REPORT DOCUMENTATION FORM
WATER RESOURCES RESEARCH CENTER
University of Hawaii at Manoa

[1]**Report Number** Technical Report No. 154	[2]**FCST Category** 05-A, B, C
[3]**Title** *Clostridium perfringens* as an Indicator of Stream Water Quality	[4]**Report Date** June 1983
	[5]**No. of Pages** viii + 37
	[6]**No. of Tables** 12 — [7]**No. of Figures** 3
[8]**Author(s)** Roger S. Fujioka, Lyle Shizumura	[9]**Grant Agency** Office of Water Policy, U.S. Department of the Interior
	[10]**Grant/Contract No.** 14-34-0001-1113; A-087-HI

[11]**Descriptors:** *Bacteria, **Clostridium perfringens*, *Water Quality, Water Pollution, Streams, Effluent, Indicators

Identifiers: *Fecal Coliform, *Fecal Streptococcus, Kipapa Stream, Ahuimanu Stream, Oahu, Hawaii

[12]**Abstract (Purpose, method, results, conclusions)**

The mCP medium devised by Bisson and Cabelli in 1979 was used to recover *Clostridium perfringens* from sewage and streams. This membrane filtration method proved to be uncomplicated and reliable. Of 98 presumptively positive colonies recovered from various stream samples, 89 or 91% were confirmed as *C. perfringens* by using biochemical tests, whereas only 1 of 29 (3%) of the presumptively negative colonies was subsequently confirmed as *C. perfringens*. The quality of streams receiving treated and chlorinated sewage effluent was determined by analyzing the effluent entering the stream as well as stream samples above and below the effluent discharge site for fecal coliform (FC), fecal streptococcus (FS, and *C. perfringens* (CP). Chlorination was shown to drastically reduce the concentrations of FC and FS, but not CP in the sewage effluent. As a result, the concentrations of FC and FS in the chlorinated effluent were less than the natural concentrations of these bacteria in the stream, whereas the same effluent contributed significantly more CP than was naturally present in the stream. Thus, analysis of stream water for CP, but not FC or FS, clearly indicated the input of effluent into the stream. Moreover, the FC:CP ratio was useful in determining the quality and distance of a major source of pollution within a stream.

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CLOSTRIDIUM PERFRINGENS
AS AN INDICATOR OF STREAM WATER QUALITY

Roger S. Fujioka
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Technical Report No. 154

June 1983

Research Project Technical Completion Report
for
A Preliminary Investigation of *Clostridium perfringens*
as an Indicator of Stream Water Quality
Project A-087-HI
Grant Agreement No.: 14-34-0001-1113
Project Investigator: Roger S. Fujioka
Prepared for
UNITED STATES DEPARTMENT OF THE INTERIOR

The work upon which this report is based was supported in part by federal funds provided by the United States Department of the Interior, as authorized under the Water Research and Development Act of 1978, as amended.

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ABSTRACT

The mCP medium devised by Bisson and Cabelli in 1979 was used to recover Clostridium perfringens *from sewage and stream. This membrane filtration method proved to be uncomplicated and reliable. Of 98 presumptively positive colonies recovered from various stream samples, 89 or 91% were confirmed as* C. perfringens *by using biochemical tests, whereas only 1 of 29 (3%) of the presumptively negative colonies was subsequently confirmed as* C. perfringens.

The quality of streams receiving treated and chlorinated sewage effluent was determined by analyzing the effluent entering the stream as well as stream samples above and below the effluent discharge site for fecal coliform (FC), fecal streptococcus (FS), and C. perfringens *(CP). Chlorination was shown to drastically reduce the concentrations of FC and FS, but not CP in the sewage effluent. As a result, the concentrations of FC and FS in the chlorinated effluent were less than the natural concentrations of these bacteria in the stream, whereas the same effluent contributed significantly more CP than was naturally present in the stream. Thus, analysis of stream water for CP but not FC or FS clearly indicated the input of effluent into the stream. Moreover, the FC:CP ratio was useful in determining the quality and distance of a major source of pollution within a stream.*

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INTRODUCTION

Waterborne diseases are transmitted by the ingestion of water contaminated with pathogenic microorganisms (Feachem 1977). The mode of transmission for waterborne diseases has been commonly described as occurring via the oral-fecal route. Table 1 lists the fecal enteric pathogens or microorganisms which may be recovered from human fecal discharges and which have been implicated in waterborne diseases. This table lists three major groups of microorganisms (bacterial, viral, protozoan) that can cause diseases in man. Because of differences in their chemical and physical structure,

TABLE 1. ENTERIC PATHOGENS IMPLICATED IN TRANSMITTING WATERBORNE DISEASES

Pathogen	Disease or Symptom
BACTERIAL	
Salmonella typhi	Typhoid fever
Salmonella spp.	Enteric fevers, gastroenteritis
Shigella dysenteriae	Bacterial dysentery
Shigella spp.	Gastroenteritis
Vibrio cholera	Cholera
Yersinia enterocolitica	Gastroenteritis
Campylobacter fetus	Enteritis
VIRAL	
Hepatitis A	Infectious hepatitis
Poliovirus	Poliomyelitis
Rotavirus	Infantile gastroenteritis
Adenovirus	Respiratory, gastrointestinal
Coxsackie, Echo-, Reoviruses	Respiratory, aseptic meningitis, myocarditis; fever, rash, paralysis
PROTOZOAN	
Entamoeba histolytica	Amoebic dysentery
Giardia lamblia	Giardiasis
Balantidium coli	Dysentery, intestinal ulcers

physiology, and genetic make-up, the methods for the recovery, identification, and growth of the three groups of microorganisms are very different. Also, even within the same genus, the number of species, variants, and serotypes may be numerous. For example, approximately 700 known species exist of Salmonella and over 30 serotypes of echo and coxsackieviruses. Moreover, some pathogens, such as hepatitis A virus, cannot be routinely detected or cultured. Since any body of water may be contaminated with any of these microbial pathogens, suspected waters must be assessed for the possible presence of these pathogens. However, it was quickly determined that it would not be feasible to test the water for all of these pathogens. Even the analysis of the water for only one or two pathogens would not be sufficient since absence of one or two pathogens does not mean that other pathogens may not be present.

In the development of tests to assess the hygienic quality of water, it was reasoned that since the etiological agents of waterborne diseases are of fecal origin, a test designed to detect for the presence of fecal components in the water could be used as an indirect measurement for the probable presence of enteric pathogens in the water. This concept is still acceptable today and the presence of coliform bacteria in water is used as the standard test to determine the hygienic quality of that water. However, since coliforms are not enteric pathogens but are used to indicate for the probable presence of pathogens in water, coliform bacteria are referred to as indicator bacteria when used for this purpose. Under current federal regulations (EPA 1978, pp. 94-95), coliform bacteria is the only indicator system required to assess the hygienic quality of waters (Table 2).

Assessment of Coliform as a Water Quality

Coliform bacteria include total coliform, fecal coliform (FC) and Escherichia coli. The total coliform group is described as gram-negative, nonspore-forming, facultatively anaerobic bacilli which ferment lactose with the production of gas within 48 hr at 35°C (APHA, AWWA, and WPCF 1975). The fecal coliform group is a subgroup which can multiply at an elevated incubation of 44.5°C and in the presence of bile salts. E. coli is considered the coliform most typically found in fecal wastes of man.

Coliform bacteria have been used since the early 1900s and continue to be used to assess the hygienic quality of water. Despite the fact that this

TABLE 2. FEDERAL WATER QUALITY STANDARDS AND CRITERIA

TYPE OF WATER	ALLOWABLE COLIFORMS/100 mℓ Total	Fecal
Standards		
Potable	5	-------
Chlorinated Effluent	--	200-400
Secondary-Treated Wastes	--	200-400
Criteria (Recreational)		
Primary Contact	--	200* 400†
General Contact	--	1000* 2000†

SOURCE: U.S. Environmental Protection Agency (1978).
*Based on geometric mean during a 30-day period.
†Based on maximum in 10% of samples during same period.

system has served a useful purpose, many recent studies have concluded that coliforms are inadequate as indicators of water quality. One of the first prominent scientists to come to this conclusion was Dutka who in 1973 pointed out that a valid indicator organism should fulfill the following four criteria:

1. Be present and occur in much greater numbers than the pathogens concerned
2. Not be able to proliferate to any greater extent than enteric pathogens in the aqueous environment
3. Be more resistant to disinfectants and to the aqueous environment than the pathogens
4. Yield characteristic and simple reactions enabling as far as possible an unambiguous identification of the group.

Dutka (1979) points out that of the four criteria, coliforms do not fulfill criteria 2 and 3. Specifically, coliforms have been shown to be able to multiply under enriched environmental conditions and thereby falsely indicate an elevated health hazard. On the other hand, coliforms have been shown to be less resistant to disinfectants, such as chlorine, or to survival in environmental conditions than some pathogens, such as human enteric viruses. In this regard, human enteric viruses have been recovered from chlorinated sewage effluent or from natural waters determined to be hygienically safe, based on low to negligible concentrations of coliform bacteria. Thus, low concentrations of coliforms may falsely indicate the absence of pathogens in natural waters.

As a result of these problems, there has been an active movement to find an alternative and a superior indicator of water quality than coliforms. For example, a conference sponsored by the American Society for Testing and Materials was held in 1976 to evaluate each of the proposed alternative indicators of water quality. The proceedings of this conference was published in a book entitled, Bacterial Indicators/Health Hazards Associated with Water (Hoadly and Dutka 1977). More recently, in Bacterial Indicators of Pollution, Pipes (1981) addressed the problem of water quality and health significance of bacterial indicators. Despite acknowledged problems with the use of coliforms to confidently assess water quality and more recent studies proposing the use of other bacteria as indicators, the coliform group of bacteria continues to be the only legally accepted indicator of water quality in the U.S.

Alternative Indicators of Fecal Pollution: Fecal Streptococcus and *Clostridium perfringens*

OVERVIEW. The list of viable alternative indicators of fecal pollution include fecal streptococcus, *Clostridium perfringens*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida albicans*, bacteriophage, and bifidobacteria. Of these microorganisms, *S. aureus*, *P. aeruginosa*, and *C. albicans* are not truly enteric microorganisms since they do not normally multiply in the intestinal tract of man. Thus, these microorganisms fail in the most basic criteria for indicator bacteria: their presence does not necessarily indicate the presence of fecal matter. Bacteriophages or viruses of *E. coli* bacteria are direct indicators of fecal pollution. However, these viruses can multiply in *E. coli* cells under environmental conditions. Thus, their concentrations may not be related to the concentrations of fecal matter in the water environment.

The group of bacteria which are truly normal inhabitants of the intestinal tract of man and animals includes total coliforms, fecal coliforms of which *E. coli* is the primary member, fecal streptococcus or enterococcus of which *Streptococcus faecalis* is the primary member, bifidobacteria, and

Clostridium perfringens. The density, source and relative stability of these fecal bacteria are summarized in Table 3. As shown in this table, bifidobacteria are found in the highest concentration and do not appear to have an extra-fecal source. However, this bacteria is a strict anaerobe and is unstable in environmental waters. Moreover, no reliable method exists to recover and identify this bacteria from sewage and natural waters containing many types of bacteria. The usefulness of bifidobacteria appears to be its unambiguous relationship to fecal matter. However, since it is very unstable, the concentrations of bifidobacteria cannot be related to the presence of enteric pathogens which are much more stable in environmental waters.

FECAL STREPTOCOCCUS. Of the many proposed alternative indicators of water quality, more studies have been conducted using fecal streptoccus (FS) than any other microorganism. Like fecal coliforms, fecal streptococci are not pathogenic to man and are normally found in the intestinal tract of man at relatively high concentrations; and techniques for their recovery and enumeration are reliable and feasible. However, the impetus for the numerous studies involving FS involves its usefulness in determining the source of the fecal pollution. In this regard, the source of most enteric pathogens (e.g., Salmonella typhosa, Shigella sp., Vibrio cholera, all human enteric viruses) are of human origin and therefore are not found in fecal discharges of animals. Consequently, streams containing high concentrations of FC of human origin are of greater public health significance to man than streams with high concentrations of FC of animal origin.

Geldreich (1976) determined that human fecal waste contains higher concentrations of FC than FS, whereas animal fecal waste is characterized by higher concentrations of FS than FC. Based on these results, Geldreich proposed that streams with FC:FS ratios of 4 or greater indicate the presence of human fecal waste, whereas streams with FC:FS ratios of less than 0.7 indicate contamination with animal fecal waste. Although this FC:FS ratio has been extensively applied to determine the origin of fecal pollution in natural bodies of water, its application often suffers because of certain limitations in the use of this ratio. Geldreich himself pointed out that the FC:FS ratio is dependent on the survival characteristics of FC and FS bacteria which are not the same. He therefore stressed that the FC:FS ratio should not be used if the fecal waste has been in the water environment for

TABLE 3. COMPARISON OF FECAL INDICATOR BACTERIA

INDICATOR BACTERIA	LOG_{10} DENSITY Feces/g		Sewage/100 mℓ		RELATIVE SURVIVAL	EXTRA-FECAL SOURCES
	Human	Animal	Influent	Cl*		
Bifidobacteria	7-8	1	7	<1	+	None
Total Coliform	6-7	4-6	6-7	<1	++	_Enterobacter_ and _Citrobacter_ from uncontaminated soil; _Klebsiella_ from vegetation and industrial effluent
Fecal Coliform	6-7	4-6	6-7	<1	++	_Klebsiella_ from vegetation and industrial effluent
E. coli	6-7	4-6	6-7	<1	++	None
Enterococci (_S. faecalis_ and _faecium_)	3-4	2-3	5	1	+++	_S. faecalis_ biotypes from insects and vegetation
C. perfringens (spores)	3-4	2-3	4	4	++++	Spore collection in soil and sediment with possible multiplication

SOURCE: Adapted from Cabelli (1979).
*Chlorinated.

over 24 hr. In this regard, when samples of most natural bodies of water are taken, the source of the fecal pollution is not known and it is not possible to determine for certain if the fecal contamination in the water sample was in the water environment for only a few hours or in excess of 24 hr.

The 24-hr limitation as imposed by Geldreich was based on the assumption that the fecal matter was directly discharged into a nontoxic, neutral water environment. Any environmental factor which actively and rapidly inactivates FC and FS at different rates can invalidate the usefulness of the FC:FS ratio within a short period of time. Some of these environmental factors include chlorination of sewage or the discharge of toxic substances (heavy metals, acid mine washes, pesticides, chemical toxins) into natural bodies of water.

Two other complicating factors should be recognized in interpreting the FC:FS ratio. First, under high nutrient load, environmental waters can support the multiplication of FC but not FS. Under these conditions the FC:FS ratio in the water sample may not reflect the true level of fecal contamination of that stream. Second, FS encompasses a heterogenous group of bacteria and some members of FS, especially those of nonhuman origin, are less stable than FC. On the other hand, the major population of human fecal streptococcus represented by Streptococcus faecalis belongs to a subgroup called enterococcus, which is considerably more stable than FC. In this regard, the most recent EPA-supported studies (Cabelli 1980) indicate that enterococcus concentrations in recreational waters correlated better than concentrations of FC to the incidence of diarrhea among active users of these waters. These recent findings indicate that FS or enterococci may be accepted as viable indicators of water quality by EPA in the near future. However, at the present time, interpretations of water quality based on concentrations of FS is not covered by any enforcing regulations. In this regard, Geldreich (1976) has cautioned that some FS recovered from stream waters have limited sanitary significance since they may be derived from plants and insects. Thus, some level of FS can be expected in any natural body of water. Geldreich concluded that concentrations of FS in natural waters should not be considered significant until they exceed 100 FS/100 mℓ of water.

CLOSTRIDIUM PERFRINGENS. Clostridium perfringens (CP) is an anaerobic spore-forming gram-positive bacillus. It is a straight rod, approximately

0.9 to 1.3 μm in width and 3.0 to 9.0 μm in length, often with oval, subterminal spores. When cultured under artificial conditions, *C. perfringens* rarely forms spores. Its colonial morphology is raised, circular, about 2 to 5 mm in diameter, and usually has a translucent, grayish-yellow color. *C. perfringens* is considered one of the fastest growing bacterium with a generation time of 8 to 10 min at an optimal growth temperature of 45°C. It can grow at temperatures between 20 to 51°C and at pH ranging between 5.5 to 8.0. Important characteristics of *C. perfringens* are listed in Table 4.

The natural habitat of *C. perfringens* is the intenstinal tract of man and warm blooded animals where it readily multiplies and forms spores. Natural waters and soil are reservoirs for *C. perfringens*, apparently because of its very stable spores. Despite the fact that *C. perfringens* is considered a human pathogen capable of infecting wounds (gas gangrene) and causing food poisoning, there is no evidence that *C. perfringens* is a water-borne disease. Soil is considered the most common environment of *C. perfringens*--a conclusion based primarily on the studies of Smith (1975, pp. 115-176) who reported the recovery of *C. perfringens* from every North American soil sample tested, at varying concentrations but as high as 5×10^4/g of soil.

In Europe and England, *C. perfringens* is accepted as a valid indicator of water quality since it is a fecal bacteria which can be consistently recovered at moderate densities from feces and sewage. In these countries, the use of *C. perfringens* is considered especially useful under the following conditions: (1) when the environment is especially harsh (e.g., chlorination, extreme pH, temperature, presence of toxins) for the survival of coliform bacteria; (2) to confirm the presence of coliforms in some water samples; and (3) to detect intermittent sources of fecal pollution, a condition which cannot be determined by analyzing the water for relatively unstable coliform bacteria. Scientists in the U.S. have not advocated the use of *C. perfringens* to assess water quality primarily because of its resistant spore form, its reported ubiquity in the soil in the U.S., and because the recovery methods used do not specifically measure only *C. perfringens*. However, Bisson and Cabelli (1979) recently developed a two-step membrane filtration method which specifically recovered *C. perfringens* from water samples. Using this new medium (mCP), which selectively recovered the spores rather than the vegetative cells of *C. perfringens*, Bisson and Cabelli (1980) determined that concentrations of coliforms (*E. coli*) in sewage de-

TABLE 4. CHARACTERISTICS OF CLOSTRIDIUM PERFRINGENS

Gram stain	Gram + rods
Nitrate reduction	Variable, depending on basal medium
Milk reaction	Stormy fermentation
Motility	Nonmotile
Gelatinase	+
Lecithinase	+
Lipase	-
Indole	-
Carbohydrate Fermentation	
Arabinose	-
Cellobiose	-
Dulcitol	-
Esculin	-
Fructose	+
Galactose	+
Glucose	+
Glycerol	Variable
Inositol	+
Inulin	Variable
Lactose	+
Maltose	+
Mannitol	-
Melibiose	-
Raffinose	Variable
Rhamnose	-
Ribose	Variable
Salicin	Variable
Sorbitol	-
Sorbose	-
Starch	+
Sucrose	+
Trehalose	Variable
Xylose	-

SOURCE: Adapted from Smith and Hobbs (1974).

creased from 10^6/100 mℓ to less than 10/100 mℓ after chlorination, whereas the concentrations of C. perfringens were barely reduced by chlorination. They concluded that a high ratio of E. coli to C. perfringens would indicate that the pollutant source was not chlorinated, while a corresponding low ratio would indicate that the pollutant source was chlorinated. Thus, Bisson and Cabelli reported that a satisfactory technique is now available to recover C. perfringens from sewage and other water samples. They also presented preliminary evidence that C. perfringens may be useful in assessing water quality even in the U.S.

Problem of Assessing Stream Water Quality in Hawai'i

In a recently completed study (Fujioka 1983), the quality of stream water in Hawai'i was determined by carefully analyzing samples of unpolluted streams, streams polluted with sewage effluent, sewage effluent, cesspool wastes, and storm drain runoff for concentrations of fecal coliform (FC) and fecal streptococcus (FS). The results of this study showed that high concentrations ($>10^3$-10^4/100 mℓ) of FC and FS could be recovered from samples of stream waters obtained from unpolluted and polluted sites. In fact, most of the samples of stream water collected upstream from the sewage effluent discharge site (unpolluted stream) contained higher concentrations of FC than the 200 to 400 FC/100 mℓ considered by state and federal laws as being polluted with fecal matter and a possible source of enteric pathogens. Only after analyzing stream samples for concentrations and the ratio of FC and FS, as well as concentrations of phosphates, were we able to determine which samples were or were not contaminated with sewage effluent.

In summary, we found that stream quality in Hawai'i could not be determined by analyzing stream water for only concentrations of FC as required by federal and state regulations. Although a means of determining stream water quality was established, it was at odds with state and federal regulations as to what constitute a polluted stream. Also, the method used was not simple and direct since it involved the measurements of three separate parameters. The results of this earlier study raised two important needs: (1) a more direct means of assessing stream water quality and (2) the reevaluation of water quality criteria for streams in semitropical environments, such as Hawai'i. The data obtained from our earlier study indicate that the same regulations and conclusions based on data obtained from studies conducted in North America may not be applicable to Hawai'i conditions.

OBJECTIVES AND EXPERIMENTAL DESIGN

The major goal of this study was to determine whether Clostridium perfringens can serve as a better indicator of fecal contamination than coliform bacteria and particularly in determining the quality of streams in

Hawai'i.

Specific objectives to attain this goal were:

1. To determine whether the newly described (Bisson and Cabelli 1979) membrane filtration method of recovering C. perfringens can be successfully applied to determine the concentrations of C. perfringens in sewage effluent, marine waters, stream waters, and soils of O'ahu
2. To establish the concentrations of C. perfringens relative to that of FC and FS in raw and chlorinated sewage, polluted and unpolluted streams, coastal waters, soil, and groundwater
3. To determine whether C. perfringens can be used to successfully and directly determine whether stream water samples in O'ahu are or are not contaminated with sewage, and to compare this method to the method recently described (Fujioka 1983) in which concentrations and ratios of FC and FS as well as concentrations of phosphates in stream water were required to assess sewage contamination of stream waters on O'ahu.

The method described by Bisson and Cabelli (1979) was followed to recover C. perfringens (CP) from water samples. Presumptively positive colonies of CP were further characterized to establish the specificity of this method of recovering C. perfringens from environmental samples.

Essentially the same sampling plan and sites used in an earlier study (Fujioka 1983) to evaluate the water quality of streams based on FC and FS analyses were used in this study. Primary emphasis was placed on evaluating the quality of 'Āhuimanu and Kīpapa streams into which sewage effluent is discharged from wastewater treatment plants (WWTP) operated by the City and County of Honolulu. Initially, sewage samples from WWTPs were analyzed for concentrations of FC, FS, C. perfringens, and phosphates to determine the expected concentrations of these sewage components in raw sewage and in the final chlorinated effluent which is discharged into streams. Stream samples were taken from selected sites upstream and downstream from the sewage effluent discharge site and analyzed for FC, FS, C. perfringens, and phosphates. All stream sites were also characterized by their surrounding environment (e.g., urban, forest reserve, agricultural uses) and known concentrations of sewage effluent. By comparing the concentrations of FC, FS, C. perfringens, and phosphates in the stream samples, the impact of sewage effluent discharge into streams were determined. The usefulness of C. perfringens as an indicator of sewage pollution of streams on O'ahu was determined by comparing its concentration in water samples wih that of FC and FS.

MATERIALS AND METHODS

Bacteriological Analysis

The membrane filtration technique was used to determine the concentrations of bacteria in all water samples. A total of 25 to 100 mℓ of the water sample (undiluted or diluted in PBW) was filtered through 0.45-μm membranes (GN-6, Gelman Company). The membranes were then placed on appropriate media for incubation and subsequent enumeration of the presumptively positive colonies. For fecal coliform (FC), the membranes were placed on mFC medium without rosolic acid, incubated for 24 hr at 44.5°C and the blue colonies counted (APHA, AWWA, and WPCF 1980). Because chlorine is known to stress coliforms resulting in low counts when the standard method is used, samples of chlorinated sewage were assayed using the resuscitation method described by Lin (1976). Briefly, the filters containing bacteria were initially placed on phenol red lactose agar and incubated for 4 hr at 35°C to resuscitate stressed bacteria. The filters were then transferred to mFC medium to selectively grow and enumerate FC. For fecal streptococci (FS), the membranes were placed on KF agar, incubated for 48 hr at 35°C and the pink to red colonies counted (APHA, AWWA, and WPCF 1980). For Clostridium perfringens (CP), the membranes were placed on mCP medium and incubated anaerobically for 24 hr at 45°C. Yellow colonies which became red to dark pink when exposed to ammonium hydroxide fumes were counted as positive for CP (Bisson and Cabelli 1979). Anaerobic conditions were created using anaerobic jars manufactured by BBL or Oxoid Company. The method for the recovery and presumptive identification of CP described by Bisson and Cabelli (1979) is outlined in Figure 1.

The standard 5 tube most probable number (MPN) technique (APHA, AWWA, and WPCF 1980) was used to determine the concentrations of bacteria in soil. A 50-g portion of soil was added to 450 mℓ of PBW containing 0.5 mℓ of sterile 0.1% tween 80 to aid in desorption of bacteria from the soil. This sample was vigorously mixed for 30 min with a magnetic stirrer and the homogenous mixture used in the assay. For FC, soil samples were inoculated into

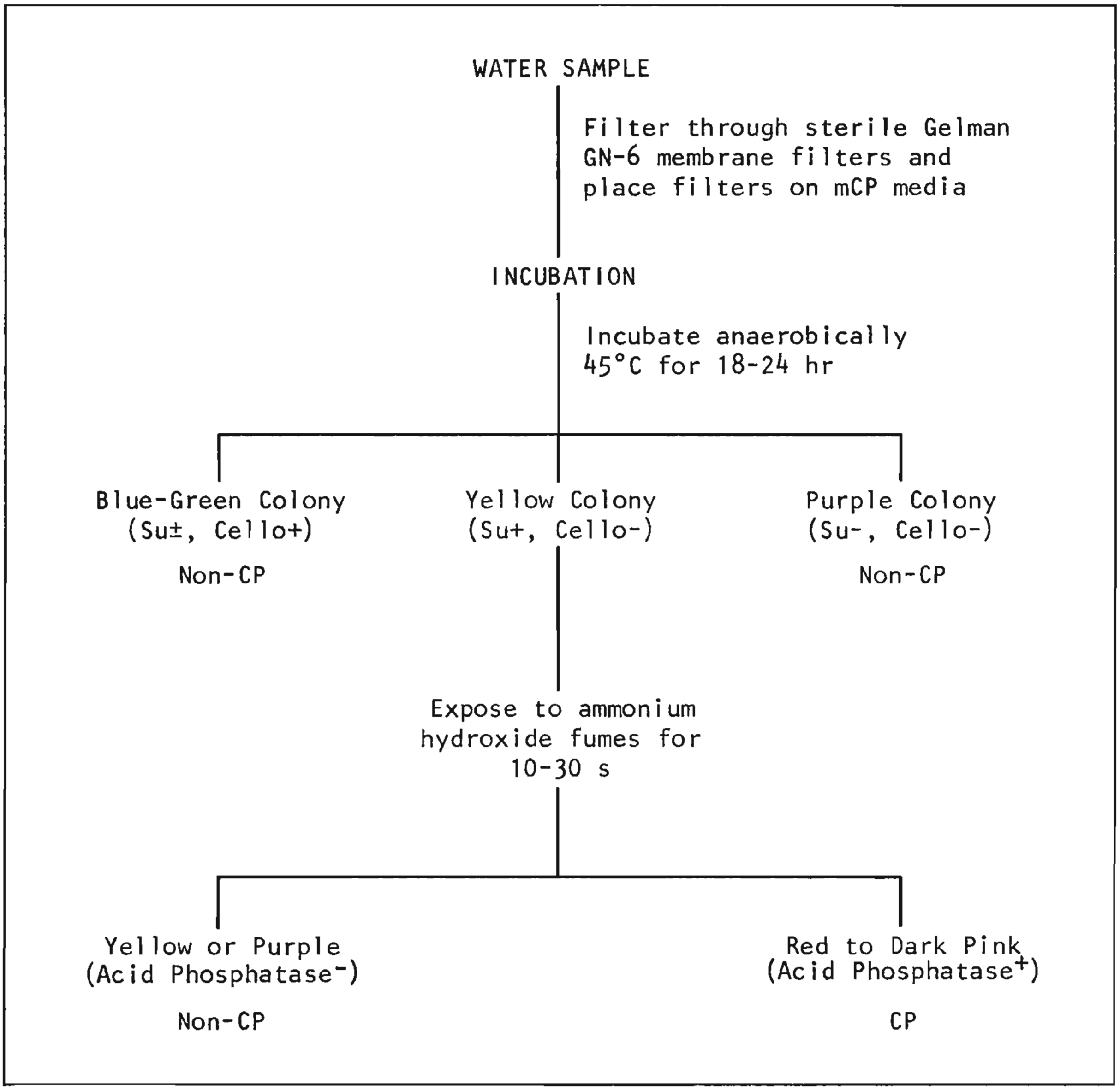

Figure 1. Flow chart for identifying Clostridium perfringens colonies on mCP media

Lauryl Tryptose Broth and incubated for 24 to 48 hr at 35°C. Under these conditions, presumptively positive samples were indicated by acid and gas production. The samples were then inoculated into EC broth and incubated for 24 hr at 44.5°C. Growth and gas production in this medium confirmed the presence of FC (APHA, AWWA, and WPCF 1980). For FS, soil samples were inoculated into Azide Dextrose Broth and incubated for 48 hr at 35°C. Under these conditions, presumptively positive samples were indicated by growth (turbidity). These samples were then streaked onto PSE (Bile Esculin Azide) agar and incubated for 24 hr at 35°C. Brown to black colonies with brown halos confirmed the presence of FS. For CP, soil samples were added to rapid perfringens medium (RPM) and incubated anaerobically for 24 hr at 45°C. Under these conditions, presumptively positively samples were indicated by stormy fermentation reaction. These samples were streaked onto mCP medium to confirm the presence of CP.

Physical and Chemical Analyses

Water turbidity was measured in nephelometric turbidity units (NTU) using the HACH Turbidimeter (Model 2100A). The pH was measured using a Corning pH meter (Model 7). Total residual chlorine was measured in mg/ℓ using the HACH DPD Total Chlorine Kit. Orthophosphate concentrations in water were measured in mg^2P/ℓ using the ascorbic acid method (APHA, AWWA, and WPCF 1980).

Sample Identification and Sampling Sites

All water samples were collected in sterile polypropylene containers and transported to the laboratory in an ice chest. Samples with known concentrations of chlorine were dechlorinated with sodium thiosulfate. All samples were assayed for bacteria within 4 hr after collection. Sewage samples were obtained from the Ala Moana Pumping Station, and the Pearl City, Ahuimanu, and Mililani WWTPs.

Two streams receiving sewage effluent discharge from WWTPs operated by the City and County of Honolulu were selected as our model stream systems. The first stream (Kīpapa Stream) is located in Central O'ahu and represents a long stream flowing through a dry area. Kīpapa Stream originates in one of the valleys of the leeward slopes of the Ko'olau Range and traverses through the populated central plains of O'ahu where it receives the effluent from the Mililani WWTP before it flows through the town of Waipahu and into West Loch of Pearl Harbor. The Mililani WWTP utilizes primary settling, activated sludge treatment, and chlorination before 7 685 m^3/day (2 mgd) of treated effluent is discharged into Kīpapa Stream.

A map of the Kīpapa-Waikele streams and the locations of the sampling sites is presented in Figure 2. Descriptions of the sampling sites are as follows:

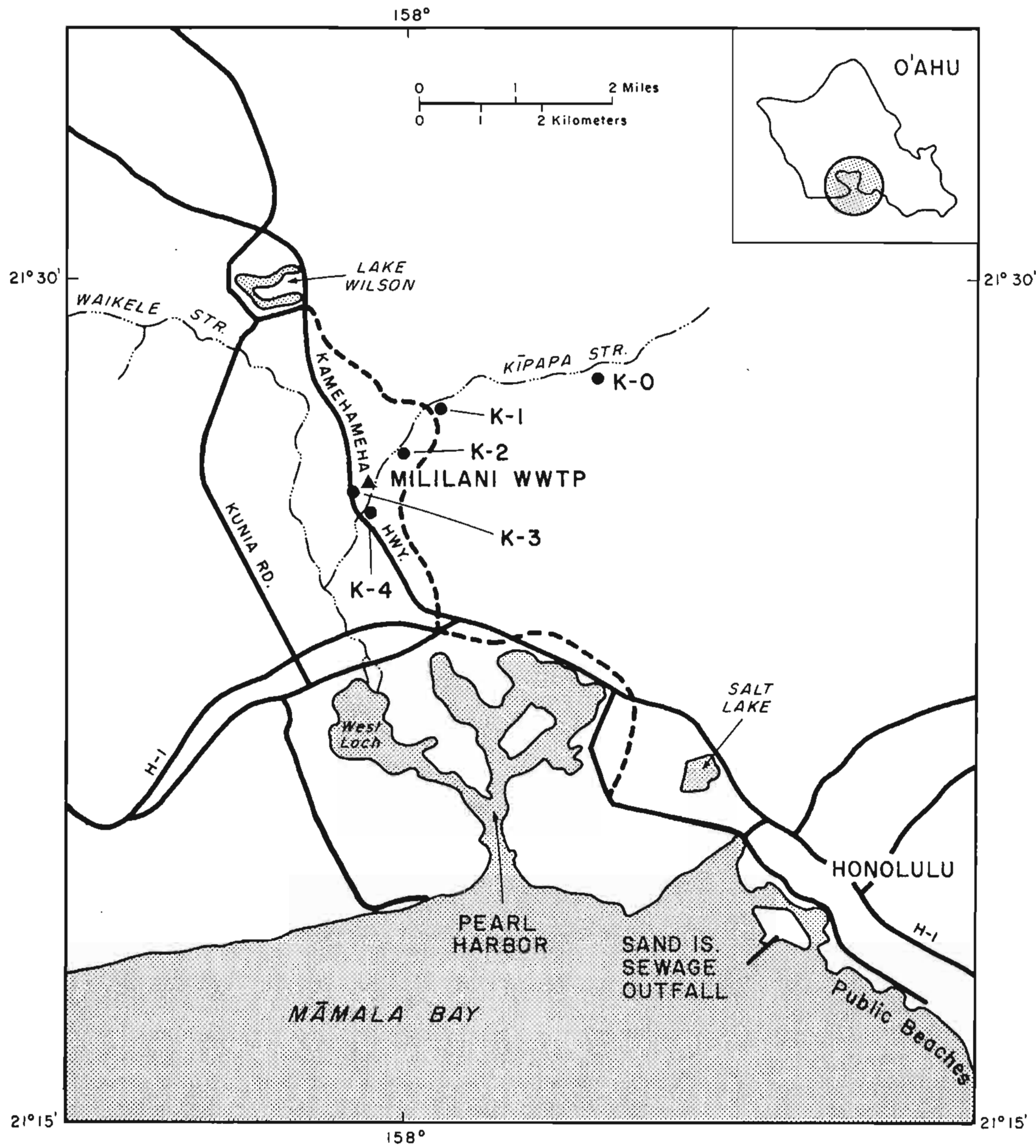

Figure 2. Kīpapa Stream sampling sites, O'ahu, Hawai'i

Site K-0 - U.S. Geological Survey gaging station located in a remote, mountainous area, far from human habitation and approximately 9 976 m (6.2) miles upstream of the sewage effluent discharge site into Kīpapa Stream

Site K-1 - Within the Kipapa Military Reservation, approximately 1 931 m (1.2 miles) upstream of the sewage effluent discharge site into Kīpapa Stream

Site K-2 - Kīpapa Stream only 274.32 m (300 yd) upstream of the Mililani sewage effluent discharge site into Kīpapa Stream

Site K-3 - Sewage effluent from Mililani WWTP being discharged into Kīpapa Stream

Site K-4 - Kīpapa Stream only 274.32 m downstream of Mililani sewage effluent discharge site into Kīpapa Stream.

The second stream (South 'Āhuimanu Stream) is located in windward O'ahu and represents a stream which collects rainfall from a wet mountainous area and flows out to sea in a relatively short period of time. The South 'Āhuimanu Stream originates at the 670.56-m (2200-ft) elevation on the windward slopes of the Ko'olau Range and is fed by rain and spring waters from the closed watershed of the Waiāhole Forest Reserve. From the approximate 121.92-m (400-ft) elevation boundary of the forest reserve, this stream flows sequentially through a small urban community, a small pasture, and past the Ahuimanu WWTP. This WWTP uses primary settling, activated-sludge, chlorination and oxidation in a pond to treat 1 740 m^3/day (0.5 mgd) of sewage effluent which is then discharged into South 'Āhuimanu Stream. This stream then merges with the major tributary of 'Āhuimanu Stream and flows through populated and more complex, mixed land-use areas before it discharges into Kāne'ohe Bay.

A map of the 'Āhuimanu Stream and the locations of sampling sites are shown in Figure 3. Descriptions of the sampling sites are as follows:

Site A-0 - Located 548.64 m (600 yd) upstream of the Byodo-In Temple (a mortuary park) in an uninhabited, forest reserve area at base of the Ko'olau Range

Site A-1 - Approximately 1 609 m (1 mile) downstream of Site A-0, just below the first small urban community in that area

Site A-2 - Discharge point of sewage from Ahuimanu WWTP

Site A-3 - Approximately 402 m (0.25 mile) downstream of Site A-2.

RESULTS AND DISCUSSION

Relative Concentrations of CP, FC, and FS in Sewage

To assess the usefulness of Clostridium perfringens (CP) as an indicator of fecal contamination of stream water, the concentrations of CP in sewage relative to that of the more common indicators (FC, FS) were determined. To address this question, the following sewage samples were obtained and analyzed for CP, FC, and FS by the membrane filtration method: (1) raw

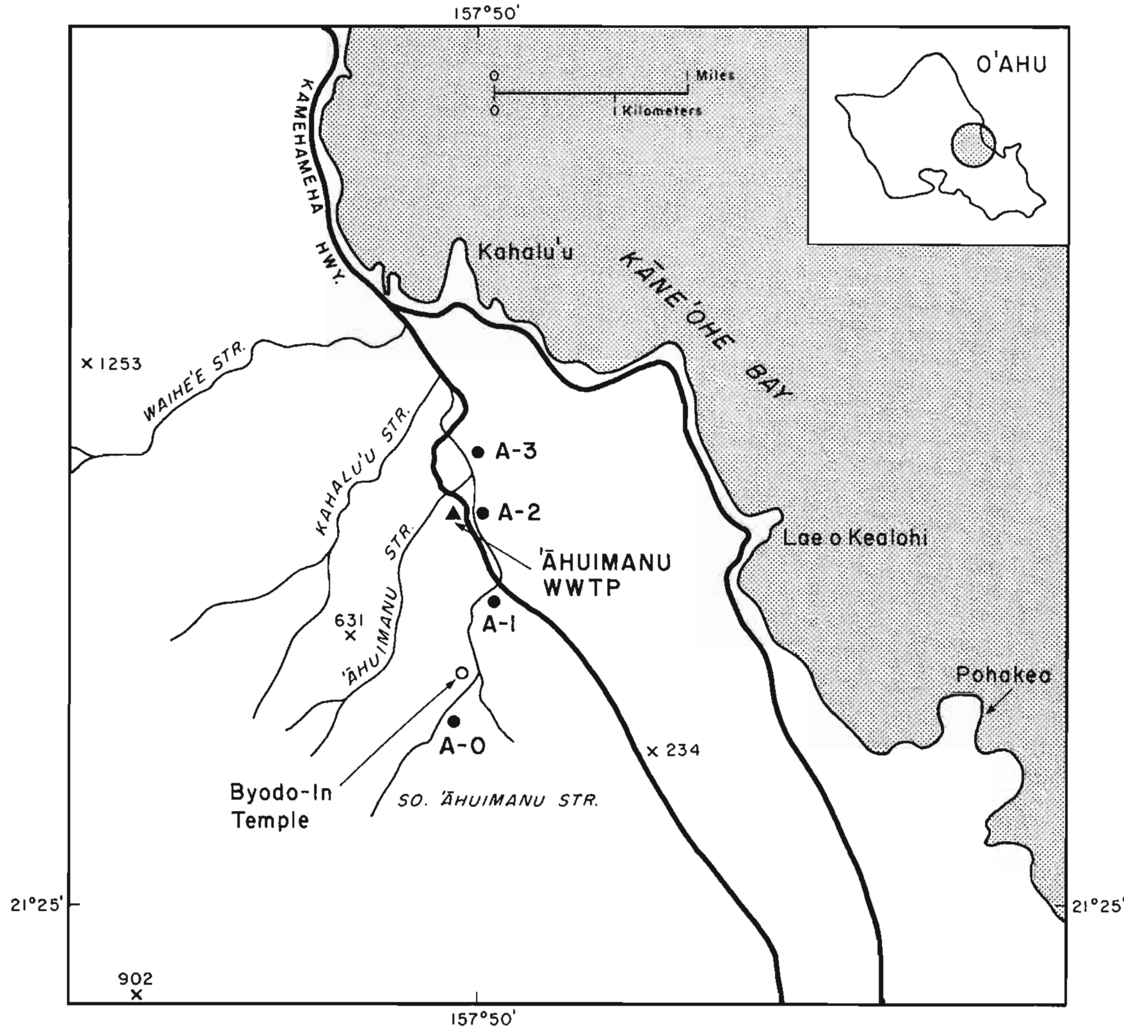

Figure 3. 'Āhuimanu Stream sampling sites, Kāne'ohe, O'ahu

sewage from the Ala Manoa Pumping Station, (2) primary treated sewage from the Pearl City WWTP, (3) secondary treated sewage from the Pearl City and from the Mililani WWTPs. The results of these analyses are summarized in Table 5.

In raw sewage samples, the concentrations of FC (10^7/100 mℓ) were the highest, followed by FS at 10^6/100 mℓ, and CP at 10^4/100 mℓ. As expected, the FC:FS ratio in the three samples were greater than 4, indicating that the source of the fecal waste was human. Moreover, the high FC:CP ratio of 200, 250, and 540 in the three samples correctly indicated that the sewage had not been chlorinated to reduce the concentrations of FC. Similar results were obtained when the two primary treated sewage samples were anal-

TABLE 5. INDICATOR BACTERIA DENSITIES IN SEWAGE, ALA MOANA STATION, PEARL CITY WWTP, AND MILILANI WWTP, O'AHU, HAWAI'I

DATE	SEWAGE		INDICATOR/100 mℓ			INDIC. RATIO	
	Type	Source	FC	FS	CP	FC:FS	FC:CP
01/15/82	Raw	AM*	1.5×10^7	1.6×10^6	2.8×10^4	9	540
01/26/82		AM	1.1×10^7	2.4×10^6	5.4×10^4	5	200
03/08/82		AM	1.3×10^7	2.2×10^6	5.3×10^4	6	250
02/18/82	Primary-Treated	PC†	1.3×10^7	9.5×10^5	1.6×10^4	14	290
04/06/82		PC	1.2×10^7	7.0×10^5	2.8×10^4	17	430
10/07/81	Secondary-Treated	MI‡	2.8×10^4	3.5×10^3	3.8×10^3	8	7
02/18/82		PC	8.4×10^6	2.4×10^4	6.0×10^3	35	1400
04/06/82		PC	2.4×10^5	3.6×10^4	1.7×10^4	7	14

*Ala Moana Station.
†Pearl City WWTP.
‡Mililani WWTP.

yzed. When activated sludge-treated sewage samples were similarly analyzed, concentrations of FC and FS were much lower than in the raw and primary treated sewage, while concentrations of CP were reduced only slightly. However, the concentrations of FC were still higher than FS and concentrations of FS higher than CP. Thus, the FC:FS ratio remained well above 4, while the FC:CP ratio fluctuated reflecting the variability of the concentrations of FC in the samples.

Based on the results of this series of tests, the technique and the mCP medium described by Bisson and Cabelli were concluded to be a practical method to recover CP from sewage. The concentrations of CP in sewage relative to the concentrations of FC and FS were also similar to that reported by Bisson and Cabelli. Although the concentrations of CP in sewage are lower than that of FC and FS, the CP levels are high enough to be useful as an indicator of fecal contamination, especially if CP is as stable as it is reported to be. Finally, the results of this study indicate that the FC:CP ratio as an indicator of the age or the degree of sewage treatment may also be useful.

Comparative Resistance of CP, FC, and FS to Chlorination

When cesspools overflow or when sewage lines are broken, untreated fecal material may enter streams. However, under current regulations, sewage effluents are properly treated and disinfected with chlorine before they are discharged into streams. Thus, the concentrations of FC, FS, and CP in chlorinated sewage effluent must be determined to assess the impact of sewage effluent discharge into streams. As a first step in this assessment, the concentrations of FC, FS, and CP in the unchlorinated and chlorinated sewage effluent samples obtained from the Mililani and Pearl City WWTPs were determined. Since FC has been reported to be stressed by chlorination, the resuscitation technique reported by Lin (1976) was also used in the analysis of chlorinated sewage effluent for FC. The results of these analyses are summarized in Table 6.

Unlike most WWTPs, the Pearl City WWTP channels portions of its primary and secondary sewage effluents into the chlorine contact chamber for disinfection. Thus, the primary and secondary sewage effluents from this plant were analyzed for concentrations of FC, FS, and CP. In the primary sewage, the concentration of FC was determined to be 1.2×10^7/100 mℓ as compared to 7.0×10^5/100 mℓ for FS and 2.8×10^4/100 mℓ for CP. In the secondary sewage, the concentrations of FC (2.4×10^5/100 mℓ) and FS (3.6×10^4/100 mℓ) were substantially lower than in the primary sewage, whereas the concen-

TABLE 6. INDICATOR BACTERIA REDUCTION BY CHLORINATION, MILILANI WWTP AND PEARL CITY WWTP, O'AHU, HAWAI'I

DATE	SITE	TOT. Cl_2 RESID. (mg/ℓ)	INDICATOR	INDICATOR COUNT/100 mℓ			LOG_{10} REDUC.
				Sewage		Chlorinated Effluent	
				Primary	Secondary		
10/17/81	MI*	3.0	FC	--------	2.8×10^4	3	4.0
			FC‡	--------	--------	9	3.5
			FS	--------	3.5×10^3	15	2.4
			CP	--------	3.8×10^3	2.3×10^3	0.2
04/06/82	PC†	3.5	FC	1.2×10^7	2.4×10^5	1.3×10^3	4.0-2.3
			FC‡	--------	--------	2.4×10^3	3.7-2.0
			FS	7.0×10^5	3.6×10^4	2.3×10^3	2.5-1.2
			CP	2.8×10^4	1.7×10^4	1.6×10^4	0.03-0.2

*Mililani WWTP.
†Pearl City WWTP.
‡Using resuscitation method of Lin (1976).

tration of CP (1.7×10^4/100 mℓ) remained similar to that observed in the primary sewage. These results are comparable to that obtained earlier (Table 5) when similar samples were analyzed. Samples of the chlorinated sewage efluent were then obtained and determined to have a total chlorine residual of 3.5 mg/ℓ. In this sample the concentration of FC was determined to be 1.3×10^3/10 mℓ (Standard Methods) or 2.4×10^3/100 mℓ (resuscitation method) as compared to 2.3×10^3/100 mℓ for FS and 1.6×10^4/100 mℓ for CP. The results show that FC and FS are effectively inactivated by chlorine, whereas CP is resistant to the effects of chlorine. It is therefore significant to note that the concentrations of CP in the chlorinated sewage is greater than that of FC and FS, and that despite the high total chlorine residual in this chlorinated effluent, the concentrations of FC were much greater than the maximum allowable limits of 200 FC/100 mℓ for a properly disinfected sewage. This high concentration of FC is undoubtedly due to the fact that primary sewage as well as secondary effluent contributes very high concentrations of FC as well as high concentrations of suspended and organic matter which are known to interfere with the chlorination process.

The Mililani WWTP differs from that of the Pearl City WWTP in that all the sewage is first treated by activated sludge before it is chlorinated. The concentrations of FC (2.8×10^4/100 mℓ), FS (3.5×10^3/100 mℓ) and CP (3.8×10^3/100 mℓ) in samples of secondary sewage obtained from this plant again reflected the substantially reduced concentrations of FC and FS, but not of CP in the activated sludge treated sewage as compared to raw or primary treated sewage. Samples of chlorinated sewage effluent from this plant were determined to have a total chlorine residual of 3.0 mg/ℓ. In this same sample the concentration of FC was determined to be 3/100 mℓ (Standard-Methods) or 9/100 mℓ (resuscitation method) as compared to 15/100 mℓ of FS and and 2.3×10^3/100 mℓ for CP. It should be noted that sufficient chlorine was determined in the chlorinated sewage effluent and that the concentrations of FC and FS were at levels far below the maximum permissible level of 200 FC/100 mℓ, indicating that the overall sewagetreatment process including chlorination was opreating effectively. However, even under these conditions, the concentrations of CP in the chlorinated effluent remained essentially similar to that found in untreated sewage.

Based on the results of analyzing the unchlorinated and chlorinated sewage effluents from two WWTPs, it was concluded that although FC at 10^7/

100 mℓ is at a high concentration in raw and primary treated sewage as compared to FS at 10^6/100 mℓ and especially to CP at 10^4/100 mℓ, the relative concentrations of the three indicator bacteria become similar in the activated sludge-treated sewage because of the reduction in the concentrations of FC and FS but not CP. During chlorination, the concentrations of FC and FS, but not CP, are usually reduced to levels of 10 to 200/100 mℓ, whereas the concentrations of CP remains unaffected and is similar to that found in the untreated sewage. Thus, in the chlorinated effluent, the concentrations of CP can be expected to be 10 to 100 times higher than the concentrations of FC and FS. Because of this acknowledged sensitivity of FC and FS to chlorination and the acknowledged resistance of CP to chlorination, the contamination of stream water by sewage effluent can be more reliably followed by analyzing the stream water for the presence of CP than FC or FS. This proposal is supported by data already collected (Fujioka 1983) which showed that much higher concentrations of FC and FS were recovered in stream water samples obtained just upstream of the sewage effluent discharge site. The results of this study also confirm that 1 to 3 times more FC can be recovered from chlorinated sewage effluent when the resuscitation technique is used as compared to the standard technique. However, chlorination irreversibly inactivates a much greater percentage of the population of FC than it stresses. Thus, the conclusion based on results obtained from analyzing water samples for FC using the standard technique was not drastically different from the conclusion based on results obtained using the resuscitation technique.

Water Quality Analysis of Kīpapa Stream

In a recent study Fujioka (1983) determined that the quality of Kīpapa Stream could be ascertained by obtaining water samples from sites above and below the sewage efluent discharge site and determining their concentrations of FC, FS, orthophosphates, and turbidity. To determine whether CP can serve as a more direct means of assessing the impact of sewage discharge into Kīpapa Stream, the same stream sites selected in the previous study were analyzed for concentrations of FC, FS, orthophosphates, turbidity, chlorine, and CP. The results of the analyses are summarized in Table 7.

The stream site (Site K-0) located farthest upstream and in a very remote, mountainous area, uninhabited by man was considered the most pris-

TABLE 7. FECAL COLIFORM, FECAL STREPTOCOCCUS, AND *CLOSTRIDIUM PERFRINGENS* ANALYSIS OF KĪPAPA-WAIKELE STREAM UPSTREAM AND DOWNSTREAM OF MILILANI WWTP DISCHARGE SITE

SAMPLING SITE	SAMPLING DATE	TURBIDITY (NTU)	TOTAL CHLORINE (mg/ℓ)	ORTHOPHOSPHATE (mg-P/ℓ)	INDICATOR BACTERIA FC	FS	CP	INDICATOR RATIO FC:FS	FC:CP
					-----(CFU/100 mℓ)-----				
K-0 (remote Kīpapa, 6.2 miles upstream of WWTP discharge)	06/03/81	ND	ND	ND	170	100	<1	1.7	>170
K-1 (upper Kīpapa, 1.2 miles upstream of WWTP discharge)	06/03/81	ND	ND	ND	150	390	5	0.4	30.0
	10/14/81	0.9	ND	0.003	430	840	3	0.5	143.0
K-2 (mid-Kīpapa, 300 yd upstream of WWTP discharge)	06/03/81	ND	ND	ND	14000	6500	40	2.2	350
	06/17/81	ND	ND	0.005	3700	8000	46	0.5	80
	06/24/81	1.3	<0.1	0.001	39000	18000	41	2.2	951
K-3 (Mililani WWTP effluent discharge)	06/17/81	ND	ND	7.2	760	650	2700	1.2	0.3
	06/24/81	3.2	0.3	5.7	2000	1700	3400	1.2	0.6
	10/14/81	3.3	0.2	5.7	3900	9400	2300	0.4	1.7
K-4 (lower Kīpapa, 300 yd downstream of WWTP discharge)	06/17/81	ND	ND	6.9	2800	2800	2100	1.0	1.3
	06/24/81	2.9	0.2	5.1	2600	1900	1500	1.4	1.7
	10/14/81	3.3	0.2	5.9	1800	5300	1800	0.3	1.0

NOTE: NTU = nephelometric turbidity unit;
ND = not done.

tine of the selected stream sites. Despite the remoteness of this area, the single stream water sample revealed a concentration of 170 FC/100 mℓ and 100 FS/100 mℓ but 0 CP/100 mℓ. The second sampling site (Site K-1) was located further downstream but still 1 930.8 m (1.2 miles) above the sewage effluent discharge site. This site was located in a military reservation and characterized by limited human habitation. The two stream samples obtained from this site revealed a substantial increase in the concentrations of FS (390-840/100 mℓ), a moderate increase in the FC (150-430/100 mℓ) and minimal increase in CP (3-5/100 mℓ) over that recovered from Site K-0. The third downstream sampling site (Site K-2) was located only 2 741 m (300 yd) upstream of the sewage effluent discharge site and the surrounding area was used for human habitation as well as for poultry farming. Three stream samples were collected from this site and the results show a dramatic increase in the concentrations of FC (3700-39,000/100 mℓ) and FS (6500-18,000/100 mℓ) but only a moderate increase in CP (40-46/100 mℓ). It should be noted that Sites K-0, K-1, and K-2 are located above the sewage effluent discharge site and that there is no evidence that human fecal wastes are being discharged into Kīpapa Stream in these areas. However, runoff from land where there are animals enters the stream. The high FC:FS ratio in these stream samples indicated that the source of these FC and FS was probably of animal rather than human origin. Moreover, the high FC:CP in these same stream samples indicated that the fecal matter was fresh and had not been treated or disinfected in any way.

To determine the concentrations of FC and FS entering the stream as a result of the sewage effluent discharge, three samples of the chlorinated sewage effluent entering the stream were analyzed. Despite the fact that the sewage effluent was chlorinated, substantial concentrations of FC (760-3900/100 mℓ) and FS (650-9400/100 mℓ) were recovered indicating that ineffective chlorination of the effluent was occurring during these periods of sampling. As expected, the concentrations of CP (2300-3400/100 mℓ) in these samples reflected the expected concentrations of CP in untreated sewage. Three stream samples were then taken from site K-4 which was located only 549 m (600 yd) downstream of the sewage effluent discharge site and represented the mixing of the sewage effluent with the stream. The concentrations of FC in the three samples ranged from 1800 to 2800/100 mℓ as compared to 1900 to 5300/100 mℓ for FS and 1500 to 2100/100 mℓ for CP.

In summary, the efficacy of using CP to assess the impact of sewage discharge into Kīpapa Stream was evaluated by comparing the concentrations of CP to that of FC, FS, and orthophosphates in stream samples upstream and downstream of the sewage effluent discharge site. A major consideration in interpreting the results of this study was that the total volume of the sewage effluent entering the stream was 2 to 4 times the volume of the stream during these sampling periods. Thus, the volume of water in the sewage effluent may dilute the constituents in the stream while adding its own constituents to the stream. In this regard it is significant to note that the concentrations of FC and FS in the chlorinated sewage effluent may vary from 0 to 10^4/100 mℓ, depending on the efficiency of the sewage treatment processes, whereas the concentrations of CP in the chlorinated sewage effluent remain fairly constant at 10^2 to 10^4/100 mℓ.

The results of this study clearly showed that the concentrations of FC, FS, and CP were lowest in stream samples taken in a remote area farthest upstream. However, even at this site, substantial concentrations (≥100/100 mℓ) of FC and FS could be recovered from stream waters. On the other hand, CP concentrations were undetectable in the stream sample. As the stream flowed downstream, progressively passing through areas used more extensively for human uses, the concentrations of FC and especially FS drastically increased to levels of 10^3 to 10^5/100 mℓ, whereas the concentrations of CP in these stream waters did not exceed 50/100 mℓ. Since there were no known discharges of sewage or human wastes into these streams and since these streams were characterized by a low FC:FS ratio and a high FC:CP ratio, the sources of these indicator bacteria in streams were concluded as not originating from humans and were most likely from animal fecal wastes. It was also significant to note that the concentrations of orthophosphates in all these upstream water samples were very low, indicating that the stream water did not contain high loads of total organic matter as was recovered in the sewage effluent and in the stream water sample just downstream of the sewage effluent discharge site. By comparing the concentrations of FC, FS, CP and orthophosphates in the sewage effluent and in samples of stream water taken immediately upstream and downstream of the sewage effluent discharge site, it was obvious that the stream site below the sewage discharge site contained sewage effluent whereas the stream site above the sewage effluent discharge site did not. Significantly, this conclusion could not be attained with confidence by analyzing the stream water for only FC and FS which are the traditional measurements of water quality. It was the additional measurement of orthophosphates which clearly indicated whether the stream waters did or did not contain sewage. The results of this study suggest that this same conclusion can be reached by analyzing stream water for concentrations of CP.

Water Quality Analysis of 'Āhuimanu Stream

The analysis of Kīpapa Stream indicated that CP can serve as a more direct means of assessing the impact of sewage discharge into streams as compared to measuring stream water for concentrations of FC, FS, and orthophosphates. To further test the utility of CP as a reliable indicator of water quality, a similar study was conducted in 'Āhuimanu Stream which is located on the windward site of O'ahu. Unlike Kīpapa Stream, 'Āhuimanu Stream is located in a wet region and the distance of the stream from its origin in the mountains to its discharge into the ocean is relatively short. However, like Kīpapa Stream, discharge of sewage effluent from a WWTP (Ahuimanu WWTP) is the only known source of fecal waste which is being discharged into 'Āhuimanu Stream. Because rainfall characterizes this study area, the effect of wet weather on the concentrations of indicator bacteria in the stream was also evaluated. Rain or wet weather was defined as sampling days when heavy rainfall was obvious during or 12 hr before the sampling period. During rainy conditions, the increase in turbidity of the stream water was apparent and could be measured. Four sampling sites were selected for this study. The first site (A-0) was at the foot of the mountains in an uninhabited area classified as forest reserve. The second site (A-1) was at the point where the stream passed through the first small subdivision after leaving the forest reserve area. The third site (A-2) was the sewage effluent discharge point from Ahuimanu WWTP, and the last site (A-3), designated lower 'Āhuimanu Stream, was located only 402.25 m (0.25 miles) downstream from the sewage effluent discharge site. The results of analyzing water samples from these sites for FC, FS, CP, orthophosphates, chlorine, and turbidity are summarized in Table 8.

Six stream samples representing three rainy days and three dry days were obtained from Site A-0. During the dry sampling days, the concentrations of FC (25-210/100 mℓ) and FS (40-300/100 mℓ) were at moderate levels,

TABLE 8. FECAL COLIFORM, FECAL STREPTOCOCCUS, AND *CLOSTRIDIUM PERFRINGENS* ANALYSIS OF 'ĀHUIMANU STREAM UPSTREAM AND DOWNSTREAM OF AHUIMANU WWTP DISCHARGE SITE

SAMPLING SITE	SAMPLING DATE	TURBIDITY (NTU)	TOTAL CHLORINE (mg/ℓ)	ORTHO-PHOSPHATE (mg-P/ℓ)	INDICATOR BACTERIA (CFU/100 mℓ)			INDICATOR RATIO	
					FC	FS	CP	FC:FS	FC:CP
A-0 (upper Ahuimanu [forest res.] 1.5 miles upstream of WWTP)	06/30/81	0.3	<0.1	0.006	25	69	1	0.4	25
	07/07/81	0.4	ND	0.011	26	40	<1	0.7	>26
	07/14/81*	0.3	ND	0.013	330	750	1	0.4	330
	07/28/81*	0.3	ND	0.014	4600	3100	<1	1.5	>4600
	08/11/81*	0.6	ND	ND	1700	4100	18	0.4	94
	09/23/81	0.3	ND	0.005	210	300	1	0.7	210
A-1 (mid-Ahuimanu [Small Subdivision] 0.6 miles upstream of WWTP	06/30/81	5.5	<0.1	0.001	40	140	7	0.3	6
	07/07/81	5.6	<0.1	0.001	140	45	5	3.1	28
A-2 (Ahuimanu WWTP Effluent discharge)	08/11/81*	4.7	0.2	ND	52	140	110	0.4	0.5
	09/23/81	4.5	ND	6.0	110	120	310	0.9	0.4
A-3 (lower Ahuimanu 0.25 miles downstream of WWTP)	06/30/81	7.0	0.2	2.5	100	40	100	2.5	1.0
	07/07/81	7.7	0.2	2.7	140	240	56	0.6	2.5
	07/14/81*	22.0	0.2	0.9	13000	11000	920	1.2	14.1
	07/28/81*	9.7	ND	2.9	20000	13000	140	1.6	142.9
	08/11/81*	14.0	0.2	ND	36000	36000	210	1.0	171.4
	09/23/81	5.4	ND	2.8	140	270	110	0.5	1.3

NOTE: NTU = nephelometric turbidity unit.
ND = not done.
*Wet weather conditions.

whereas the concentrations of CP (0-1/100 mℓ) were very low. During wet weather days, the concentrations of FC (330-4600/100 mℓ) and FS (750-4100/100 mℓ) increased dramatically while there was only a slight increase in the concentratrions of CP (0-18/100 mℓ). It is significant to note that the turbidity of the water did not increase significantly at this stream site even during wet-weather conditions. This was believed to be due to a number of factors, such as the heavy underbrush and the wet, well-packed soil in this area. Moreover, much of the rain water entering the stream at this site represented subterranean sources, such as water emerging from the stream bottom as springs and from the sides of some stream banks. The low ratio of FC:FS and the high ratio of FC:CP indicated that the source of fecal organisms was not from man and was probably fresh animal fecal waste which entered the stream. The low orthophosphates in the stream samples indicated that the organic load in this stream was also very low.

Two stream samples obtained from Site A-2 during dry days yielded moderate concentrations of FC (40-140/100 mℓ) and FS (45-140/100 mℓ), and low concentrations of CP (5-7/100 mℓ). Although these water samples had moderate turbidities (5.5-5.7 NTU) their orthophosphate concentrations were still very low. Overall, the quality of water at Site A-2 was similar to that at Site A-1. Analyses of two samples of the chlorinated sewage effluent from Ahuimanu WWTP yielded concentrations of FC (52-110/100 mℓ) and FS (120-140/100 mℓ) which were similar to that observed in the upstream water samples. However, the high concentrations of CP (110-310/100 mℓ) and the high concentration of orthophosphate (6.0 mg/ℓ), as well as the low FC:CP ratio, indicated that this sample was very different from that of the upper stream water samples.

Finally, six samples representing three wet-weather days and three dry sampling days were obtained from Site A-3. During the dry sampling day the concentrations of FC (100-140/100 mℓ) and FS (40-270/100 mℓ) were at moderate levels, while the concentrations of CP (56-110/100 mℓ) were in the range expected in sewage effluent. During wet-weather days, the turbidity and concentrations of FC (13,000-36,000/100 mℓ) and FS (11,000-36,000/100 mℓ) increased dramatically, whereas the concentrations of CP (140-920/100 mℓ) increased significantly in only one of the three samples. It is significant to note that all samples from Site A-3 contained low but definite concentrations of chlorine (0.2 mg/ℓ) and relatively high concentrations of orthophosphates (0.9-2.8 mg/ℓ), indicating that these samples contained significant volumes of sewage effluent. The data also indicated that during wet-weather days, the dramatic increase in the concentrations of FC and FS in stream samples from Site A-3 is due to the input of the very high concentra tions of FC and FS in the stream from Site A-1 upstream from the sewage effluent discharge site. Thus, during wet-weather days, the FC:CP ratio in these samples also increased dramatically.

In conclusion, the results from the analysis of 'Āhuimanu Stream were very similar to that obtained from the Kīpapa Stream survey. That is, by utilizing three measurements (FC, FS, orthophosphates), the contribution of sewage effluent to stream water can be assessed. However, when the single measurement of CP was utilized, the same conclusion can be made. Thus, CP appears to be a reliable, feasible and direct means of assessing the impact of sewage discharge into streams. The results of this study also showed that wet-weather conditions dramatically increases the concentrations of FC and FS but not of CP in the stream. Undoubtedly, this is due to the run-off of FC and FS accumulated on the land into the stream. The only slight increase in CP concentrations in streams following rainy conditions indicate that CP concentrations in the land may be very low. However, this is contrary to the reports of Smith (1975) who reported high concentrations of CP in all North American soil samples tested. It is possible that the concentrations of Hawai'i are different. Alternatively, these results may suggest that CP does not wash off the soil as readily as FC or FS.

Densities and Adsorptive Capacity of Indicator Bacteria to Soil

The finding of dramatic increases in the concentrations of FC and FS but not of CP in stream water following a rain raised the possibility that CP was not found in high concentrations in the natural soil of Hawai'i. To determine the relative densities of FC, FS, and CP in soil, two soil samples obtained adjacent to the 'Āhuimanu Stream site (A-0) were analyzed for the three indicator bacteria by using the MPN method. The results (Table 9) show that the concentrations of CP ($1.1\text{-}14 \times 10^4$/100 g) were greatest in the soil followed by that of FS ($2.2\text{-}35 \times 10^3$/100 g) and finally that of FC ($1.4\text{-}5.4 \times 10^3$/100 g). The relative concentrations of CP, FS, and FC correspond to the known greater stability of CP, followed by that of FS and finally that of FC under environmental conditions. It should be cautioned,

TABLE 9. INDICATOR DENSITIES IN SOIL ADJACENT TO 'ĀHUIMANU STREAM (SITE A-0)

DATE	INDICATOR BACTERIA FC	FS	CP
	--------(MPN/100 g)--------		
3/02/82	5.4×10^3	2.2×10^3	1.1×10^4
3/20/82	1.4×10^3	3.5×10^4	1.4×10^5

however, that the indicator concentrations in the soil may not be directly comparable to the indicator concentrations in the water since the MPN method, as opposed to the membrane filtration method, was used to assess the concentrations of indicator bacteria in soil. This was particularly true for CP since the MPN medium (RPM) differs from that of the mCP medium used in the membrane filtration method. Significantly, the RPM medium is known to be capable of recovering vegetative cells of CP to a greater degree than the mCP medium. However, since only the top 12.7 mm (1/2 in.) of soil was sampled, an obligate anaerobe like CP would be unlikely to multiply and to form vegetative cells.

Based on the results obtained, CP was tentatively concluded to be more tightly bound to particulate matter than FC or FS. This conclusion was supported by the subsequent observation that when sewage was clarified by filtration through a course pre-filter (AP-20), the concentrations of CP in the clarified sewage were reduced by factors of 10 to 100, whereas the concentrations of the FC and FS in these same clarified sewages were not appreciably reduced.

Solid evidence that CP readily adsorbs onto soil was obtained when sewage effluents were percolated and pumped through 0.3 and 0.9 m (1 and 3 ft) of soil. In this experiment, the concentrations of FC, FS, and CP in the sewage effluent were compared with their concentrations after having been pumped through the soil layer. The results (Table 10) show that the concentrations of CP were drastically reduced in the soil filtrate, whereas substantial concentrations of FC and FS were recovered in these same soil filtrates. These results indicate that CP can be expected to be adsorbed out of the water phase by the soil more efficiently than FC and FS.

In conclusion, high concentrations of FC, FS, and CP can be expected in soils of Hawai'i. During wet-weather conditions, considerably more FC and

TABLE 10. RECOVERY OF INDICATOR BACTERIA FROM SEWAGE EFFLUENT PUMPED 1 FT AND 3 FT BELOW SOIL SURFACE

DATE	INDICATOR*	INDICATOR COUNT/100 mℓ		
		Effluent Stream	Range @ Tube Depth	
			1 ft	3 ft
12/14/81	FC	17,000	2600-8400	60-400
	FS	11,000	1400-2200	340-540
	CP	9,000	<1-50	<1-2
12/14/81	FC	5,200	1100-1500	140-620
	FS	3,800	1800-1900	480-640
	CP	5,400	5-15	<1-<1
12/21/81	FC	700	240-260	81-440
	FS	2,800	630-940	40-43
	CP	3,200	<1-5	<1-<1
12/21/81	FC	470	110-720	25-340
	FS	3,000	780-840	160-180
	CP	950	5-6	<1-2

NOTE: ft x 0.304 8 = m.
*FC = fecal coliform,
FS = fecal streptococcus,
CP = *Clostridium perfringens*.

FS as compared to CP are carried in runoff into streams, resulting in significant increases in the concentrations of FC and FS but not of CP in stream water samples. Thus, the lower concentrations of CP in the stream water sample following wet-weather conditions were concluded to be due to the greater adsorption of CP to soil and other particulate matter.

Assessment of mCP Medium Specificity

The conclusion of this study is based on the reliability of the mCP medium to specifically recover CP. Bisson and Cabelli (1979) reported that 93% of the colonies which were counted as presumptively positive for CP were subsequently confirmed as CP by identifying these colonies. To verify the reliability of the mCP medium in this study, 98 colonies of presumptively positive CP were obtained from mCP used to recover CP from three different streams (Kīpapa, Mānoa, 'Āhuimanu). The 98 colonies were tested for the following characteristics: (1) presence of gram-positive rods with no apparent spores, (2) sulfite reduction (blackening of the SPS stab medium); (3) stormy fermentation in litmus milk, (4) strict anaerobicity, (5) fermentation of lactose with gas and acid production, (6) presence of gelatinase, (7) nitrate reduction to nitrite, and (8) non-motility. A total of 89 of the 98 colonies or 91% were positive for the eight tests and were therefore determined to be true CP (Table 11). Thus, the reliability of the mCP medium as tested was determined to be approximately 91%. As a further test of the specificity of the mCP medium, 29 colonies which were presumptively negative for CP on the mCP medium were similarly tested. Only 1 of the 29 colonies or 3% was subsequently identified as CP. This low rate of false positive also supports the reliability of the mCP medium.

Although the data indicated that the mCP medium is specific for CP, high concentrations occasionally occurred of non-CP colonies in some samples. This was especially apparent when water samples from Site K-2 in Kīpapa Stream were analyzed. To quantitate the expected frequency of non-CP to CP colonies recovered on mCP medium, water samples from Sites K-2, K-3, and K-4 were analyzed by the mCP medium and the numbers of CP colonies to non-CP colonies determined by analyzing two samples from each of the three sampling sites. The results (Table 12) show that CP comprised 85–88% of the colonies recovered from the Mililani WWTP sewage effluent (Site K-3) and 74 to 76% of the colonies recovered from Kīpapa Stream water (Site K-4) which was an approximate 4:1 mixture of sewage effluent and Kīpapa Stream water.

TABLE 11. CONFIRMATION OF NATURAL ISOLATES FROM mCP MEDIA AS CLOSTRIDIUM PERFRINGENS

Date	Stream	No. CP Isolates	No. Confirmed	No. Non-CP Isolates	Confirmed
06/03/81	Kīpapa	19	13	13	1
06/10/82	Mānoa	19	19	-	-
06/24/81	Kīpapa	18	17	3	0
06/30/81	'Āhuimanu	12	12	3	0
07/07/81	'Āhuimanu	10	9	5	0
08/04/81	Mānoa	10	10	-	-
10/14/81	Kīpapa	10	9	5	0
Total		98	89	29	1
Percent Confirmed			91		3

TABLE 12. COMPARISON OF PERCENT CLOSTRIDIUM PERFRINGENS COLONIES FOUND IN SAMPLES FROM KĪPAPA STREAM SITES, O'AHU, HAWAI'I

Date	Site	No. CP Colonies*	Total No. Colonies†	% CP
6/17/81	K-2	10	127	8
6/24/81		26	407	6
6/17/81	K-3	135	154	85
6/14/81		274	313	88
6/17/81	K-4	156	212	74
6/13/81		139	183	76

*Colonies showing typical Clostridium perfringens (CP) morphology (yellow growth which turns pink to red when exposed to ammonium hydroxide fumes) on mCP media.
†Total mCP plate count of CP and non-CP colonies.

In contrast only 6-8% of the colonies were CP when Kīpapa Stream water from Site K-2 were analyzed. Subsequent characterization of the non-CP colonies indicated that most of these colonies resulted from the growth of bacteria which reduced sulfite and were anaerobic, spore-forming, gram-positive rods. Therefore, these non-CP colonies were concluded to represent other species of Clostridium present in the water.

Although the extremely low percentage of CP colonies were peculiar to water samples obtained from Site K-2, it was generally true that high percentages of CP to non-CP colonies were recovered when samples of sewage were analyzed, and low percentages of CP to non-CP were recovered when stream waters were analyzed. This same phenomenon was reported by Cabelli (1977). In this regard, although it is possible that the overabundance of non-CP colonies may adversely affect the growth of CP, this was not considered likely because of the fast growth rate of CP at the selectively high incubation temperature of 45°C. Even on plates with a heavy background growth of non-CP colonies, CP colonies were noticeably larger than the non-CP colonies.

SUMMARY AND CONCLUSIONS

Federal, state and county governments are responsible for monitoring, and maintaining the aesthetic, aquatic habitat, and recreational quality of streams. In assessing the hygienic quality of streams, it is not feasible to analyze the streams for the presence of the many pathogenic microorganisms which may be present in the water. Instead, stream waters are analyzed for the presence of coliform bacteria as an indicator for the presence of fecal matter in the stream and, thus, indirectly predict the presence of pathogenic microorganims. To assess the hygienic quality of recreational waters, federal and state regulations require that samples of the water be tested only for concentrations of fecal coliform (FC) bacteria based on the assumption that all unpolluted streams will not contain FC in concentrations exceeding 200/100 mℓ of stream water. In this regard the Hawaii Public Health Regulations on Water Quality Standards (Dept. of Health 1982, §11-54-08) specify

> Fecal coliform content shall not exceed a geometric mean of 200 per 100 mℓ in 10 or more samples collected during any 30-day period and not more than 10% of the samples shall exceed 400 per 100 mℓ in the same period.

This same regulation is applied to every state in the U.S. and assumes that natural environmental quality of all streams in the U.S. are similar. However, in a recent and carefully conducted study, Fujioka (1983) documented that FC in excess of 200/100 mℓ could be frequently recovered from streams flowing through a forest reserve area with no known sources of contamination and the absence of nearby human habitation. Moreover, when streams flowed through suburban or agricultural areas, the concentrations of FC in these streams increased dramatically to levels ranging from 800 to 10,000/100 mℓ. Thus, the common practice of assessing the impact of sewage effluent discharge into a stream by analyzing the streams below the sewage effluent discharge site for FC was not meaningful, even when the sewage treatment plant was not operating efficiently and was discharging sewage effluent with concentrations of FC exceeding the maximum permissible levels of 200 to 400 FC/100 mℓ. Two significant conclusions were made based on the results of this earlier study. First, the measurement of FC alone cannot be used to assess the impact of sewage effluent discharge into streams on O'ahu. Moreover, because of the high concentrations of FC in all streams on O'ahu, the usefulness of FC as a means of determining the hygienic quality of stream water on O'ahu is questionable. Second, the presence of sewage effluent discharge into streams can be determined by determining the concentrations of FC, fecal streptococcus (FS), and orthophosphates in stream samples obtained from above and below the sewage effluent discharge site. Moreover, the source of the high concentrations of FC and FS in all streams on O'ahu did not appear to be of human origin but most probably of animal origin.

The purpose of this present investigation was to determine whether Clostridium perfringens (CP) could serve as a better indicator of fecal contamination than coliform bacteria and, in particular, to be useful in determining the hygienic quality of streams in Hawai'i. To assess the usefulness of CP, the same sampling sites utilizing the same two streams (Kīpapa and 'Āhuimanu) used in the earlier study were selected. As in the earlier study, these stream waters were analyzed for concentrations of FC, FS, and orthophosphates and compared to that of CP. The results of this investigation show that the same conclusion regarding the presence or absence of sewage effluent in stream waters could be reached by analyzing the stream water for the three measurements (FC, FS, orthophosphates) or only CP. The usefulness of CP in assessing the hygienic quality of stream was especially encouraging because the concentrations of CP in stream water were directly correlated with the predicted concentrations of fecal matter in that stream. In streams located in remote areas uninhabited by man, the concentrations of CP ranged from 0 to 10/100 mℓ and only increased to levels of 20 to 50/100 mℓ in streams located in urban and agricultural areas. However, in sewage effluent (unchlorinated or chlorinated) the concentrations of CP ranged from 1,000 to 10,000/100 mℓ. Moreover, CP at relatively high concentrations could be readily recovered from sampling sites downstream of the sewage effluent discharge site.

As pointed out by Dutka (1973) a good indicator of water quality should fulfill four major criteria. Thus, the evaluation of CP should be based on Dutka's four criteria.

1. Be present and occur in much greater numbers than the pathogens concerned. The concentrations of CP in raw sewage were determined to average 10^4/100 mℓ as compared to 10^7/100 mℓ for FC and 10^6/100 mℓ for FS. Although CP is at a lower concentration than that of FC and CP, it is still at an acceptable concentration which is greater than the expected concentrations of pathogens.
2. Not be able to proliferate to any greater extent than enteric

pathogens in the aqueous environment. CP is a strict anaerobe and as such will multiply only in the absence of oxygen. Since oxygenation of water environments is a normal and desirable characteristic of aqueous environment, CP will not proliferate in the aqueous environments. In this regard, CP will be similar to human enteric viruses which cannot multiply in aqueous environments.

3. Be more resistant to disinfectants and to the aqueous environments than the pathogens. CP forms spores which are one of the most resistant biological forms. Evidence from this investigation as well as from others have clearly shown that CP will resist common sewage treatment procedures, including disinfection with chlorine. Thus, the concentrations of CP in secondary treated as well as in chlorinated sewage were still at 10^3 to 10^4/100 mℓ. In contrast the concentrations of FC and FS were reduced to 10^4 to 10^6/100 mℓ in secondary treated sewage and to levels of 0 to 100/100 mℓ in properly chlorinated sewage effluent. Salmonella, a bacterial pathogen, is similar to FC and is expected to respond in a similar manner. Human enteric viruses are known to be much more resistant to chlorination and to survive better in the aqueous environment than FC. However, CP is even more stable than these pathogenic viruses under similar conditions.

4. Yield characteristic and simple reactions enabling as far as possible an unambiguous identification of the group. The method of Bisson and Cabelli (1979) is a new two-step membrane filtration technique to selectively recover CP from sewage and environmental waters. This technique was evaluated by our laboratory and determined to be a practical, feasible, and reliable method. We determined that 91% of the presumptively positive CP colonies could subsequently be confirmed as CP and only 3% of the presumptively negative CP colonies were subsequently determined to be true CP.

In summary, CP fulfills the four major criteria for a good indicator of water quality. Field data to support the usefulness of CP as a good indicator of water quality was obtained by analyzing two streams which were receiving sewage effluent discharge. CP was determined to be a superior indicator compared to FC of stream water quality in Hawai'i. Therefore, CP is proposed to be used by all government agencies to assess the quality of streams in Hawai'i. In this regard, pristine streams can be expected to have less than 10 CP/100 mℓ, whereas streams in urban and agricultural areas can be expected to contain less than 50 CP/100 mℓ. On the other hand, streams contaminated with sewage can be expected to contain CP in concentrations greater than 50/100 mℓ. The ratio of FC:CP is also proposed to be used to determine whether the source of fecal matter in the stream was fresh or underwent treatment. Thus, a FC:CP ratio exceeding 100 is indicative of a fresh, untreated sewage source. On the other hand, a FC:CP ratio of less than 10 is definitely indicative that the source of sewage was either old or highly treated or disinfected.

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