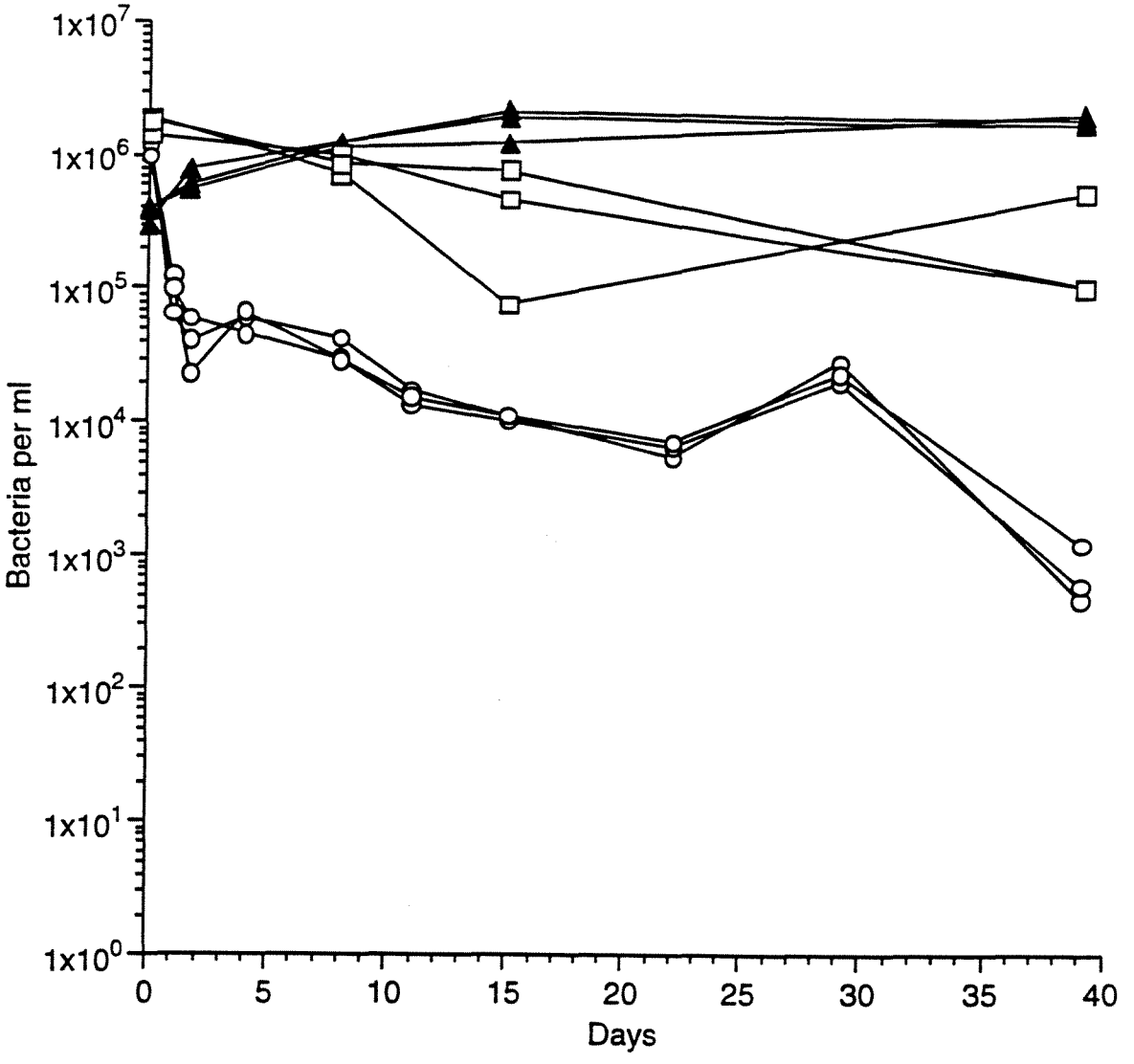

Fig. 8.5 Culturable, viable and total counts of enterotoxigenic *E. coli* in triplicate microcosms containing filtered water (28°C). Culturable counts (open circles) were determined by plating on nonselective medium, viable counts (open squares) by the method of Kogure et al. (1979), and total counts (closed triangles) by microscopic enumeration of acridine orange-stained cells.

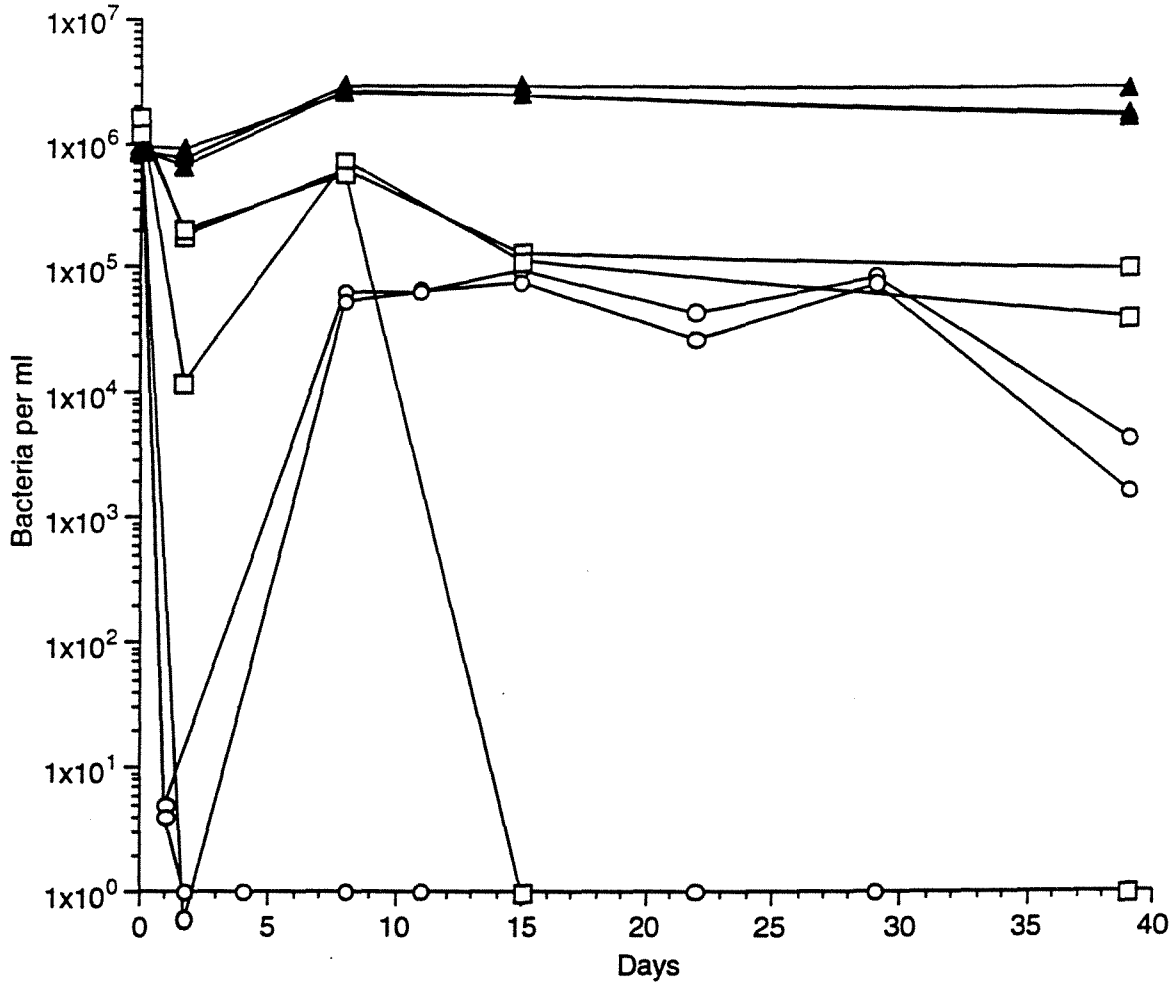

Fig. 8.6 Culturable, viable and total counts of *S. flexneri* in triplicate microcosms containing filtered water (28°C). Culturable counts (open circles) were determined by plating on nonselective medium, viable counts (open squares) by the method of Kogure et al. (1979), and total counts (closed triangles) by microscopic enumeration of acridine orange-stained cells.

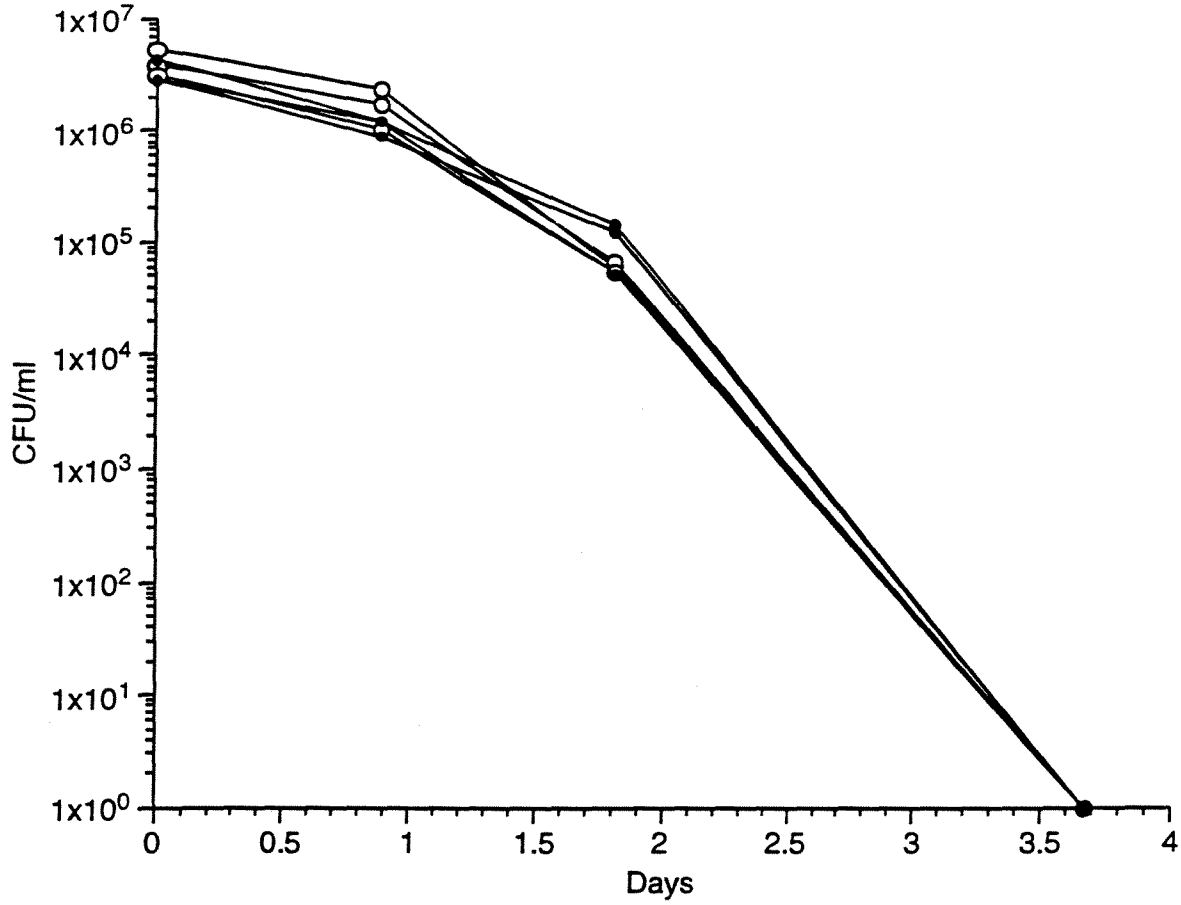

Fig. 8.7 Culturable counts of *S. flexneri* inoculated into triplicate microcosms containing filtered water at 22°C (closed circles) and 28°C (open circles).

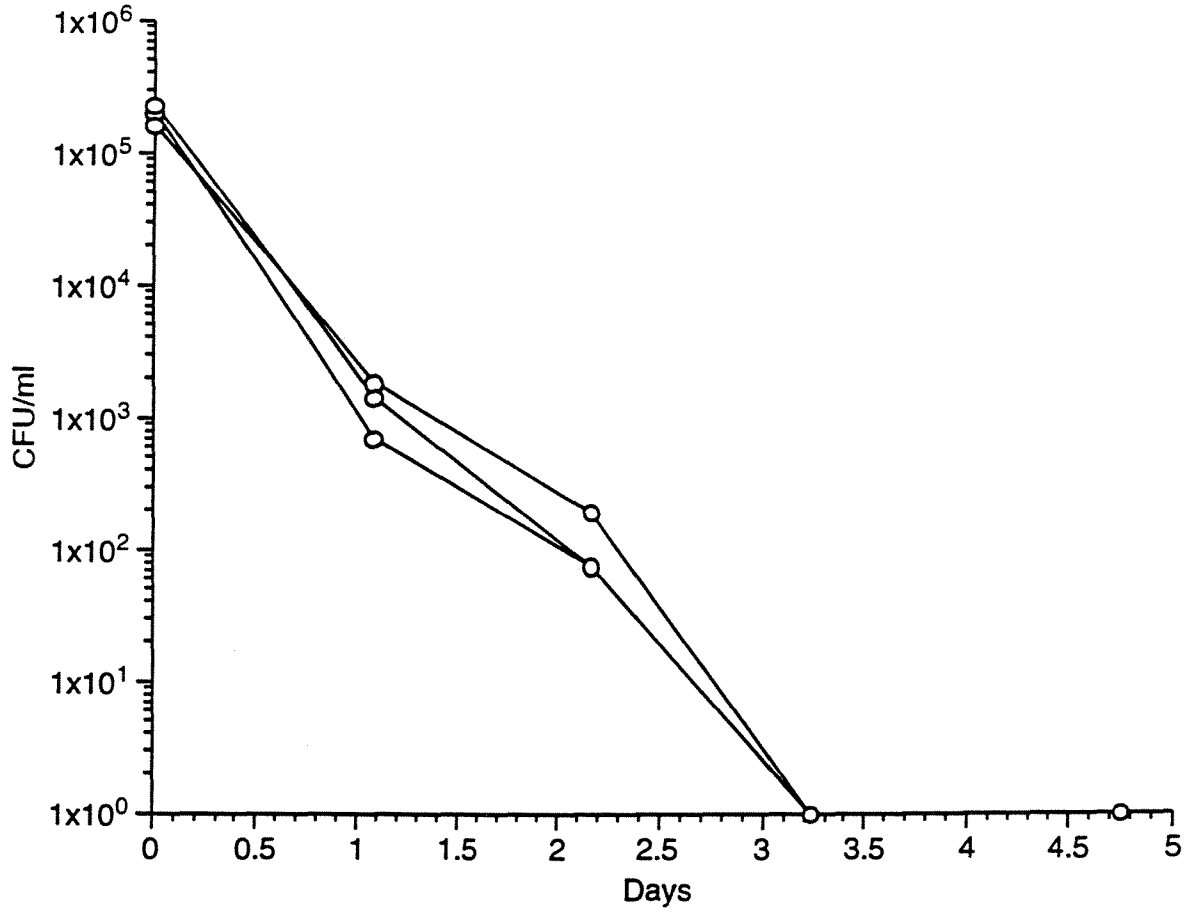

Fig. 8.8 Culturable counts of *V. cholerae* in triplicate microcosms of unfiltered water from station D2B (28°C) (open circles).

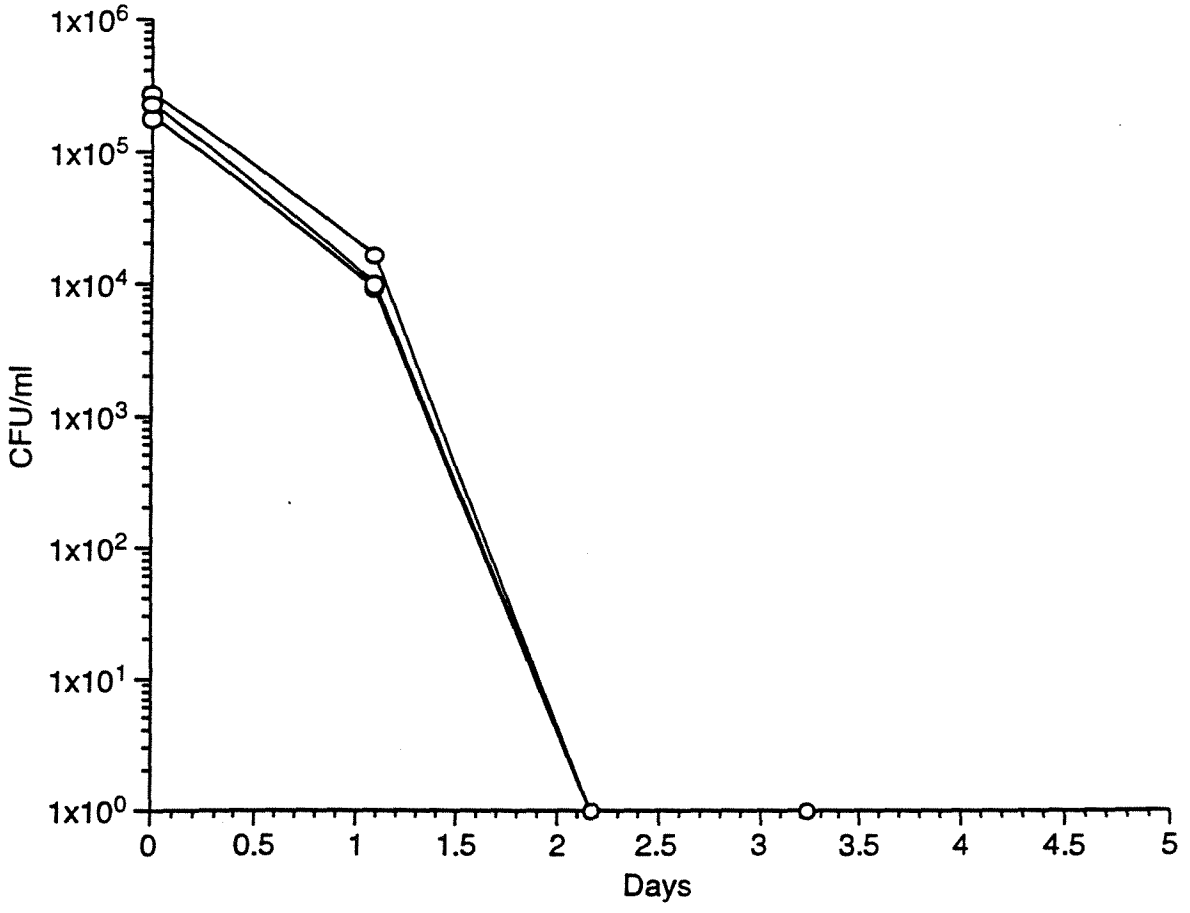

Fig. 8.9 Culturable counts of enterotoxigenic *E. coli* in triplicate microcosms of unfiltered water from station D2B (28°C) (open circles).

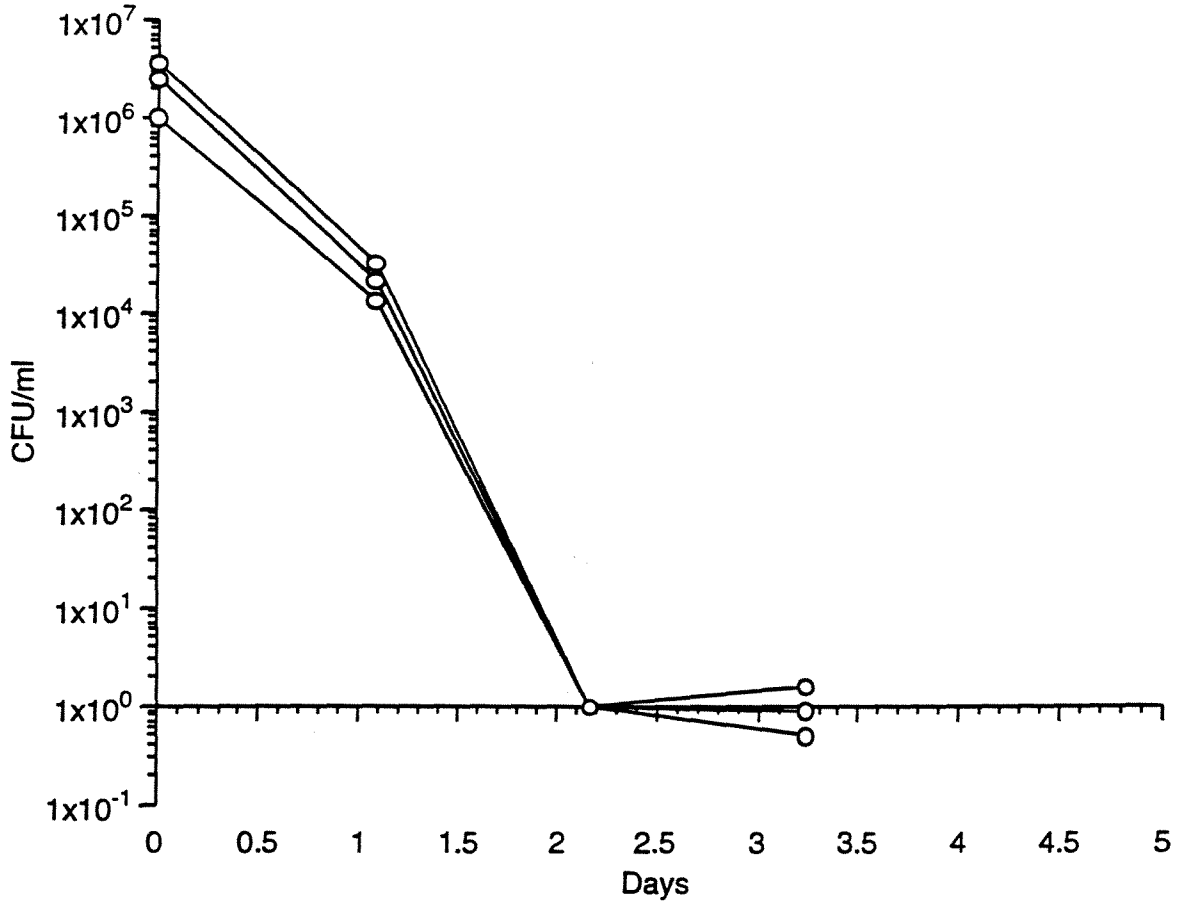

Fig. 8.10 Culturable counts of S. flexneri in triplicate microcosms of unfiltered water from station D2B (28°C) (open circles).