MODIFICATION OF FUNG DOUBLE TUBE AND CP ANASELECT OXYPLATE METHODS TO IMPROVE THEIR PERFORMANCE IN ENUMERATING *CLOSTRIDIUM PERFRINGENS* FROM SEWAGE AND ENVIRONMENTAL WATERS

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ABSTRACT

The Fung Double Tube (FDT) and the newly developed CP AnaSelect Oxyplates methods can enumerate concentrations of Clostridium perfringens *without need for external anaerobic generating systems. Because* Clostridium perfringens *is a reliable indicator of fecal contamination and it is one of the fastest growing fecal bacteria, these two methods were evaluated as feasible methods to screen recreational waters for sewage contamination. To increase the sensitivity and selectivity of these methods, three modifications were evaluated. The first modification was to pretreat the water samples using a microwave oven to attain high temperature (70C) for a short time (2.5 min) to reduce the interfering growth of non-*C. perfringens *colonies on the selective media. The second modification was the addition of phosphatase reaction, thus enumerated colonies could be confirmed as* C. perfringens. *The third modification was to increase the sample volume for the FDT test from 5 to 10 mL/tube. The data collected showed that these modifications improved the selectivity and sensitivity of these two methods to enumerate* C. perfringens *from sewage-contaminated water samples as well as environmental water samples such as streams, harbors, canals and coastal swimming beaches. The recovery efficiencies of the FDT and experimental CP AnaSelect Oxyplate for* C. perfringens *were similar to traditionally used membrane* C. perfringens, *tryptose sulfite cycloserine and Shahidi Ferguson perfringens agar media by membrane filtration technology, followed by incubation in anaerobic cham-*

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bers. These results show that the modified FDT and CP AnaSelect Oxyplate methods are feasible and reliable methods to monitor environmental waters for C. perfringens. *The FDT method is especially promising because it is simple, inexpensive and can produce results in 5–6 h.*

PRACTICAL APPLICATIONS

Clostridium perfringens is currently being evaluated as a reliable indicator of sewage contamination in recreational waters. However, traditional methods to assay for *C. perfringens* are expensive and cumbersome because of the need for external anaerobic generating systems. In addition, target colonies must be confirmed by a second test. In this study, two methods are described, with capabilities to self-generate anaerobic conditions and to immediately confirm the target colonies as *C. perfringens*. Moreover, one of the methods meets the difficult criterion of obtaining results in 6 h so decision on closing the beach can be reached on the same day the beach water sample is tested. Since these two methods are feasible and reliable, it will encourage many laboratories to assay their recreational waters for *C. perfringens* and a national database to determine the quality of recreational waters based on concentrations of *C. perfringens* can be developed.

INTRODUCTION

In the U.S.A., all states must routinely monitor swimming beaches for fecal indicator bacteria (FIB) such as *Escherichia coli* or enterococci to determine whether recreational waters meet water quality standards and are safe for swimmers. However, numerous studies (Fujioka and Byappanahalli 2000, 2003; Byappanahalli *et al.* 2003; Byappanahalli and Fujioka 2004; Whitman *et al.* 2005) have shown that these FIB are able to occur, persist and multiply in environmental sites (soil, plants, sediments, sand) and therefore are not reliable indicators of fecal or sewage contamination. As a result, the Environmental Protection Agency (EPA) is currently reevaluating its recreational water quality standards and *Clostridium perfringens* is one of the alternative FIB being considered (USEPA 2007). *C. perfringens* is an anaerobic spore-forming bacteria whose normal habitat is the gastrointestinal tracts of human and some warm-blooded animals. Because its spores are found in high concentrations in sewage and can be enumerated using the reliable membrane *C. perfringens* (mCP) medium (Bisson and Cabelli 1979), *C. perfringens* was proposed as a conservative tracer of sewage-borne pathogens in environmental waters (Bisson and Cabelli 1980). Using the mCP medium, monitoring studies

conducted in Hawaii have shown that *C. perfringens* is a reliable indicator of sewage contamination because it was determined to be present in high concentrations in sewage, at low concentrations in ambient streams and its concentrations increased when streams were contaminated with sewage (Fujioka and Shizumura 1985; Roll and Fujioka 1997; Luther and Fujioka 2004). More recently, EPA reported the need for a rapid beach monitoring method, which can provide results in a few hours so decisions on closing the beach can be made before the swimmers are exposed to that water. To address this need, we (Fung *et al.* 2007) applied the Fung Double Tube (FDT) method to enumerate *C. perfringens* from environmental water samples and reported that counts could be obtained within 5–6 h after incubation.

In the development of methods to enumerate *C. perfringens* from water, the following ideal criteria should be achieved: (1) it must be sensitive and specific enough to enumerate target colonies as *C. perfringens* and not be interfered with growth of nontarget colonies; (2) the enumerated counts should be confirmed for *C. perfringens*; (3) it should be able to process sufficient sample volume (10–100 mL) to measure low concentrations in water; (4) enumerative counts should be obtained within a few hours so decisions on closing beaches can be made before the swimmers are exposed to contaminated waters; and (5) the method should be feasible enough to be completed at the field site without dependence on complex equipment, including many external anaerobic generating systems. None of the methods available today can meet all five ideal criteria. Most of the established methods used to enumerate *C. perfringens* require special equipment to create anaerobic growth conditions. Two methods can meet the criterion of creating anaerobic conditions without reliance on anaerobic chamber. The first is the FDT method, which uses a unique technology of creating anaerobic conditions by forming a thin layer of agar medium between two tubes. The second is the experimental CP AnaSelect Oxyplate method (Oxyrase, Inc., Mansfield, OH), which creates anaerobic conditions in each plate using oxyrase enzyme and a specially designed culture dish.

The goal of this study was to evaluate and modify the FDT method and the CP AnaSelect Oxyplate method to feasibly and reliably monitor sewage-contaminated as well as environmental waters for *C. perfringens* as evidence of sewage contamination. To improve on the performance of these two methods, two procedures were implemented. The first procedure was to pretreat water samples using a microwave oven to achieve high temperature (70C) for a short time (2.5 min). This high-temperature and short-time (HTST) pretreatment procedure was designed to inactivate the potentially interfering concentrations of vegetative bacterial population but not spores of *C. perfringens* in the water sample. Moreover, the HTST treatment was expected to activate the germination of *C. perfringens* spores to shorten

the time to visualize colony formation. The second procedure was to incorporate a phosphatase test to these two methods, so the enumerative counts can be confirmed as *C. perfringens*. The phosphatase test is one of the most reliable tests to confirm for *C. perfringens* (Sartory *et al.* 2006), and phosphatase test such as use of 4-methylumbelliferyl-phosphate (MUP) was successfully added to tryptose sulfite cycloserine (TSC) medium to confirm for *C. perfringens* colonies (Adcock and Saint 2001; Araujo *et al.* 2001, 2004).

MATERIALS AND METHODS

Identification of Water Samples

Water samples were obtained from the island of Oahu, state of Hawaii and included sites designated for swimming, such as 23 coastal marine beaches (BW1-BW23) and seven fresh water streams (SW1-SW7). However, sites not designated for swimming such as three harbors (HW1-HW3) and two canals (CW1-CW2) were also included. From each of these sites, 1,000-mL samples were collected in sterile plastic bottles (Nalgene, Rochester, NY) and transported to the laboratory preserved in coolers and analyzed within 6 h of collection. Raw sewage is a natural source of *C. perfringens* and is the most likely source to contaminate environmental waters. Raw sewage from Sand Island wastewater treatment plant was collected and diluted 1:1,000 with tap water to simulate sewage-contaminated water samples. This diluted sewage was used in the evaluation of various methods for the recovery of *C. perfringens*.

HTST Pretreatment of Water Samples Using Microwave Oven

The microwave oven was chosen to preheat water samples to quickly reach water temperatures (60–70C) that are known to inactivate and perhaps kill off most bacteria but not spore-forming bacteria such as *C. perfringens*. For this study, one 500 mL of tap water sample in a plastic sampling (high-density polypropylene) bottle was placed in the center of the rotating glass tray of a Panasonic microwave oven (Model No NN-L930BA, power output 1100W, Matsushita Home Appliance Corporation of America, Danville, KY). Each sample was heated at the high setting for 1, 2, 2.5, 3, 3.5 and 4 min based on preliminary measurements that these time settings would raise the water temperature below and above temperatures known to rapidly kill most bacteria. The temperature of post-microwave water samples was immediately recorded using a thermometer. The results of this experiment showed a corresponding temperature reading of 40, 60, 70, 75, 80 and 90C, respectively. The

time of the 2.5-min treatment resulting in water temperature (70 ± 2C) was chosen as the HTST pretreatment condition because it reached the maximum desired temperature of 70C. Sewage samples diluted 1:10, 1:100, 1:1,000 and 1:10,000 with tap water were then treated with the HTST procedure and *C. perfringens* enumerated on Shahidi Ferguson perfringens (SFP), mCP and TSC media. The results showed substantially reduced growth of background-interfering bacteria in the sewage sample. In this regard, microwave treatment was clearly more effective when the sample was more highly contaminated, such as 1:10 and 1:100 dilution of sewage.

Confirmation of *C. perfringens* Colonies by Phosphatase Test in SFP and TSC Media

The SFP and TSC media have been used to enumerate *C. perfringens*, but the target black colonies are considered sulfite-reducing *Clostridium* group and presumptive *C. perfringens* colonies. For this study, the phosphatase test to confirm colonies as *C. perfringens* using MUP was added to SFP and TSC media as previously reported (Adcock and Saint 2001; Araujo *et al.* 2001, 2004). Briefly, 50 mg of MUP was initially dissolved in 5 mL of sterile water and added to 500 mL of TSC or SFP sterile liquid agar media. MUP is a fluorogenic substrate, which is cleaved by acid phosphatase produced by *C. perfringens* colonies. Thus, black colonies which take on a blue fluorescence after exposure to long-wave ultraviolet (UV) light (320–400 nm) are considered confirmed as *C. perfringens*.

Enumeration of *C. perfringens* from Water Samples Using the Modified Fung Double System

The method, as previously described by Fung *et al.* (2007), showed promise but was limited by interference with growth of nontarget bacteria, low sample volume and presumptive rather than confirmed counts for *C. perfringens*. To address these limitations, three modifications were made. First, the water sample was pretreated with microwave oven as previously described. Second, the sample volume being assayed was increased from 5 to 10 mL per tube. This was accomplished by mixing pre-warmed, 12 mL of melted double strength (2×) SFP media to a large, sterile screw cap tube (outer diameter [OD] 25 × 150 mm). After mixing, a smaller inner tube (OD 16 × 150 mm) was then inserted into the large tube. This allowed the mixed sample with SFP agar to fill the space between the two tubes to form a thin layer of agar. The cap to the large tube was immediately screwed on to maintain anaerobic condition, and the tubed samples were incubated under aerobic conditions at 42C. The third modification was to add MUP to SFP medium as previously described. After 5–6 h of incu-

bation, black colonies were enumerated as presumptive *C. perfringens*. In tubes containing SFP medium plus MUP, the black colonies were exposed to long-wave UV light (320–400 nm). Black colonies that fluoresced blue were enumerated as confirmed *C. perfringens* colonies, whereas black colonies that did not fluoresce were enumerated as non-*C. perfringens* colonies. For each sample, duplicate tubes were counted resulting in the analysis of 20 mL of sample, and the counts were expressed as colony forming units, cfu/100 mL.

Enumeration of *C. perfringens* Using the CP AnaSelect Oxyplate

The CP AnaSelect Oxyplate (Oxyrase, Inc.) was obtained as prepared medium containing proprietary ingredients in the specially designed culture plates. Briefly, this medium contains oxyrase enzyme, a biocatalytic oxygen-reducing agent, which removes dissolved oxygen from aqueous, gaseous and semisolid environments. The Oxyplate is a self-contained anaerobe chamber in which a sealing ring in the lid of the plate rests on the agar surface to create an airtight seal and serves as a self-contained anaerobic chamber (Wiggs *et al.* 2000). For this test, up to 100 mL of the water samples were initially filtered through a 47-mm-diameter mixed cellulose ester membrane with 0.45 μm pore size (Pall Corporation, Ann Arbor, MI). These membranes were then placed upright on the CP AnaSelect Oxyplate agar, sealed and incubated under aerobic conditions at 42C. After 12–15 h of incubation, the target straw-yellow-colored colonies were counted as *C. perfringens* based on manufacturer's instruction. However, as this method did not have a phosphatase confirmatory step, a second step to confirm the yellow colonies as *C. perfringens* was completed by layering this filter onto a Whatman membrane (47 mm), which was saturated with 5-bromo-4-chloro-3-indolyl phosphate, p-toluidine salt/nitro blue tetrazolium (BCIP/NBT) reagents following the procedure as reported by Senn and Wolosiuk (2005). BCIP is a chromogenic substrate which is hydrolyzed by phosphatase to form an intermediate that undergoes dimerization in the presence of NBT, to produce an indigo dye (NBT-formazan). As a result, the straw-yellow colonies that turn blue/indigo within 10–15 min are considered acid phosphatase-positive and confirmed as *C. perfringens*.

Because the CP AnaSelect Oxyplate is new, its recovery efficiency for *C. perfringens* was compared with the recovery efficiency using the mCP medium, which has been the method of choice in the U.S.A. since 1979 (Bisson and Cabelli 1979). When mCP medium is used, the straw-colored colonies are enumerated as presumptive *C. perfringens*. The mCP medium incorporates phenolphthalein diphosphate as the substrate used for the detection of acid phosphatase. Yellow colonies, which change to purple colonies

upon exposure to ammonium hydroxide fumes, are enumerated as confirmed *C. perfringens* colonies.

Application of FDT and CP AnaSelect Oxyplate Methods to Enumerate *C. perfringens* from Environmental Water Samples

All the environmental samples were initially treated by the HTST method and enumerated for concentrations of *C. perfringens* by FDT and CP AnaSelect Oxyplate method, and the results were compared with the more traditional membrane filtration methods using mCP, SFP and TSC media. All methods included an acid phosphatase reaction and only acid-positive colonies were enumerated as *C. perfringens*.

RESULTS AND DISCUSSION

Recovery of *C. perfringens* Using the Modified FDT System

The FDT method is the only method able to meet the two difficult ideal criteria of a simple, feasible method that can enumerate *C. perfringens* within a few hours (5–6 h) and will not require an external anaerobic generating system. In the current study, we modified the previously described FDT test for *C. perfringens* to overcome some of the limitations of that method. The modifications included HTST pretreatment of water samples, increasing the sample volume from 5 to 10 mL/tube and addition of phosphatase test as MUP to the SFP medium. The effects of these modifications on the recovery of *C. perfringens* from diluted sewage sample are summarized in Table 1. The

TABLE 1.
EFFECT OF HTST PRETREATMENT OF SEWAGE SAMPLE AND INCORPORATION OF PHOSPHATASE TEST (MUP) TO SFP MEDIUM ON THE RECOVERY EFFICIENCY OF *CLOSTRIDIUM PERFRINGENS* USING THE MODIFIED FUNG DOUBLE SYSTEM AFTER 5–6 h OF INCUBATION UNDER AEROBIC CONDITIONS AT 42C

SFP media			SFP media + MUP phosphatase test	
Sample*	cfu/100 mL	Target colony	cfu/100 mL	Target colonies
Sewage-contaminated water	85	Black colonies	60	Blue fluorescing black colonies
			30	Black colonies
HTST-treated sewage	75	Black colonies	65	Blue fluorescing black colonies

*The tested sewage samples were of the dilution 10^{-3}.
SFP, Shahidi Ferguson perfringens; MUP, 4-methylumbelliferyl-phosphate; HTST, high-temperature, short-time; cfu, colony forming units.

TABLE 2.
COMPARATIVE EFFECT OF HTST PRETREATMENT OF SEWAGE SAMPLE AND INCORPORATION OF PHOSPHATASE TEST (MUP) TO SFP AND TSC MEDIA ON THE RECOVERY EFFICIENCY OF *CLOSTRIDIUM PERFRINGENS* USING THE MEMBRANE FILTRATION METHOD AFTER 12–15 h OF INCUBATION IN AN ANAEROBIC CHAMBER AT 42C

SFP media			SFP media + MUP phosphatase test	
Sample*	cfu/100 mL	Target colony	cfu/100 mL	Colony morphology
Sewage-contaminated water	96	Black colonies	82 9	Blue fluorescing black colonies Black colonies
HTST-treated sewage	92	Black colonies	95	Blue fluorescing black colonies
TSC media			**TSC media + phosphatase test**	
Sample*	cfu/100 mL	Target colony	cfu/100 mL	Target colonies
Sewage-contaminated water	90	Black colonies	78 8	Blue fluorescing black colonies Black colonies
HTST-treated sewage	84	Black colonies	81	Blue fluorescing black colonies

*The tested sewage samples were of the dilution 10^{-3}.
SFP, Shahidi Ferguson perfringens; TSC, tryptose sulfite cycloserine; MUP, 4-methylumbelliferyl-phosphate; HTST, high-temperature, short-time; cfu, colony forming units.

results show that the recovery efficiencies of presumptive *C. perfringens* as black colonies were 85 cfu/100 mL in untreated sewage and 75 cfu/100 mL in the microwave (HTST)-treated sewage. When the untreated sewage sample was assayed on SFP + MUP medium, the recovery efficiency of presumptive *C. perfringens* was 90 cfu/100 mL of which 60 cfu/100 mL could be confirmed as *C. perfringens* (blue fluorescing black colonies). When the HTST-treated sewage sample was assayed on SFP + MUP, all 65 cfu/100 mL of black colonies were confirmed as *C. perfringens*. As stated earlier, the HTST treatment reduced the interfering growth of nontarget colonies. Moreover, the addition of MUP to SFP media using the FDT method was successful in confirming the concentrations of *C. perfringens* colonies.

Because the addition of MUP to SFP in the FDT system is a new procedure, the recovery efficiency of *C. perfringens* by FDT method was compared with the recovery efficiency of the standard membrane filtration method using SFP and TSC media incorporated with MUP. The results (Table 2) show that the recovery efficiency of presumptive *C. perfringens* as black colonies on SFP medium were 96 cfu/100 mL in untreated sewage and 92 cfu/100 mL in the microwave (HTST)-treated sewage. When the untreated sewage sample was assayed on SFP + MUP medium, the recovery efficiency of presumptive *C. perfringens* was 91 cfu/100 mL, of which 82 cfu/100 mL could be confirmed

as *C. perfringens* (blue fluorescing black colonies). When the HTST-treated sewage sample was assayed on SFP + MUP, all 95 cfu/100 mL of black colonies were confirmed as *C. perfringens*. These results confirm that the addition of MUP test to SFP and to TSC medium was effective in confirming the black presumptive colonies as *C. perfringens*. The results also suggest that the HTST pretreatment of water samples may also inactivate some target black colonies, which are not *C. perfringens*. The recovery efficiency of *C. perfringens* colonies was similar for both SFP and TSC media using the membrane filtration method, and the results appear to be slightly higher than the recovery efficiency of *C. perfringens* using FDT method. The most likely explanation for this observation is that membrane filtration methods analyzed 100 mL of sample and incubation was 12–15 h, as compared with analyzing a total of 20 mL of sample and incubation for only 6 h by the FDT method.

Recovery of *C. perfringens* Using the CP AnaSelect Oxyplate Method

The CP AnaSelect Oxyplate system (Oxyrase, Inc.) represents a new experimental selective growth medium for *C. perfringens* and a new technology to generate its own anaerobic condition. This method was used to enumerate the concentrations of *C. perfringens*, from untreated and HTST-treated sewage samples. The results (Table 3) show that the recovery efficiencies of *C. perfringens* as target straw-colored colonies were 91 cfu/100 mL from untreated sewage sample and 88 cfu/100 mL from the HTST-treated sample. When the membranes containing the straw-colored colonies were exposed to the phosphatase test using BCIP/NIT reagents, all 91 target colonies recovered from untreated sewage and all 88 target colonies recovered from HTST-treated sewage were confirmed as *C. perfringens*.

Because the CP AnaSelect Oxyplate method uses a proprietary medium, its recovery efficiency for *C. perfringens* was compared with the recovery efficiency when the same samples were assayed by the mCP media method. The results (Table 3) show that the mCP recovery efficiencies for presumptive *C. perfringens* (yellow colonies) were 99 cfu/100 mL from untreated sewage and 84 cfu/100 mL from treated sewage. When the 99 straw-colored colonies from the untreated sewage were exposed to ammonium hydroxide, only 76 (purple colonies) were confirmed and 23 colonies were not confirmed as *C. perfringens*. When the 84 straw-colored colonies from HTST-treated sewage were exposed to ammonium hydroxide fumes, all 84 straw-colored colonies were confirmed as *C. perfringens*. Taken together, these results suggest that the recovery efficiencies for yellow or presumptive *C. perfringens* colonies by the AnaSelect Oxyplate method and mCP method were similar. However, the results suggest that the CP AnaSelect Oxyplate medium appears to be more selective for *C. perfringens* than the mCP medium.

TABLE 3.
COMPARATIVE EFFECT OF HTST PRETREATMENT OF SEWAGE SAMPLE AND INCORPORATION OF PHOSPHATASE TEST (MUP) TO CP ANASELECT OXYPLATE METHOD AND mCP METHOD ON RECOVERY EFFICIENCY OF *CLOSTRIDIUM PERFRINGENS* AFTER 12–15 h OF INCUBATION AT 42C

CP AnaSelect Oxyplate			CP AnaSelect Oxyplate + phosphatase test†	
Sample*	cfu/100 mL	Target colony	cfu/100 mL	Colony morphology
Sewage-contaminated water	91	Straw yellow colonies	91	Dark blue colonies
HTST-treated sewage	88	Straw yellow colonies	88	Dark blue colonies
mCP media			mCP media + phosphatase test‡	
Sample*	cfu/100 mL	Target colony	cfu/100 mL	Colony morphology
Sewage-contaminated water	99	Straw yellow colonies	76 23	Purple colonies Blue colonies
HTST-treated sewage	84	Straw yellow colonies	84	Purple colonies

*The tested sewage samples were of the dilution 10^{-3}.
†5-bromo-4-chloro-3-indolyl phosphate, p-toluidine salt was used as phosphatase substrate.
‡Phenolphthalein diphosphate was used as phosphatase substrate.
CP AnaSelect Oxyplates were incubated under aerobic conditions.
mCP plates were incubated under anaerobic conditions.
mCP, membrane *C. perfringens*; MUP, 4-methylumbelliferyl-phosphate; HTST, high-temperature, short-time; cfu, colony forming units.

Effect of HTST Treatment and Phosphatase Test on Recovery Efficiency *C. perfringens* from Environmental Waters

The newly developed method becomes valid only if it is practically applicable in the environment for monitoring different classes of water samples and if the results are classified into some category of pollution level. We (Fung *et al.* 2007) previously developed the following four categories of pollution based on the concentrations of *C. perfringens* recovered from environmental waters using the FDT method:

(1) Category I classifies water as uncontaminated (<10 cfu/100 mL);
(2) Category II classifies water as nonpoint contamination (10–100 cfu/100 mL);
(3) Category III classifies water as contaminated with sewage (110–500 cfu/100 mL); and
(4) Category IV classifies water as highly contaminated with sewage (>500 cfu/100 mL).

These categories of pollution were applied to the results of recovering *C. perfringens* from 23 swimming beaches (BW1-BW23), seven streams (SW1-SW7), three harbors (HW1-HW3) and two canals (CW1-CW2) using the FDT and CP AnaSelect Oxyplate methods. As controls, the results of these two methods were compared with the recovery efficiencies on mCP, SFP and TSC methods using the membrane filtration method and external systems to produce anaerobic conditions. All samples were initially treated by the HTST procedure before they were analyzed. The results (Table 4) show that all stream samples contained 10–48 cfu/100 mL of *C. perfringens* and were classified as Pollution Category II or impacted by nonpoint sources of contamination. In this regard, all streams in Hawaii are presumably contaminated by urban run-off, which likely includes animal feces. Some of these animal feces, such as ducks that live in streams, are known sources of *C. perfringens* (Roll and Fujioka 1997). Water samples from harbors (HW1-HW3), canals (CW1, CW2) and some beach sites (BW7, BW17, BW18) contained lower recoveries of 10–31 cfu/100 mL of *C. perfringens* and were also classified as Pollution Category II or impacted by nonpoint sources of contamination. Because these sites receive stream water discharge, it was concluded that streams are the source of *C. perfringens* at these sites. Significantly, *C. perfringens* was undetectable at most of the beaches on Oahu, which were classified as Pollution Category I or relatively uncontaminated. These results indicate that the quality of water at these beaches is good and is not contaminated with sewage or stream water. In summary, the results show that all methods measured comparable concentrations of *C. perfringens*. These results indicate that the FDT and CP AnaSelect Oxyplate methods, which do not require an external anaerobic generating system, can be relied upon to monitor environmental waters for *C. perfringens*.

CONCLUSION

C. perfringens is currently being evaluated in the development of new recreational water quality standards because this fecal bacterium is not likely to multiply under most aerobic conditions and therefore its presence in a water sample is more reliably related to fecal or sewage contamination. Moreover, because of its short doubling time of 7–10 min (Johnson 1990), *C. perfringens* is a good candidate for a rapid beach monitoring method. This study focused on improving the FDT method and the CP AnaSelect Oxyplate method to recover *C. perfringens*, because these are the only two methods that meet the difficult ideal criteria of a feasible method that can be completed at the field site and can enumerate *C. perfringens* without dependence on external, anaerobic generating systems. Based on the results of the current study, the

TABLE 4.
COMPARATIVE RECOVERY OF *CLOSTRIDIUM PERFRINGENS* BY FUNG DOUBLE TUBE (FDT) AND CP ANASELECT OXYPLATE (OXYPLATE) METHODS AS COMPARED WITH MEMBRANE FILTRATION METHOD USING mCP, SFP AND TSC MEDIA FROM ENVIRONMENTAL WATER SAMPLES

Sample ID	Sampling sites	*C. perfringens* (cfu/100 mL)					Fung/Fujioka scale of pollution
		mCP	SFP	TSC	Oxyplate	FDT	
SW1	Nuuanu stream	48	33	39	43	40	II
SW2	Kahala stream	21	19	20	26	20	II
SW3	Manoa stream	21	12	10	18	10	II
SW4	Makiki stream	43	31	29	35	40	II
SW5	Palolo stream	28	23	19	32	20	II
SW6	Kahana stream	23	31	18	26	30	II
SW7	Punaluu stream	36	16	25	31	40	II
CW1	Ala Wai canal	27	21	26	31	30	II
CW2	Kaelepulu canal	28	36	20	26	30	II
HW1	Honolulu harbor	0	0	0	0	0	I
HW2	Fishermen's wharf	0	0	0	0	0	I
HW3	Ala Wai harbor	19	16	15	12	20	II
BW1	Kakaako waterfront	0	0	0	0	0	I
BW2	Ala Moana beach	0	0	0	0	0	I
BW3	Magic Island beach	0	0	0	0	0	I
BW4	Sans Souci beach	0	0	0	0	0	I
BW5	Kuhio beach	0	0	0	0	0	I
BW6	Waikiki beach	0	0	0	0	0	I
BW7	Waialae beach	16	19	10	14	20	II
BW8	Maunalua beach	0	0	0	0	0	I
BW9	Sandy beach	0	0	0	0	0	I
BW10	Makapuu beach	0	0	0	0	0	I
BW11	Waimanalo beach	0	0	0	0	0	I
BW12	Lanikai beach	0	0	0	0	0	I
BW13	Kailua beach	0	0	0	0	0	I
BW14	Kualoa beach	0	0	0	0	0	I
BW15	Kaaawa beach	0	0	0	0	0	I
BW16	Swanzy beach	0	0	0	0	0	I
BW17	Kahana Bay beach	18	16	23	19	15	II
BW18	Punalulu beach	21	13	12	12	15	II
BW19	Hauula beach	0	0	0	0	0	I
BW20	Kokololio beach	0	0	0	0	0	I
BW21	Laie beach	0	0	0	0	0	I
BW22	Hukilau beach	0	0	0	0	0	I
BW23	Malaekahana beach	0	0	0	0	0	I

mCP, membrane *C. perfringens*; SFP, Shahidi Ferguson perfringens; TSC, tryptose sulfite cycloserine; cfu, colony forming units; SW, stream water; CW, canal water; HW, harbor water; BW, beach water.

following conclusions were made: (1) The HTST pretreatment procedure successfully reduced the nontarget colonies which grew as interfering colonies on most of the selective media for *C. perfringens*; (2) the addition of phosphatase test to FDT and CP AnaSelect Oxyplate methods allowed these methods to meet the criterion of enumerating for confirmed *C. perfringens* colonies; (3) the advantages of the CP AnaSelect Oxyplate methods is that it can process a large sample volume (100 mL) and does not require external anaerobic generating systems. However, this method still requires 12–15 h of incubation to enumerate *C. perfringens*, and the additional cost of this technology has not been determined; (4) the performance of the FDT method was improved by increasing the inoculum size to 10 mL/tube. Multiple tubes per sample may be needed to increase the sensitivity of this test; (5) the FDT method is the simplest, least expensive method and the only method capable of providing data in 5–6 h; (6) the recovery efficiencies of *C. perfringens* from sewage-contaminated water as well as environmental waters using the FDT and experimental CP AnaSelect Oxyplate system were similar to each other and were comparable to the traditional methods using membrane filtration methods and incubation in anaerobic chambers. These results indicate that the anaerobic conditions generated by FDT and CP AnaSelect Oxyplate are adequate and these methods can be reliably used to monitor environmental waters; (7) streams as well as harbors, canals and beaches, which receive stream discharges, were classified as Pollution Category II based on low to moderate levels of *C. perfringens.* The source of *C. perfringens* in streams represents nonpoint source contamination, most likely animal feces; and (8) most coastal beaches in Hawaii do not receive stream discharges and were classified as Pollution Category I or uncontaminated because these sites did not contain measurable concentrations of *C. perfringens*. These results indicate that most beaches in Hawaii are relatively free of sewage contamination as well as nonpoint source contamination.

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