DEVELOPMENT OF POLYMERASE CHAIN REACTION TECHNIQUES FOR THE DETECTION OF WATERBORNE PATHOGENS IN ENVIRONMENTAL WATERS

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Abstract

Currently water quality indicator bacteria (*Escherichia coli,* enterococci) are utilized to determine the sanitary quality of recreational waters. These indicators are normal inhabitants of the intestinal tract of warm-blooded animals and their presence in recreational water is considered indicative of fecal contamination. However, in tropical climates *E. coli* and enterococci appear to be naturally present in tropical fresh waters and soil. This has questioned the use of *E. coli* and enterococci as water quality indicators for tropical climates and demonstrates the need for alternative means to determine the sanitary quality of recreational waters. The development of the polymerase chain reaction (PCR) method has offered an attractive alternative to the problematic cultural isolation of indicator bacteria and pathogens. The study utilize PCR to detect waterborne pathogens and indicator bacteria in environmental waters. In addition survival experiments were conducted to determine the persistence of PCR-target gene sequences in ocean water. A pair of PCR primers were chosen targeting a *Salmonella* spp. gene sequence. These primers were specific for 25 *Salmonella* spp. and did not cross react with 27 different non Salmonella bacteria. In seeding experiments the sensitivity of these primers was 12 CFU (*Salmonella typhimurium*) in primary treated sewage and 1 CFU in ocean water. In addition, field studies were conducted using PCR and standard culture method. A total of six field samples were tested with these primers

and 3 demonstrated the presence of *Salmonlla* spp. Two of the PCR positive samples were positive for culturally viable *Salmonella* spp. A set of PCR primers were developed to detect *Bacteroides* spp., the most common inhabitant of the human intestinal tract. These primers were specific for 4 *Bacteroides* spp. and did not cross react with 27 non Bacteroides isolates. These primers were able to detect *Bacteroides* spp. in a variety of environmental waters. Survival experiments using *E. coli* and *Salmonella typhimurium* were conducted in ocean water and demonstrated a loss of *E. coli and S. typhimurium* gene sequences after exposure for 14 and 16 days, respectively. In summary this research supports the use of PCR as a water quality monitoring tool.

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Literature Review

I. Types of Diseases Transmitted by Water

There are an estimated 250 million new cases of waterborne illness reported every year, resulting in over 10 million deaths with 75% of these deaths occurring in tropical climates (Snyder and Merson, 1982). Most of these disease outbreaks stem from fecal contamination of water and subsequent ingestion by humans. This form of disease transmission is termed the fecal oral route and is the major route of transmission for waterborne diseases. Fecal contamination of water is most often associated with sewage that is discharged into waters which serve as drinking or recreational waters. In Hawaii, pollution derived from sewage may pose a health risk to individuals who contact contaminated recreational water. This risk comes from contact and ingestion of contaminated water. The risk of contracting an illness from polluted water is dependent on the type of exposure. Activities such as swimming, surfing and scuba diving are often termed primary exposure activities since the individual is in intimate contact with the water. Other activities such as sailing, boating and fishing pose less of a risk since exposure to the water is not as frequent or intimate. The risk of contracting illness from these forms of exposure is dependent on the health of the individual, duration of exposure, and the concentration and type of pathogen present in the water. As seen in Tables 1 and 2 there is a variety of bacterial, viral and protozoan pathogens transmitted by water. Illnesses contracted from recreational waters can be divided into two categories, water contact disease and waterborne disease from the ingestion of contaminated waters.

Table 1. Water-Transmitted Infectious Bacteria

Organism	Disease
A. Water Contact Diseases	
Aeromonas hydrophila	Wound infection
A. sobria	Wound infection
A. caviae	Wound infection
Citrobacter spp.	Wound infection
Acinetobacter calcoaceticus	Nosocomial infections
Enterobacter spp.	Nosocomial infections
Francisella tularensis	Tularemia
Klebsiella pneumoniae	Nosocomial, pneumonia
Fusobacterium necrophorum	Liver abscesses
Klebsiella pneumoniae	Nosocomial, pneumonia
Leptospira icterohemorrhagia	Leptospirosis
Legionella pneumophila	Legionellosis
Mycobacterium tuberculosis	Tuberculosis
M. marinum	Granuloma
Morganella morganii	Nosocomial
Pseudomonas pseudomallei	Meliodosis
Staphylococcus aureus	Wound infections
Vibrio alginolyticus	Wound infections
Vibrio vulnificus	Wound infections
B. Waterborne Diseases	
Aeromonas hydrophila	Enteritis
A. sobria	Enteritis
A. caviae	Enteritis
Campylobacter jejuni	Enteritis
C. coli	Enteritis
Chromobacterium violaceum	Enteritis
Citrobacter spp.	Nosocomial infections
Clostridum perfringens type C	Enteritis
Enterobacter spp.	Nosocomial
Escherichia coli serotypes	Enteritis
Flavobacterium meningosepticum	Nosocomial, meningitis
Plesiomonas shigelloides	Enteritis
Salmonella enteritidis	Enteritis
S. montevideo B	Salmonellosis
S. paratyphi A & B	Paratyphoid fever
S. typhi	Typhoid fever
S. typhimurium	Salmonellosis
Serratia marcescens	Nosocomial
Shigella dysenteriae	Dysentary
Staphylococcus aureus	Food poisoning
Vibrio cholerae	Cholera dysentery
V. fluvialis	Enteritis
V. mimicus	Enteritis
V. parahaemolyticus	Enteritis
Yersinia enterocolitica	Enteritis

(adapted from Hazen, 1988)

Table 2. Water-Transmitted Viral and Parastic Pathogens

Organism	Disease
Viruses	
A. Water Contact Diseases	
Adenovirus	Pharyngitis, eye infections
B. Waterborne Diseases	
Adenovirus	Enteritis
Calicivirus	Enteritis
Norwalk virus	Enteritis
Coronavirus	Enteritis
Coxsackievirus A & B	Meningitis, myocrditis
Echo virus	Enteritis,meningitis
Hepatitis A	Hepatitis
Poliovirus	Poliomyelitis
Rotavirus	Enteritis
Astrovirus	Enteritis
Protozoa	
A. Water Contact Diseases	
Naegleria fowleri	Meningoencephalitis
Acanthamoeba spp.	Meningoencephalitis
B. Waterborne Diseases	
Balantidium coli	Balantidiosis
Cryptosporidium spp.	Cryptosporidiosis
Giardia lamblia	Giardiasis
Entamoeba histolytica	Dysentery

(Hazen, 1988)

A. Water Contact Diseases

The first category, water contact diseases, are those illnesses contracted from contact with recreational waters. Most of the diseases that fall into this

category are characterized by diseases that results from skin and wound infections. Pathogens that causes these types of diseases have both human and environmental sources. The pathogens associated with human sources are generally opportunistic pathogens and are considered normal inhabitants of humans. One example, *Staphylococcus aureus,* has been found to be the most numerous pathogen shed by swimmers (Robinton and Mood, 1966). *S. aureus* is a pathogenic bacterium which often becomes a normal inhabitant of many individuals. Among swimmers, *S. aureus* is capable of causing a variety of infections, usually targeting the skin (e. g. scratches, boils, and other skin infection) and less frequently the eyes and throat. Another example of a pathogen having a human source is *Pseudomonas aeruginosa* which is the most frequent pathogen isolated from people affected by the disease termed "swimmer's ear" (Calderon, 1981). Numerous epidemiological studies have confirmed an association between swimming and ear infection caused by *P. aeruginosa* (Hoadely and Knight, 1975; Seyfried and Cook, 1984).

In addition to human sources, the environment often contains non-human sources of pathogens associated with water contact. These pathogens may be indigenous to the aquatic environment or excreted by animals found in the vicinity of the recreational waters. Many *Vibrio* species are indigenous to the marine environment and are capable of causing a variety of contact diseases. These diseases include ear infections and septicemias with the majority being caused by *V. parahaemolyticus, V. vulnificus,* and *V. alginolyticus. V. vulnificus* has been associated with the septicemia resulting from contact with swimming waters and has a high fatality rate (Bonner, 1983). *V. parahaemolyticus* is usually associated with food borne transmission but may also be transmitted through water contact. The mode of transmission for most of the marine vibrios is through cuts, abrasions and

wounds. In addition to the naturally occurring pathogens, animal urine can be a source of water contact disease. *Leptospira* is probably the best example of a contact disease resulting from excreted urine of infected animals. A variety of animals including rats, dogs, cats, pigs and cattle may carry *Leptospira* and excrete this pathogen into recreational waters. *Leptospira* gains entry via skin abrasions so swimming and water contact activities are the major means of exposure to humans. Numerous outbreaks have occurred in the United States (Table 3). Safer reported an outbreak associated with recreational waters with an attack rate of 63% (Safer, 1961). In this study, the source of infection was a local swimming hole fed by a stream in which dead hogs had been floating. Serological testing was positive in hogs and other animals found in the vicinity of the stream. Since *Leptospira* are not associated with fecal contamination, current water quality standards will not always predict the possible presence of this pathogen.

Table 3. Outbreaks of Leptospirosis Associated with Swimming Water

Outbreak Location	Etiologic Agent	No. of Cases	Possible Source	Reference
Geneva, Ala	*L. pomona*	50	Hogs	Cockburn et al.,1954
Jackson Hole, Wyo	*L. canicola*	24	Dogs	Schaffer et al., 1951
Standing Rock, S.D.	*L. pomona*	5	Livestock	Jellison et al., 1958
Kennewick, Wash	*L. pomona*	61	Cattle	Nelson et al., 1973
Tennessee	*L. interrigans*	7	"unkown"	CDC, 1976
Cedar Rapids, Iowa	*L. pomona*	40	Cattle	Tjalma et al.,1965

The State of Hawaii has the highest incidence of leptospirosis in the United States and accounts for 29% of the total cases reported (Sasaki et al., 1991). Leptospirosis is by far the most serious water-borne disease in Hawaii and has been

associated with swimming, wading and working in fresh water streams (Fujioka et al., 1991). Although freshwater streams in Hawaii are considered the source of *Leptospira,* a lack of available methodology for isolating pathogenic *Leptospira* has made it very difficult to monitor recreational water for *Leptospira.*

B. Waterborne Diseases

Unlike water contact diseases, waterborne diseases result from the ingestion of a pathogen in recreational waters and most result in gastroenteritis, although viral infections cause hepatitis, poliomyelitis, meningitis, and myocarditis. The pathogens involved in gastroenteritis are a large group of microorganisms which include bacteria, viruses and protozoans. For the most part these pathogens are associated with human and animal feces and the fecal oral route of transmission. There are however, pathogens that are naturally occurring (e.g. *V. parahaemolyticus*) in the environment and can cause gastroenteritis from ingestion of contaminated waters (Colwell et al., 1973).

1. Human and Animal Feces : A Source of Pathogens

Human and animal feces are the major sources of pathogens causing waterborne diseases. Feces may enter recreational water by a variety of mechanisms, but one of the most frequent routes is the discharge of sewage by outfalls located near recreational waters. Although waterborne recreational water outbreaks of gastroenteritis are a rare occurrence in the U. S. and most industrialized countries, the potential health threat is always present. This risk is

greatly amplified when sewage discharges occur in close proximity to recreational waters. When considering documented cases of waterborne diseases, however, the range of illnesses is much more limited with the majority being caused by gastroenteritis. Documented disease outbreaks of gastroenteritis are limited mainly to three types of pathogens, bacterial, viral and protozoan.

Although there is a large number of bacterial pathogens that can be transmitted by water, the reported incidence of recreational water outbreaks is limited to typhoid fever, salmonellosis and shigellosis. For the most part, diseases caused by these bacteria are characterized by nausea, vomiting, diarrhea, and abdominal cramps. In the case of typhoid fever, there is also entry into the bloodstream resulting in a persistent fever and often a chronic carrier state.

Typhoid fever was one of the earliest reported diseases associated with recreational water. In 1888 Discher (1963) reported on an outbreak involving 49 cases from swimming in the Elbe River in Germany. Another outbreak occurred ten years later in Walmer, England, and was associated with marine recruits that swam in sea water-filled pools as part of their training (Reece, 1954). In this case a nearby sewage discharge was responsible for contaminating sea water that was used to fill the pool. The first documented outbreak in the United States occurred in 1923 in New Haven, Connecticut. Ciampolini (1921) reported that the cause of typhoid fever among swimmers was the contaminated water in New Haven Harbor. Unlike the previous two cases in England, this report was unable to show a direct relationship between disease and swimming. Water contact was implicated since food, milk and drinking water did not appear contaminated. Through deductive reasoning Ciampolini was able to show an association with swimming in the harbor. As is often the case with waterborne outbreaks the disease causing

organism was not isolated from swimming waters and therefore only a casual relationship is demonstrated.

The second bacterial pathogen associated with gastroenteritis, *Salmonella* spp., has been a rare occurrence when compared to food borne transmission of this pathogen. Only 3 % of waterborne outbreaks between 1971 and 1978 was caused by *Salmonella* spp. (Craun, 1981). The largest outbreak occurred in 1965 in Riverside, California, and over 16,000 people were infected (Craun, 1986). The causative agent of this particular outbreak was determined to be *S. typhimurium* from a contaminated ground water source. Subsequent chlorination appeared to eliminate this problem.

Although the infectious dose of *Shigella* is only 10 to 100 bacilli (Dupont, 1973) as compared to the infectious dose of 10^3 to 10^4 for *S. typhi* and *S. typhimurium* (Mims, 1987), there have been very few cases of *Shigella* outbreaks associated with swimming in contaminated waters. This may be due to the fact that this bacterium does not survive long in the aquatic environment (Dunlop, 1957). The only reported outbreak caused by *Shigella* occurred in Dubuque, Iowa, in 1974 when *Shigella* was isolated in swimming waters frequented by infected swimmers (Rosenberg, 1976). One strain isolated from a swimmer had an antibiotic sensitivity pattern similar to isolates from the swimming waters. An upstream sewage discharge was likely the cause of this particular outbreak. Waters downstream of this discharge point had fecal coliform levels of between 400,000 and 5,000,000 per 100 ml.

The second category of microorganisms, viral pathogens, have been well documented in recreational water outbreaks. As with many bacterial pathogens, it has often been difficult to isolate viral pathogens from recreational waters at the

time of the outbreak. As a result, there are very few cases of direct relationships between swimming in infected waters and disease. Three of the more common causes of viral outbreaks are those involving hepatitis A, the coxsackie viruses and norwalk viruses. In 1969 an outbreak among a Boy Scout troop was attributed to swimming exposure in a South Carolina Lake (Bryran et al., 1974). In this case an association with scouts swimming in the lake and hepatitis A was determined since a large number of swimmers contracted this illness. Although the hepatitis A virus was never recovered, the lake was shown to have fecal contamination. Food did not appear to be a source of fecal contamination. It is important to note, however, the scouts may have contracted this disease from drinking water obtained from the same lake water. As with the majority of cases involving infectious hepatitis, contaminated drinking water is implicated most often (Craun, 1986).

In addition to hepatitis A viruses, coxsackie viruses have been implicated in outbreaks associated with swimming in contaminated waters. In Nirot, France coxsackie A virus was isolated from stool specimens from infected swimmers as well as from a lake water sample (Denis et al., 1974). In addition, samples from the lake also contained *Escherichia coli* levels between 50 and 1000 per 100 ml. A similar outbreak occurred in Michigan in 1979 when coxsackie B virus was isolated from lake water as well as infected swimmers (Hawley et al., 1979). In both of these cases the pathogen was isolated from swimming waters used by infected swimmers.

Recently the Norwalk-like viruses have been considered the major cause of the incidences of swimming-associated gastroenteritis. Many public health officals feel Norwalk virus is responsible for most swimming associated gastroenteritis (Cabelli and Debartolomeis, 1991). Most of the symptoms associated with

waterborne gastroenteritis illnesses indicate that many of these illnesses show symptoms similar to the symptoms expressed by the Norwalk viruses.

Recreational water outbreaks of waterborne protozoan diseases associated gastroenteritis are a rare occurrence when compared to drinking water outbreaks. One such protozoan, *Giardia lamblia*, has been implicated as the most commonly identified pathogenic intestinal protozoan in waterborne outbreaks involving drinking water. The disease caused by this protozoan, Giardiasis, is characterized by nausea, anorexia, and explosive diarrhea. *Giardia* cysts are capable of surviving for long periods in the environment (Rentorff and Holt, 1954) and the infectious dose is as low as 10 cysts (Rentorff, 1954). These factors contribute to the health risk involved with swimming in fecally polluted waters. Giardiasis contracted by contact with water has been documented in a case involving police and fire department divers working in the Hudson River near New York City (Craun, 1986). Another case showing an association occurred in a swimming pool in Washington (Craun, 1986). Both cases were by association since *Giardia* was not isolated from the contacted waters.

2. Environmental Sources of Gastroenteritis

In addition to the pathogens associated with feces, the environment can also be a source of pathogens capable of causing gastroenteritis. Many naturally occurring marine *Vibrio* species are capable of causing gastroenteritis. *V. cholerae* and *V. parahaemolyticus* are human pathogens capable of causing gastroenteritis. *V. parahaemolyticus* is most often associated with the consumption of contaminated shellfish (Colwell et al., 1973).

3. Unknown Sources of Waterborne Outbreaks

Although careful investigation is able to determine the causes of many waterborne outbreaks, the agents responsible for the majority of waterborne outbreaks are never identified. Waterborne gasterenteritis of undetermined etiology accounted for about 77% of all waterborne illnesses reported in the United States between 1920 and 1980. Many factors may contribute to this group of outbreaks. Lack of methodology to isolate pathogens has been, and continues to be a major problem when isolating bacteria, protozoan and viral pathogens, although many new techniques have been developed in the last decade.

II. Monitoring Water for Hygienic Quality: The Indicator Concept

Because of the difficulty in isolating pathogens from the environment and the wide variety of pathogens that may be transmitted through recreational waters, an indicator system for determining the hygienic quality of water was developed. Since the majority of disease causing microorganisms transmitted via water are found in sewage or human fecal material, an indicator of feces was established. While there is no ideal water quality indicator, the best indicator system is one whose densities correlate best with the risk associated with a given type of pollution (Cabelli, 1978). When considering risk analysis, the ideal indicator should possess the following characteristics:

1) The ideal indicator should always be present and occur in much greater numbers than the pathogens concerned.

2) The indicator should not be able to proliferate to a greater extent than pathogens in the aqueous environment.
3) The indicator should be more resistant to disinfectants and to the aqueous environments than pathogens.
4) The indicator should be easy to isolate, identify, and enumerate.

(Dutka, 1973)

Since no microorganism fits all of these criteria, microorganisms that best fit these criteria are utilized knowing that each has positive and negative attributes. Some of the popularly accepted indicators for recreational waters include total coliforms, fecal coliform, *Escherichia coli,* enterococci, *Clostridium perfringens,* and *Bacteroides fragilis* which are normally found in the feces of all humans (Table 4). As seen in Table 5 each of these indicators has different sources and potential uses. It is important to note that these indicators were developed in temperate climates and thus take into account the behavior of these indicators under temperate climate conditions. These characteristics may not apply to tropical climates (Fujioka, 1985).

Table 4. Human Fecal Flora[a]

Group	Genus	Average (CFU/g[b])
Total Bacteria	-----	1.5×10^{10}
Total aerobic bacteria	-----	7×10^{9}
Aerobic Bacteria, Gram negative		
	Escherichia	4×10^{8}
	Citrobacter	1×10^{6}
	Klebsiella	5×10^{4}
	Enterobacter	1×10^{5}
Aerobic bacteria, Gram positive		
	Enterococcus	2×10^{8}
	Staphylococcus	8×10^{6}
	Bacillus	3×10^{4}
Anaerobic bacteria, Gram negative		
	Bacteroides	1×10^{10}
	Lactobacillus	1×10^{9}
Anaerobic bacteria, Gram positive		
	Clostriduim	4×10^{6}
Yeasts	-----	5×10^{4}
Molds	-----	4×10^{4}

[a] Adpated from Oliveri (1981)
[b] Colony forming units/ gram of dry weight of human feces

A. Coliform Bacteria: The First Indicator

In the late 19th century Escherich (1885) first proposed the concept of indicator bacteria. This group, the "coliforms", is an ecologically heterogeneous group of bacteria, some of which consistently and exclusively are associated with the feces of warm-blooded animals. This group is defined by *Standard Methods* (1992) as consisting of aerobic and facultatively anaerobic, gram-negative, nonspore-forming, rod-shaped bacteria that ferment lactose with gas and acid formation within 48 hours at 35°C. Included in this group are the genera *Escherichia, Citrobacter, Enterobacter,* and *Klebsiella.* These bacteria are normally

found in the human intestine, but also occur in soils, on vegetation, and in natural waters (Fujioka et al., 1988; Hazen, 1988). Since these bacteria are found in sources other than sewage, their significance as water quality indicators is questionable.

Table 5. Water Quality Indicators, Significant Sources, and Potential Uses (adapted from Cabelli. 1978)

Indicator	Significant Source[a]	Potential Use[b]
Coliforms	F S I R A	S
Escherichia coli	F S [c]	P F S A
Klebsiella sp.	S I R A	P S N
Enterobacter sp.	S I R A	S
Citrobacter sp.	F S I R A	S
Fecal coliforms	F S [c]	F S
Enterococci	F S [c]	F S A D
Clostridium perfringens	F S [c]	F S D
Candida albicans	F S	P F S
Bacteroides fragilis	S	F S A D
Coliphage	[d]S I R A	S
Pseudomonas aeruginosa		P S N

[a]Relative to other sources: F, feces of warm-blooded animals; S, sewage; I, industrial wastes; R, run-off from uncontaminated soils; A, fresh and marine waters.

[b]Potential use: P, pathogen;F, fecal indicators; S, sewage indicator; A, separation of human from lower animals sources; D, proximity to fecal source; N, indicator of nutrient pollution.

[c]May occur in tropical climates

[d]Questionable

According to Olivieri (1992), there are a number of deficiencies with using total coliform bacteria as sanitary indicators. Coliform bacteria tend to die off faster than many non-bacterial pathogens like the enteric viruses and protozoans like *Giardia.* In addition, coliform bacteria are more sensitive to disinfection than are many non-bacterial pathogens such as enteric viruses and many protozoans.

Another deficiency is the ability of coliform bacteria to survive and replicate in the environment (Hazen, 1988; Fujioka et al.,1988) whereas pathogenic enteric viruses and protozoans are unable to replicate in the environment. In this case coliforms would not necessarily represent fecal contamination. Although total coliform bacteria are still widely used as indicator organisms, there is currently a trend which favors the use of those coliform genera that more accurately represent fecal pollution.

B. Fecal Coliform: A More Specific Indicator of Feces

The fecal coliform are a subgroup of the total coliform group. Research in the use of fecal coliforms was stimulated by findings that showed some total coliforms members included coliforms from non-fecal sources. Fecal coliform are more specifically associated with the feces of warm-blooded animals and were primarily used (1968-1986) for determining the level of fecal material in recreational waters (Geldreich et al.,1962). This group is comprised of four genera, *Escherichia, Klebsiella, Citrobacter,* and *Enterobacter* which are capable of growth at a temperature of 44.5 ^{o}C. Only a few species of *Citrobacter* and *Enterobacter* are capable of growth at 44.5^{o}C. It is the ability to grow at 44.5^{o}C that separates fecal coliform from other coliforms. Of this group, *Escherichia coli* and *Klebsiella spp.*are the species most often isolated from human feces (Table 6) with *Escherichia coli* being the largest percentage (Dufour,1977). As seen in Table 6, *Escherichia coli* appears to best represent coliforms derived from human feces.

Table 6 Percentage of Coliforms in Human Feces

Study Year	Number of samples	Number of Colonies Examined	Percent of Coliforms		
			E. coli	*Klebsiella* species	*Enterobacter/ Citrobacter*
1975	13	438	99.99	0.01	0
1976	15	285	89	5	6
Totals	28	723	96.8	1.5	1.7

(Dufour, 1977)

As with the total coliforms, the fecal coliform also have deficiencies when used as water quality indicators. Like the total coliforms, they have the same survival and sensitivity to disinfection. In addition, regrowth of fecal coliforms and the presence of non fecal members have also contributed to this problem (Knittel, 1975). Although this group may better represent fecal contamination they still have certain deficiencies that make them questionable as indicators.

C. *Escherichia coli:*The Major Coliform in Human Feces

As discussed earlier, *Escherichia coli* is the major coliform found in human feces. Although it would appear that this bacterium would best represent human waste, there is still the problem associated with the survivability of this organism in the environment. Enteric viruses and pathogenic protozoans are able to survive longer in the environment. This problem is partially alleviated by the high numbers of these bacteria in human feces. *Escherichia coli* can be easily distinguished from other coliforms because it lacks the enzymes necessary to degrade urea. This characteristic has made methods developed by Dufour et al. (1981) simple and 90 % specific for isolation of *E. coli* from recreational waters. *E. coli* has shown

promise in prospective epidemiological studies used to determine health risks involved in recreational waters (Dufour,1984).

D. Enterococci: More Stable Than Coliforms

Enterococci are comprised of a subgroup of the Group D streptococci and include the genera *S. faecalis, S. faecium, and S. avium* (*Standard Methods*, 1989). The bacteria in this group are common inhabitants of the intestinal tract of warm-blooded animals and therefore are often used to detect fecal pollution. Although enterococci are more stable in marine waters than coliform bacteria, pathogens, such as the enteric viruses and protozoans are more stable. The enterococci have, however, shown great promise in epidemiological studies relating exposure to recreational waters and the incidence of illness. Studies performed by Cabelli (1984) show enterococci have a better correlation coefficient for total gastrointestinal symptoms than *E. coli*, fecal coliforms and *Clostridium perfringens* in marine environments. Enterococci have become the indicator of choice for regulatory agencies for determining the water quality of marine recreational water.

F. *Clostridium perfringens*: An Alternative Indicator

Clostridium perfringens is a strict anaerobe capable of producing an environmentally stable spore. These characteristics have made *C. perfringens* an attractive indicator. Since it is a strict anaerobe it would not be able to replicate to any great extent in recreational waters. There are problems however with its ubiquity in soils. In 932 soils samples tested by Sidorenko (1967) 85.7 % contained

C. perfringens. Although this has been the major argument against using *C. perfringens* as an indicator, there are attractive attributes. The spore of *C. perfringens* is very stable in the environment and therefore has survival pattern more similar to the pathogenic viruses and protozoans. *C. perfringens* is currently used in Europe as an indicator of sewage contamination (Cotruvo and Vogt, 1990). It has been proposed as an indicator for recreational waters and water recently contaminated with sewage (Fujioka and Shizumura, 1985; Bisson and Cabelli, 1979).

G. *Bacteroides*: The Most Common Human Intestinal Inhabitant

Bacteroides spp. are nonsporulating, obligate anaerobic Gram-negative bacteria and are the predominat genus of the human intestinal tract (Table 4). Currently there are four genera of *Bacteroides* that are dominant in the human intestinal tract, *B. vulgatus, B. thetaiotaomicron, B. fragilis,* and *B. distasonis*. *Bacteroides* is an important potential indicator of fecal pollution since its habitat is restricted to the feces of humans and warm-blooded animals (Kator and Rhodes, 1991). In addition *Bacteroides* spp. are not found in high numbers in a variety of warm blooded animals (i.e. 10^5-10^{10}-fold lower) in common domestic animals such as cattle horses, pigs, chickens, and sheep (Allsop and Strickler, 1985.) Cultural isolation of *Bacteroides* spp. has been demonstrated by Allsop and Stickler (1984) but to date this method has not gained widespread use. This is most likely a reflection of the fact that *Bacteroides* is a strict anaerobe and its survival in environmental waters is questionable.

III. Development of Recreational Water Quality Standards

As mentioned earlier the ideal indicator is one whose densities correlate best with health hazards associated with a given type of pollution (Cabelli, 1982). The ideal indicator will represent the health risk involved in water contact and can be associated with pathogens that may be present. In order to fulfill this requirement two approaches could be used. The first approach would involve determination of indicator densities in each type of pollution, and at the same time, determination of the densities of all pathogens found in each type of pollution. Next the infectious dose would have to be determined. At this point an association between indicator and pathogens would be able to predict the health risk associated with swimming in waters contaminated with each type of pollution. Because of the large number of pathogens that may be transmitted through water and the lack of methodology for determining each of them, this type of relationship is impossible to establish at the current time. The second, and preferable approach, utilizes the densities of the candidate indicators and the illnesses contracted that are specifically associated with the use of a particular water. This comparison is obtained through the use of prospective epidemiological studies.

A. Epidemiological Studies

The first epidemiological study performed with recreational water was done by Stevenson in the 1950s. In this study total coliforms were used to determine the health risk involved in swimming waters. From this study the United State Public Health Service determined that an excess of swimming associated diseases

occurred in fresh water swimmers when the total coliform density exceeded 2300 per 100ml. Based on the results of this study, the first recreational water quality standard was established at 1, 000 total coliform / 100 ml. When fecal coliforms was determined to be a more specific indicator, a fecal coliform standard was established based on the expected ratio of fecal coliform to total coliform. From this study and studies conducted with the Ohio River, the 2300 per 100ml value was related to fecal coliforms. In the Ohio River study the proportion of fecal coliform to total coliform ratio was about 18%. The National Technical Advisory Committee (NTAC) then extrapolated that 400 fecal coliforms represented the same health risk as the 2300 total coliforms per 100 ml (National Technical Advisory Committee, 1968). An additional built-in safety factor was added by dropping this Standard to 200 fecal coliforms per 100 ml. At this point the NTAC recommended the recreational water standard should not exceed a geometric mean of 200 fecal coliforms per 100 ml. Thus, prior to 1972 the recreational standards were based on total coliforms. From 1972 until 1986, the fecal coliform standard was used.

This approach at determining water quality standards was highly criticized since fecal coliforms were not used directly in relating the occurrence of illness (Cabelli, 1974). In 1972 this problem was addressed by a series of studies conducted by the U.S. Environmental Protection Agency which examined the relationship between swimming-associated illness and water quality. Epidemiological studies were used to determine water quality standards for marine and fresh waters (Cabelli, 1983; Dufour, 1984) One of the objective of these studies was to examine several biological indicators of fecal pollution to determine

which had the strongest correlation with swimming-associated health effects. These studies were the first studies dealing with risk assessment in recreational waters.

The results of the studies by Cabelli and Dufour demonstrated that fecal coliforms were not adequate indicators of water quality. Enterococci levels correlated best with swimming associated gastrointestinal illness in both fresh and marine waters. In marine waters *E. coli* did not correlate with the incidence of gastroenteritis in swimmers. Explanations for this lack of association include a decrease in survival during sewage treatment, sensitivity to disinfection, and instability in marine waters (Cabelli, 1983). Although *E. coli* did not correlate with the incidence of gastroenteritis in marine waters, it did correlate equally with enterococci in fresh waters.

The EPA proposed a new set of standards based on these findings. After public comments the final revised recommendations were published the Federal Register in 1986. For fresh water, the new criteria proposed were 126 *E. coli* per 100ml or 33 enterococci per 100 ml. The proposed marine standard was 35 enterococci per 100 ml. In Hawaii the marine standard was lowered to 7 enterococci per 100 ml. These standards are applied to a series of, at least, five samples taken at equally spaced intervals over a thirty day period. The geometric mean of these samplings is then calculated in order to determine compliance with the before mentioned standards. The geometric mean is the antilogarithm of $\overline{\log x}$ calculated by the following equation, where X is the observation value and n is the number of observations.

$$\overline{\log x} = \frac{1}{n}\sum_{i=1}^{n} \log x_i$$

The geometric mean is utilized so that any one event (e.g. rain, sewage discharges) will not greatly influence a series of samplings. Moreover, the epidemiological data of Cabelli and Dufour are based on the geometric mean.

B. Indicators in Tropical Climates

The majority of studies dealing with indicator organisms have been conducted in temperate climates and represent the behavior of these indicators in temperate climates. In contrast, indicator bacteria behave very differently in tropical climates and therefore many of the assumptions made about indicators in temperate climates are not applicable. One basic assumption that is not applicable to tropical climates is the ability of coliform bacteria to survive and replicate in the environment. A number of studies have shown that *E. coli* is capable of surviving and even replicating in tropical environments. In Puerto Rico, *E. coli* was able to survive and replicate in rain forest streams (Carrillo et al.,1985;Lopez-Torres et al., 1987; Valdes-Collazo et al.,1987). Other studies by Hardina and Fujioka (1988) indicate that fecal coliform, *E. coli,* and fecal streptococci are naturally occurring in Hawaii's streams and soils. Once introduced, *E. coli* could remain and become part of the normal flora. The survival and replication of *E. coli* would impede its usefulness as an indicator of the possible presence of pathogens.

IV. Point Source vs. Non-point Source Pollution

A. Point Source Pollution

Point source pollution is pollution derived from a known source, most often associated with practices like municipal waste discharge into marine environments. These discharges are regulated by the Clean Water Act (CWA) of 1972 and a series of permits that allow municipalities to discharge sewage in marine environments. These permits are authorized under the National Pollutants Discharge Elimination System (NPDES). The purpose of these regulations is to protect the marine environment and recreational waters. In 1972, the Clean Water Act mandated the implementation of secondary treatment for municipal waste water facilities. As a result, the City and County of Honolulu were required to implement secondary treatment at their waste facilities. However, NPDES granted waivers to the City and County of Honolulu. These waivers are called the 301 h waivers and exempted qualified treatments facilities from being required to perform secondary treatment as long as they were able to meet marine water quality guidelines. The 301 h waivers are granted primarily to municipalities discharging effluent into deep, cold marine waters.

B. Nonpoint Source Pollution

In addition to point source pollution, there is also a category of pollution that is termed nonpoint source pollution. Nonpoint source pollution is generally the result of runoff during rainstorms. This runoff can transport pollutants via storm drains to recreational waters. The pollutants most often associated with nonpoint

pollution are agricultural fertilizers, pesticides and animal feces. In the past, NPDES permits have not been used to regulate the discharge of nonpoint sources of pollution. In 1987, however, Congress mandated a timetable for the regulation of nonpoint pollution. This required certain municipalities to obtain permits for storm drain discharges. These regulations were implemented in October 1992 (Clean Water Act of 1987).

V. Direct Detection of Waterborne Pathogens

With the limitations and problems associated with the current indicators, we must question their ability to predict the disease potential of Hawaii's recreational waters. Direct detection of pathogens may be a more accurate means to assess the disease potential of our recreational waters. Of the variety of pathogens that can be transmitted through water only a few are responsible for the majority of waterborne outbreaks in the United States. Of the bacterial pathogens, *Shigella, Salmonella,* and pathogenic *Escherichia coli* are among the most often reported bacterial agents associated with waterborne outbreaks. In addition, there are viral (norwalk and hepatitis A) and protozoan (*Giardia* and *Cryptosporidium*) pathogens that are also frequently responsible for waterborne outbreaks (Craun, 1992).

Currently, the direct detection of pathogens in water is extremely difficult and time consuming. There are a number of contributing factors that have made the direct detection of pathogens in water difficult: 1) Pathogens are present in waters intermittently and in low concentrations, 2) There are no standardized methods for detecting all pathogens, 3) Available methods to detect pathogens are often time consuming and expensive, 4) Available methods to detect pathogens have limited

sensitivity, and 5) Many waterborne pathogens are difficult to cultivate in the laboratory and some cannot be cultivated (Ajaib and McFeters, 1992). Recent advances in molecular biology, however, have opened a new door for the direct detection of pathogens in environmental waters. In particular, the use of gene probes and the polymerase chain reaction (PCR) to rapidly amplify pathogen gene sequences in environmental waters (Olson and Tsai, 1992).

A. Detection of Waterborne Pathogens: A Molecular Approach

The unique genetic sequences of microorganisms and the specificity with which these sequences hybridize to complementary sequences have been exploited to produce a set of powerful techniques for the detection and identification of microorganisms. These include DNA:DNA hybridzation, DNA: RNA hybridization, DNA fingerprinting, DNA and RNA sequencing, and the use of gene probes for detecting specific gene sequences. These technquies have been used in clinical settings and, more recently, in the detection of waterborne pathogens. The genetic material of microorganisms (DNA and RNA) has been an attractive target for the detection of bacterial indicators and waterborne pathogens. These techniques are of particular interest since many pathogens are difficult to detect by conventional cultural techniques. Since these techniques target gene sequences, time consuming cultural isolation can be avoided.

Prior to the development of the polymerase chain reaction (PCR) various hybridization techniques were utilized to detect waterborne pathogens. Unfortunately these techniques have not shown the sensitivity desired by many public health officials and therefore their application for the direct detection of

waterborne pathogens has been met with marginal success. Many of the problems associated with the sensitivity of hybridization techniques have been solved by the development of new techniques like PCR which show a high degree of sensitivity.

Since its discovery in 1983 (Mullis et al., 1983) PCR has made an enormous contribution to the field of molecular biology, human genetics, and infectious disease diagnosis. PCR is an in vitro method of amplifying defined DNA sequences to very high copy number in a very short period of time. PCR allows for the amplification of very low target sequences to high numbers that can then be readily detected. PCR has become an invaluable tool for detecting rare sequences in heterologous mixtures of DNA. In this regard its usefulness in detecting waterborne pathogens has recently become an important research topic in the field of water quality microbiology.

Since PCR targets gene sequences, it does not require the cultivation of the target pathogen. In addition, PCR is extremely sensitive and is able to detect as few as 1-2 bacterial cells in environmental waters (Bej, et al., 1990). Although PCR has been a valuable tool in clinical and molecular laboratories, to date, it has yet to be accepted as a method to monitor environmental waters for pathogens. This is likely due to the fact that PCR does not distinguish between live and dead cells and for public health reasons, only live cells are relevant. New advances in PCR technology, however, may soon distinguish live and dead cells by detecting messenger RNA by reverse transcriptase PCR (Stephan and Atlas, 1991). In

addition, there are a number of unique environmentally-derived problems not seen in other clinical and molecular PCR applications. These problems include environmental related inhibitors of PCR and an inability to recover target sequences from environmental samples (Stephan and Atlas, 1991). High background signals caused by mispriming and formation of primer dimers have also compounded this problem (Olson and Tsai, 1992).

PCR involves three steps: 1) the DNA is melted to convert double stranded DNA to single stranded DNA; 2) specific primers are annealed to target sequences flanking the desired gene sequences; and 3) DNA is extended via the aid of Taq polymerase in the presence of free deoxynucleotide triphosphates resulting in a duplication of the starting sequence. In order for this process to occur, a PCR reaction mixture is required to supply all the vital components in the correct concentration needed for this reaction.

B. PCR Reaction Mixtures

The major components of the PCR reaction mixture are, Taq polymerase, oligomer primers, template DNA, deoxynucleotides (dNTPs), and magnesium ions. Most PCR reactions are carried out in 10-50 mM Tris-HCL (pH8.3-8.8) buffer. Generally the recommended concentration of Taq polymerase is between 1.0-2.5 units for a 100 ul reaction (Lawyer et al., 1989). Concentration in excess of this amount can lead to non-specific background products while low concentration often lead to low levels of product (Steffan and Atlas, 1991).

Primer concentrations are generally between 0.1- 0.5 μM. Higher concentrations lead to non-specific binding and may promote the generation of non-specific products. In addition a number of decisions must be made when designing primers to assure specific amplification of the target sequence. Most primers are 15 to 28 nucleotides long with 50-60 % G + C composition and must have similar melting temperatures (Tms). Primers should not have complementation at the 3'end since this type of complementation will favor the production of primer dimers and reduce product yield. In addition runs of three or more guanines or cytosines at the 3' end can lead to nonspecific binding. And finally primers should not have complementation among three or more pairs of nucleotides to prevent primer dimer formation among primer pairs. To make the process of primer design easier, a number of computer programs have been developed that assist in primer selection.

Nucleotriphosphate concentrations for PCR reactions are generally between 20-200uM to give optimal specificity. The lowest primer concentration should be utilized to avoid mispriming at target sites and reduce the chances of extending misincorporated nucleotides (Innis et al., 1988).

Magnesium concentrations play an integral role in a number of reactions occurring during PCR. These reactions include, primer annealing, strand dissociation, PCR product formation, product specificity, and enzyme activity and fidelity. Generally PCR reaction mixtures contain magnesium concentrations between 0.5-2.5 mM.

C. PCR denaturation, annealing and extension

The process that governs the PCR amplification of DNA is temperature cycling. The first step in PCR is the denaturation step where double-stranded DNA is converted to single-stranded DNA. Generally this reaction occurs at 94-95 °C and the time required may vary from 15 sec to 2 min depending on the length of the target sequence. Longer sequence may require a longer denaturation step. The next step in this process is the annealing step which is dependent on the melting points (T_{ms}) of the primer pairs. Generally annealing is performed at a temperature 5°C below the T_{ms}. With an increase in annealing temperature, the specificity of the primers for the target increases and helps avoid mispriming. For environmental samples, the annealing temperature is usually the highest temperature which does not compromise the amount of product generated. The next step in this reaction is the extension step which is usually run at 72°C and will vary in duration depending on the length of the sequence being amplified. Estimates for the rate of nucleotide incorporation vary from 35 to 100 base pairs per second depending on the length of the sequence, buffer, pH, salt concentration and the nature of the target sequence (Innis et al., 1988).

D. Environmental Related Consideration of PCR

The vast majority of PCR applications have utilized pure cultures or samples with limited microbial diversity. In contrast, samples from environmental sources

contain a diversity of microorganisms. Traditional methods that rely on bacterial cultivation identify a small fraction (0.1 to 10 %) of the total microbial biomass (Ferguson et al., 1984; Torsvik et al., 1990). As would be expected, the genetic contribution of all microorganisms in an environmental samples must be considered since unknown organisms may contribute genetic sequences having similarities with those of the target organism.

VI. Survival of Indicators and Pathogens

When considering the health risk involved with the discharge of sewage into marine waters, it is important to consider the environmental factors that influence the survival of both indicators and pathogens. A variety of environmental factors have been shown to influence the survival of indicator and pathogens in marine environments. These factors include predation, parasitism, competition, and antagonism, as well as a variety of physical/chemical factors including sunlight, temperature, and salinity (MacCambridge and McMeekin, 1980, 1981). Together these factors influence the ability of indicators and pathogens to remain viable in marine water. Compounding this problem is the ability of indicators and pathogens to enter a physiological state termed viable but nonculturable. This term is used to describe a physiological state where these organisms cannot be detected by cultural methods but still remain viable and capable of causing disease (Colwell, et al.,

1985). This is of public health significance since cultural isolation may underestimate the actual disease causing potential of waters harboring viable but nonculturable indicators and pathogens.

Sunlight has been cited on numerous occasions as an agent responsible for the inactivation of waterborne pathogens and bacterial indicators. In studies by Fujioka et al. (1987) visible and UV-A light appear to reduce the number of culturally viable indicator bacteria in ocean water. Fujioka et al. (1987) demonstrated a 90% reduction of culturally viable fecal coliforms and fecal streptococci in seawater within 0.5 to 2 hours of exposure to naturally-occurring sunlight. This same study however, demonstrated a resistance by these same indicator bacteria when they were placed in freshwater. In other studies by Rhodes and Kator (1990), *E. coli* appeared to be sublethally inactivated by UV-A and visible light. In addition these studies indicated autochthonous microbiota enhanced the inactivation of *E. coli*. Using in vitro experiments, Barcina et al. (1989) reported that *E. coli* populations exposed to simulated sunlight in filtered fresh water enter a nonculturable state. In addition, visible light has been shown to retard growth and inhibit biosynthetic processes (Barcina *et al.*, 1989). Using ^{14}C-labeled glucose Barcina et al. demonstrated that visible light may be responsible for inhibition of glucose uptake in *E. coli*. Although none of these studies determined the actual mechanism of sunlight inactivation, they do indicate the detrimental

effects of sunlight on indicator bacteria. To date photooxidation appear to be the major contributor to visible light inactivation (Gourmelon et al., 1994).

Trying to determine the effect of solar radiation on aquatic systems is much more difficult than assessing the impact of radiation on terrestrial systems. Spectral irradiance changes dramatically with depth in the water column and most aquatic microorganisms will be moving up and down in the upper water column. This is of particular interest when one considers the movement of water quality indicator bacteria after discharge from deep ocean outfalls. In ocean water, visible light, and to a lesser degree UV-A, light are able to penetrate as far as 50 meters below the surface. With increasing depth the intensity of visible and UVA radiation decreases dramatically. These problems influence our ability to predict the impact of visible and UV-A light on indicator bacteria as well as pathogens.

Chapter 2

The Proposed Study

I. Problems and Needs in Water Quality Assessment

A. Inadequacy of Current Indicators of Water Quality in Hawaii

Currently *Escherichia coli* and enterococci are the USEPA endorsed water quality indicators used to determine the hygienic quality of recreational waters. The premise behind the use of these indicators is that their numbers in recreational waters is predictive of the incidence of diarrheal disease among swimmers. The research leading to the use of these indicators was generated from studies conducted in temperate climates and assumes these indicators behave in a similar fashion in all climates (Cabelli, 1983; Dufour, 1984). Unfortunately, numerous studies conducted in tropical climates have demonstrated a number of non-fecal sources of *E. coli* and enterococci. These sources include pristine tropical waters (Carrillo et al.,1985;Lopez-Torres et al., 1987; Valdes-Collazo et al.,1987) and tropical soils (Hardina and Fujioka, 1988). Since *E. coli* and enterococci have a number of non-fecal related sources in tropical climates, we must question the ability of these indictors to predict the hygienic quality of recreational waters in tropical climates. Because of the limitations of the current indictor system, a variety of alternative indicators have been proposed.

B. Limitations of Proposed Alternative Indicators

A number of alternative indicators have been proposed which include *Clostridum perfringens* (Bisson, 1980; Fujioka, 1985), coliphage (Kott et al., 1981) and *Bacteroides fragilis* (Allsop and Strickler, 1985). Although these alternative indicators have a number of advantages over the existing water quality indicators they also have limitations.

The use of *C. perfringens* as a water quality indicator has a number of advantages over existing indicators. Since it is a strict anaerobe it should not be able to replicate to any great extent in environmental waters (Bisson and Cabelli, 1980). In addition, *C. perfringens* is able to produce an environmentally stable spore which has a survival pattern similar to pathogenic viruses and protozoans (Bission and Cabelli, 1980). There are, however, disadvantages to the use of *C. perfringens* as water quality indicator. These disadvantages include its ubiquity in soils (Sidorenko, 1967) and the lower levels of *C. perfringens* (4×10^6 CFU/ gram of feces) in human feces as compared to *E. coli* (4×10^8 CFU/ gram of feces) and enterococci (2×10^8 CFU/gram of feces).

Another proposed indicator, coliphage, has a number of advantages over existing water quality indicators. Coliphage survive longer than *E. coli* in environmental waters and may better represent the survival pattern of pathogenic enteric viruses (Kott, 1981). In addition, the cultivation of coliphage is much faster and easier than the cultivation of pathogen viruses (Gerba, 1987). The disadvantage

for use of coliphage include a lack of data supporting the incidence of coliphage in environmental waters as compared to pathogenic enteric viruses. In addition, coliphage may be able to replicate in the environment (Borrego, 1990).

Because of their high levels in human feces (10^{10} per gram of feces), *Bacteroides* spp. have been proposed as alternative indicator (Allsop and Stickler, 1984; Fiksdal et al., 1985). The levels of *Bacteroides* spp. in human feces is at least 100 to 1000 fold higher than the indicator *E. coli* (Allsop and Stickler, 1984). In addition, the level of *Bacteroides* spp. in animals feces is much lower than the levels in humans which indicates that *Bacteroides* spp are species specific (Allsop and Stickler, 1984). The major disadvantage of the use of *Bacteroides* spp. as an indictor is the fact that it is a strict anaerobe and does not survive as long as *E. coli* in environmental waters. Moreover, culture methods for their recovery are unreliable (Fikdal, et al., 1995).

A major limitation in the current use of all water indicators is the time required to isolate and identify water quality indicators. Currently cultural methods are utilized to detect indicator in recreational waters and require a minimum of 24 hour to obtain results. This is of concern to public health officials who are charged with assuring public safety and the closing of recreational waters contaminated with human feces. The time required to cultivate indicators can impede our ability to respond to events which may immediately impact human health. During the time required to cultivate indicators many individuals may be exposed to contaminated

recreational waters. Because of the limitation associated with the cultural isolation of indicators, we must seek a more rapid means of detecting water quality indicators.

C. Need to Recover Pathogens From Water

In order to understand the public health significance of the presence of water quality indicators in recreational waters, we must be able to correlate their presence with the presence of waterborne pathogens. This requires the concomitant detection of water quality indicators and waterborne pathogens. This relationship is vital information and will help establish the human health risks involved with exposure to recreational waters. To date it has been very difficult to establish this relationship because of an inability to accurately detect pathogens in environmental waters. Currently the isolation of pathogens from environmental waters is plagued by a lack of standardized methods and the time consuming process of isolating and identifying waterborne pathogens. Cultural isolation of waterborne pathogens can take days and even weeks to render results. Because of the limitations with the cultural isolation approach, we must seek alternative methods that are capable of quickly and accurately detecting waterborne pathogens.

D. Need to Determine the Fate of Indicator and Pathogens in Water

The survival of indicators and pathogens in marine environments is of critical importance in order to determine the possible health risks associated with exposure to sewage contaminated recreational waters. Studies relating these factors and their

influence on indicator bacteria and pathogens are of importance to public health officials who must assess the validity and suitability of indicators to predict the sanitary quality of recreational waters. The survival and decline of enteric bacteria in marine waters are attributed to a variety of interacting chemical, physical and biological factors. These factors include sunlight (Fijioka et al., 1981; Bacina, 1988), temperature (McCambridge and McMeekin, 1980, Rhodes and Kator, 1988; Stuart et al. 1994;), predation (Mitchell et al., 1967; McCambridge and McMeekin, 1980), and nutrient availability (Carlucci and Pramer,1960; Jannasch, 1968). To date the vast majority of studies relating the survival of indicators and pathogens in environmental waters have relied on cultural isolation to predict survival. Unfortunately many indicators and pathogens are capable of entering a number of physiological states which cannot be detected by cultural isolation.

When submitted to environmentally related stresses, indicator bacteria (i.e. *E. coli*) and pathogens (i.e. *Salmonella* spp.) are capable of entering a series of physiological states termed "sublethally stressed" and "viable but nonculturable". If a bacterium is unable to form colonies on a nonselective growth medium but not on a medium which contains selective agents to which the bacterium is normally resistant, it is termed injured (James et al., 1994). Numerous survival studies have termed these injured bacteria "sublethally stressed" (Smith et al, 1994; Rhodes and Kator, 1988). Currently selective media like mTEC (*Standard Methods*, 1992) are utilized to isolate *E. coli* from recreational waters. Because of mTEC's selective nature, sublethally

stressed *E. coli* may not be detected. This would lead to an underestimation of the actual number of *E. coli* present in marine waters. In addition to sublethally stressed bacteria, as defined by selective and nonselective media, there is also another classification of stressed bacteria that are unable to demonstrate growth on either selective or non selective media but are still considered viable and capable of causing disease. This group of stressed bacteria is termed "viable but nonculturable" is of public health significance since both *E. coli* and the pathogen *Salmonella* spp. have been shown to enter a viable but nonculturable state in marine waters (Colwell, et al., 1983). The presence of sublethally stressed and viable but nonculturable indicators and pathogens demonstrates a need for developing methods capable of detecting bacteria in these physiological states.

II. PCR: A Promising New Approach to Assess Water Quality

Recently the development of the polymerase chain reaction (PCR) method has offered an attractive alternative to the problematic cultural isolation of water quality indicators and waterborne pathogens. Since the PCR method targets gene sequences, time consuming cultural isolation can be avoided and results can be obtained in a fraction of the time required by cultural methods. In addition, PCR has the potential of detecting sublethally stressed and viable but non-culturable indicators and pathogens. PCR has proven to be a sensitive and specific technique capable of detecting as few as one bacterial cell per 100 ml of water (Bej, et al.

1991) and has been utilized to detect a variety of waterborne pathogens including *Legionella* spp.(Palmer et al. 1995), *Shigella* spp. (Bej et al. 1991), *Campylobacter* spp. (Oyofo and Rollins, 1993) and enteric viruses (Morteza et al. 1993).

Although PCR has a number of advantages over the cultural isolation of indicators and pathogens, it has yet to be accepted as a method to monitor environmental waters. This is most likely due to the fact that PCR does not distinguish between live and dead cells and, for public health reason, only viable cells are relevant. In order for PCR technology to be applicable for the detection of indicators and waterborne pathogens we need to know the persistence of target gene sequences and the relationship between PCR detection and cellular viablity. In addition to the problems associated with viability, there are a number of unique environmentally derived problems encountered when performing PCR on environmental samples. These problems include environmental inhibitors of PCR and an inability to recover target sequences from environmental samples (Stephan and Atlas, 1991). High background caused by mispriming and formation of primer dimers have also compounded this problem (Olson and Tsai, 1992).

III. Dissertation Goals and Objectives

A. Goal

The overall goal of this research is to develop and evaluate the usefulness of PCR-based methods to address the existing problems and needs in water quality.

B. Design and Scope of the Study

The design of this study will be to always determine the quality of water to be tested by existing, and approved cultural methods for the presence of indicators and pathogens. Then, to apply PCR-based methods to the same sample. Interpretation will be based on comparisons between cultural and PCR results.

The scope of this research will be to initially evaluate published PCR-based methods already applied in water and determine if these methods can be feasibly applied to water in Hawaii. Specifically to evaluate the PCR-development method for *E. coli* and compare its sensitivity to traditional culture method for *E. coli* and fecal coliforms. However, the major focus of thus study will be the development and evaluation of new PCR-based methods to assess identified water quality problems and needs.

C. Study Objectives:

To achieve the goals of this study, PCR methods will be developed and evaluated to address the earlier identified water quality problems. Thus the objectives of this research are as follow;

1) To develop and evaluate a new PCR method for the detection of *Bacteroides* spp. as a reliable and sensitive means to determine whether environmental water are contaminated with human feces or sewage.

2) To develop a new PCR method for the direct detection of a bacterial pathogen (*Salmonella* spp.) in sewage and environmental waters.

3) To use PCR methods to determine the fate (culturability, viability, and structural integrity) of indicator bacteria and *Salmonella* bacteria exposed to marine waters.

D. Expected Significance and Contribution of this Study

To date no studies have been conducted in Hawaii using PCR to detect water quality indicators and pathogens. This study will be the first of its kind to utilize PCR to detect water quality indicators and pathogens in Hawaii's recreational waters. In addition, no studies have been conducted relating the persistence of PCR-targeted gene sequences to cellular viability. In this study an attempt will be made to determine the persistence of indicator and pathogen gene sequences in environmental waters. By utilizing currently accepted cultural techniques in conjunction with PCR-based detection system we will be able to assess the usefulness and feasibility of using PCR to detect water quality indicators and pathogens in Hawaii's recreational waters.

Chapter 3

Development of a PCR Detection System for *Salmonella* spp.

I. Introduction

Currently the water quality indicator bacteria (*Escherichia coli,* enterococci) are utilized to determine the sanitary quality of recreational waters. These indicators are normal inhabitants of the intestinal tract of warm-blooded animals and their presence in recreational water is considered indicative of fecal contamination and their concentrations in water correlates to the incidence diarrheal diseases among swimmers (Cabelli, 1983). The United States Environmental Protection Agency (USEPA) studies supporting the use of *E. coli* and enterococci as water quality indicators were conducted in temperate climates where environmental sources of these indicators bacteria are insignificant. However, in tropical climates such as Hawaii, Puerto Rico and Guam, *E. coli* and enterococci appear to be naturally present in high concentrations in tropical fresh waters and soil (Hardina and Fujioka, 1991). A number of studies have shown that *E. coli* is capable of surviving and even replicating in tropical environments. In Puerto Rico, *E. coli* was able to survive and replicate in rain forest streams (Carrillo et al.,1985;Lopez-Torres et al., 1987; Valdes-Collazo et al.,1987). Other studies by Hardina and Fujioka (1988) indicate that fecal coliform, *E coli* and fecal streptococci are naturally occurring in Hawaii's streams and soils. Together this evidence has led to questioning the use of

E. coli and enterococci as water quality indicators for tropical climates and demonstrates the need for alternative means to determine the sanitary quality of recreational waters.

Ideally, the direct detection of waterborne pathogens would best represent the disease potential of sewage contaminated recreational waters. Unfortunately the direct detection of pathogens, such as *Salmonella* spp., has been problematic because of a lack of standardized methods to adequately detect this pathogens in environmental waters (*Standard Methods* 19th ed., 1992). Isolation of *Salmonella* spp. using conventional cultural methods require presumptive enrichment and confirmatory steps in conjunction with biochemical tests and serological testing *(Standard Methods* 19th ed., 1992) This approach often requires 5 to 7 days to render results. More recently, *Salmonella* spp. were reported to enter a viable but nonculturable state which can not be detected by conventional cultural methods (Colwell, et al., 1985). Compounding these problems is a need to sample large volumes of water since the presence of pathogens in environmental waters is often intermittent and occur in low concentrations (Ajaib and McFeters, 1992). A variety of methods have been utilized to sample large volumes of water which include epoxy resin filters (Levin et al., 1974), membrane filtration (Presenell, et al. 1976) and more recently, vortex flow filtration (VFF) (Jiang et al., 1992; Paul et al., 1992; Tasi et al., 1994). VFF has been shown to be an efficient method for concentrating bacteria and viruses from large volumes of waters (Jiang et. al.,1992). Because of

the problems associated with conventional cultural methods and the low concentration of pathogens in environmental waters, new methods need to be developed that are both sensitive and rapid.

Recently the development of the polymerase chain reaction (PCR) method has offered an attractive alternative to the problematic cultural isolation of waterborne pathogens. Since the PCR method targets gene sequences, time consuming cultural isolation can be avoided and results can be obtained in a fraction of the time required by cultural methods. In addition, PCR has the potential of detecting viable but non-culturable pathogens. PCR has proven to be a sensitive and specific technique capable of detecting as few as one bacterial cell per 100 ml of water (Bej, et al. 1991) and has been utilized to detect a variety of waterborne pathogens including *Legionella* spp.(Palmer et al. 1995), *Shigella* spp. (Bej et al. 1991), *Campylobacter* spp. (Oyofo and Rollins, 1993) and enteric viruses (Morteza et al. 1993).

II. Study Objectives

The purpose of this study is to develop a set of PCR primers capable of detecting *Salmonella* spp. in various environmental waters including sewage, ocean water and fresh waters. Previous research by Tsen et al. (1991) indicated that a 1.8 kb restriction fragment of *Salmonella typhimurium* was highly conserved and gene probes generated from this fragment did not hybridize with closely related enteric

bacteria. A PCR method was developed using this 1.8 kb fragment to generate *Salmonella*-specific PCR primers capable of detecting *Salmonella* spp. in various environmental waters. This new PCR-based method will then be compared to existing cultural methods to determine the feasibility of using this technology to detect *Salmonella* spp. in environmental waters.

III. Experimental Design

A pair of *Salmonella* spp. specific primers were chosen from a previously described *Salmonella typhimurium* sequence (Tsen et al., 1991). In order to determine the specificity of these primers a library of *Salmonella* spp. as well as a variety of other Gram-negative and Gram-positive bacteria were submitted to PCR amplification using these primers. The sensitivity of these primers was determined by seeding sewage and ocean water with serial dilutions of *S. typhiumurim*. Finally, this PCR method was field tested on a variety of environmental waters and compared to existing cultural methods.

VI. Materials and Methods

A. Bacterial Strains and Growth Conditions.

Bacterial isolates were obtained from the Department of Health, State of Hawaii, and American Type Culture Collection, Rockville, Md. All *Salmonella* isolates obtained from the Hawaii Department of Health were isolated from clinical,

humans and food samples and were serotyped. Isolates were grown in brain heart infusion broth (Difco Laboratories, Detroit, Michigan) for 24 hrs at 37°C. For sewage seeding experiments, mid-log-phase *Salmonella typhimurium* (ATCC 14028) was collected, washed twice with phosphate-buffered saline, and serially diluted (10^{-2} to 10^{-9}). Plate counts were performed on the serial dilutions to determine the number of culturally viable *Salmonella typhimurium* using Hektoen enteric agar (Difco). A 1 ml portion from the serial dilutions was seeded into 9 ml primary-treated sewage obtained from the Sand Island Treatment facility. For ocean water seeding experiments *S. typhmurium* cultures were prepared as described in sewage seeding experiments except ocean water was substituted for primary treated sewage. *Salmonella* spp. were isolated from environmental samples using a 5 tube most probable number method with tetrathionate broth (Difco) and incubated for 24 hrs at 37°C. Tubes showing growth after 24 hrs were streaked on xylose lysine deoxycholate agar (Difco) and Hektoen enteric agar (Difco) and incubated for 24 hrs at 37°C. Characteristic Salmonella-like colonies were then inoculated into triple iron agar (TSA, Difco) and lysine iron agar (LIA, Difco) slants. Isolates having the correct growth characteristics on TSI and LIA were confirmed with polyvalent antisera specific for *Salmonella* spp. O-group antigens (Set A-1, Difco Laboratories,). Confirmed *Salmonella* spp. isolates were then used to determine the MPN index for a given sample.

B. Collection and Concentration of Environmental waters

The Sand Island Sewage Treatment Facility located on the island of Oahu discharges primary-treated sewage through a sewage outfall located 9000 ft from shore at a depth of 243 ft. Twenty-liter samples were collected from an ocean site near this outfall. Primary-treated sewage from this facility and ocean water from Waikiki beach were collected and utilized for seeding experiments and for the direct detection of *Salmonella* spp. In addition, 20 liter water samples were collected from Manoa Stream, a fresh water urban drainage system serving the suburbs of Honolulu located on the island of Oahu. Twenty liter samples were concentrated using a vortex flow filtration (VFF) device (Membrex Inc., Garfield, N. J.) at 6-8 lb/in^2 with a 100-kDa filter and rotor speed of 1,000 to 1,500 rpm. VFF-concentrated samples were further concentrated with a centriprep 100 concentrator (Amicon, Beverly, MA.) to a final volume of 2-4 ml.

C. DNA Extraction

For primer specificity testing, DNA from pure isolates of both *Salmonella* spp. and non-Salmonella were extracted using the GNOME DNA isolation kit, (BIO 101 Inc., La Jolla, CA). For sewage seeding experiments, the previously prepared seeded serial dilutions (10 ml) were filtered through a through a 0.5 μm pore size FHLP filter (Millipore) as described by Tsai et al. (1993) and crude DNA was extracted using a rapid freeze thaw method described by Bej. et al. (1991). For

ocean water seeding experiments, seeded serial dilutions (10 ml) were filtered through a 0.5μm pore size FHLP filter (Millipore) as described by Tsai et al. (1993). DNA was then extracted by placing each filter in 200 μl sterile water with 5% (w/v) chelex 100 (Biorad) and boiled for 15 min. Samples were then cooled to room temperature prior to purification and concentration. Crude DNA from VFF-concentrated samples was extracted using a rapid-freeze thaw method as described by Bej. et al. (1991).

D. DNA Purification and Concentration

Crude DNA extracts obtained from VFF-concentrated samples were submitted to extraction with an equal portion of a mixture of chloroform: isoamyl alcohol (24:1) (Sigma, St. Louis, MO). The aqueous phase was then removed and further concentrated using a centricon 100 concentrator (Amicon) to a final volume of 20 μl. Crude extracts from seeded sewage experiments were purified with Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, N. J.) spun columns by a method previously described by Tsai et al. (1991). Briefly , 100 ul of crude DNA extract was loaded onto a Sephadex G-200 column which was then centrifuged twice for 10 min. at 1,100 x *g* to obtain 100 ul of eluate. This eluate was then concentrated using a centricon-100 (Amicon) concentrator to a final volume of 20 μl. Crude DNA extracts from ocean water seeding experiments were loaded into a centricon 100 concentrator (Amicon) and concentrated to a final volume of 20 μl.

E. DNA Primers and PCR Amplification

Primer BRSALa (5'-CTG GAT GAG ATG GAA GAA TG- 3') and BRSALb (5'-ACG GTT GTT TAG CCT GAT AC-3') were chosen using Olgio 4.0 computer software (National Biosciences, Inc.) from a previously published *Salmonella* restriction fragment sequence (Tsen, et al., 1991). A second nested primer set SALIa (5'-GTT CGG CAT TGT TAT TTC T-'3) and SALIb (5'-CTG AGG GTC ATC GTT ATT C-'3) was also chosen from the same sequence. As seen in Table 7 primer set BRSALa and BRSALb generated a 526 bp PCR product and nested primers SALIa and SAIIb generated a 282 bp product. Primers were produced with an automatic DNA synthesizer (ABI 381A; Applied Biosytems Internatinal, Foster City Calif.) PCR reaction mixture contained 1 x PCR buffer (10 mM Tris-HCL [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM (each) deoxynucleoside triphosphate, 0.3 μM (each) primer, and 0.5 μl Taq DNA polymerase per 100 μl (Perkin-Elmer Cetus, Norwalk, Conn.). In the first PCR step primers (BRSALa & BRSALb) and template DNA were initially denatured at 94°C for 5 min and then subjected to 35 cycles each consisting of 120s at 94°C, 20s at 57.5°C, and 60s at 72°C; the synthesis was completed at 72°C for 7 minutes. The second nested PCR step (Primers SALIa & SALIb) was carried out in a total volume of 100 ul. A 2-ul sample of the first round of PCR was used as the template. The cycle profile consisted of DNA denaturation at 95°C for 5 min followed by twenty cycles, each consisting of 30 s at 94°C, 60 s at 54°C, and 60 s at 72°C; the synthesis was

completed at 72°C for 10 min. PCR products were analyzed by gel electrophoresis on a 2 % SeaKem GTG agarose gel (FMC BioProducts, Rockland, Maine) stained with ethidium bromide (0.5 μg/ml) and were identified using a model 6-63 UV transilluminator (Ultra-Violet Products, San Gabriel, Calif.).

Table 7. Primers Used For PCR Detection of *Salmonella* spp.

Primer Set	Sequence and Sequence position[a]	Product length (bp)
BRSALa	5' CTG GAT GAG ATG GAA GAA TG 3' (1124-1144)	526
BRSALb	5' ACG GTT GTT TAG CCT GAT AC 3' (1630-1650)	
SALIa	5'-GTT CGG CAT TGT TAT TTC T-'3 (1280-1299)	282
SALIB	5'-CTG AGG GTC ATC GTT ATT C-'3 (1543-1562)	

[a] Position of primers within the 1.8-kb *S. typhimurium* DNA sequence (Tsen et al, 1991).

F. DNA Hybridization

An internal probe, TS21 (5'-TAC ATC GTA AAG CAC CAT CGC AAA T-3') was chosen from a previously described *Salmonella*-specific sequence and used to confirm the identity of the PCR products (Tsen et al. 1991). The internal probe was 3' labeled with digoxigenin-11-ddUTP using a Genius 5 nonradioactive DNA labeling kit (Boehringer Mannheim, Indianapolis, Ind.). PCR products were

transferred onto Hybond-N+ positively charged nylon membranes (Amersham, Arlington Heights, Ill.) using a PosiBlot pressure blotter (Stratagene, La Jolla, Calif.) for the Southern analysis (Southern, 1975). DNA was fixed to the nylon membranes by UV irradiation at 254 nm for 2 min using a CL-1000 ultraviolet crosslinker (UVP, San Gabriel, Calif.). Hybridization was performed at 55°C in presence of a labeled internal probe (2 pmol/ml). The hybridized filters were washed twice with a high-salt solution (2 x SSC in 0.1% sodium dodecyl sulfate) at 50°C. A Genius 3 nucleic acid detection kit (Boehringer Mannheim) was used to prepare the hybridized filters for chemiluminescent detection. The hybridization signals were visualized on X-OMAT film (Eastman Kodak, Rochester, N. Y.) using autoradiography.

V. Results and Discussion

A. Specicifty of Primers

In order for PCR to demonstrate a high degree of specificity, primers and target gene sequences must be conserved among different *Salmonella* spp. and not share similarities with other Gram-negative and Gram-positive bacteria. To determine the specificity of primers BRSALa and BRSALb, 25 different enteric bacteria (e.g. *E. coli, Shigella* spp. and *Enterobacter* spp.) were utilized to test the specifity of primers BRSALa and BRSALb (Table 8). In addition, primers were tested with a variety of 27 different *Salmonella* spp., of human origin, obtained

from the Hawaii Department of Health (Table 8). As an example, Figure 1 shows the PCR amplification results for a variety of *Salmonella* spp. and non-Salmonella isolates. As seen in Table 8, all 56 *Salmonella* isolates tested, generated a 526 bp PCR product while no products were detected for any of the non-Salmonella isolates including genetically similar species like *E. coli* and *Shigella flexneri.* These results indicate that the primers BRSALa and BRSALb consistently generated a 526 bp product for a variety of *Salmonella* serotypes and the sequence targeted by these primers appears to be conserved in many *Salmonella* serotypes.

B. Sensitivity of PCR primers in Primary Treated Sewage

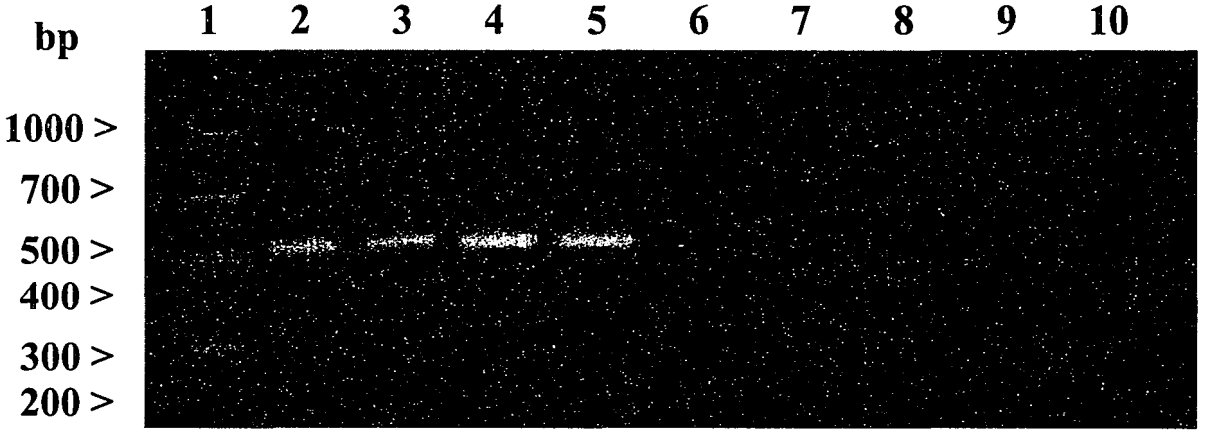
Although these primers appear to be conserved among *Salmonella* spp. and did not cross-react with other Gram-negative and Gram-positive bacteria, the genetic contributions of the diverse microbial populations found in environmental waters must be considered. In addition, it is critical to know the sensitivity of these primers in a diverse microbial population in order to determine the feasibility of using these primers to detect *Salmonella* spp. in environmental waters.

In order to determine the specificity and sensitivity of these primers in a diverse microbial population, seeding experiments were conducted using primary-treated sewage. This involved seeding primary treated sewage with serial dilutions of a mid-log phase culture of *S. typhimurium.* Total DNA was extracted from seeded sewage and purified with sephadex G-200 in order to remove PCR-inhibiting humic

Table 8. Isolates Tested with Primer Set BRSALa and BRSALb

Organism	# Isolates Tested	Source	526 bp PCR Product
Salmonella typhimurium	1	ATCC 14028	+
Salmonella typhimurium	5	DOH, Honolulu, Hawaii[a]	+
Salmonella bredency	1	DOH, Honolulu, Hawaii[a]	+
Salmonella heidelberg	1	DOH, Honolulu, Hawaii[a]	+
Salmonella infantis	1	DOH, Honolulu, Hawaii[a]	+
Salmonella sandiego	1	DOH, Honolulu, Hawaii[a]	+
Salmonella muenchen	1	DOH, Honolulu, Hawaii[a]	+
Salmonella anatum	2	DOH, Honolulu, Hawaii[a]	+
Salmonella hadar	2	DOH, Honolulu, Hawaii[a]	+
Salmonella agona	1	DOH, Honolulu, Hawaii[a]	+
Salmonella tuebingen	1	DOH, Honolulu, Hawaii[a]	+
Salmonella cerro	1	DOH, Honolulu, Hawaii[a]	+
Salmonella give	1	DOH, Honolulu, Hawaii[a]	+
Salmonella alachua	1	DOH, Honolulu, Hawaii[a]	+
Salmonella welteureden	12	DOH, Honolulu, Hawaii[a]	+
Salmonella newport	4	DOH, Honolulu, Hawaii[a]	+
Salmonella lanka	3	DOH, Honolulu, Hawaii[a]	+
Salmonella olso	7	DOH, Honolulu, Hawaii[a]	+
Salmonella singapore	1	DOH, Honolulu, Hawaii[a]	+
Salmonella nottingham	1	DOH, Honolulu, Hawaii[a]	+
Salmonella tennessee	1	DOH, Honolulu, Hawaii[a]	+
Salmonella montevideo	1	DOH, Honolulu, Hawaii[a]	+
Salmonella stanley	1	DOH, Honolulu, Hawaii[a]	+
Salmonella houten	2	DOH, Honolulu, Hawaii[a]	+
Salmonella derby	1	DOH, Honolulu, Hawaii[a]	+
Salmonella cubana	1	DOH, Honolulu, Hawaii[a]	+
Salmonella dublin	1	DOH, Honolulu, Hawaii[a]	+
Escherichia coli	1	ATCC 25922	-
Escherichia coli	1	ATCC 35401	-
Escherichia coli	1	ATCC 43886	-
Escherichia coli	1	ATCC 43889	-
Escherichia coli	1	ATCC 43890	-
Escherichia coli	1	ATCC 43894	-
Escherichia coli	1	ATCC 43895	-
Escherichia coli	1	ATCC 43896	-
Enterobacter aerogenes	1	ATCC 13048	-
Citrobacter freundii	1	ATCC 8090	-
Klebsiella pneumoniae	1	ATCC 13883	-
Shigella flexneri	1	ATCC 12022	-
Shigella sonnei	1	ATCC 25931	-
Bacillus cereus	1	ATCC 14579	-
Bacillus subtilis	1	ATCC 6051	-
Pseudomonas aeruginosa	1	ATCC 27853	-
Staphylococcus aureus	1	ATCC 25923	-
Streptococcus faecalis	1	ATCC 29212	-
Acinetobacter calcoaceticus	1	ATCC 19606	-
Serratia marcescens	1	ATCC 8100	-
Streptococcus pyogenes	1	ATCC 19615	-
Enterobacter cloacae	1	ATCC 23355	-
Proteus vulgaris	1	ATCC 13315	-
Staphylococcus epidermidis	1	ATCC 12228	-

[a] Isolates obtained from Hawaii Department of Health (clinical isolates)



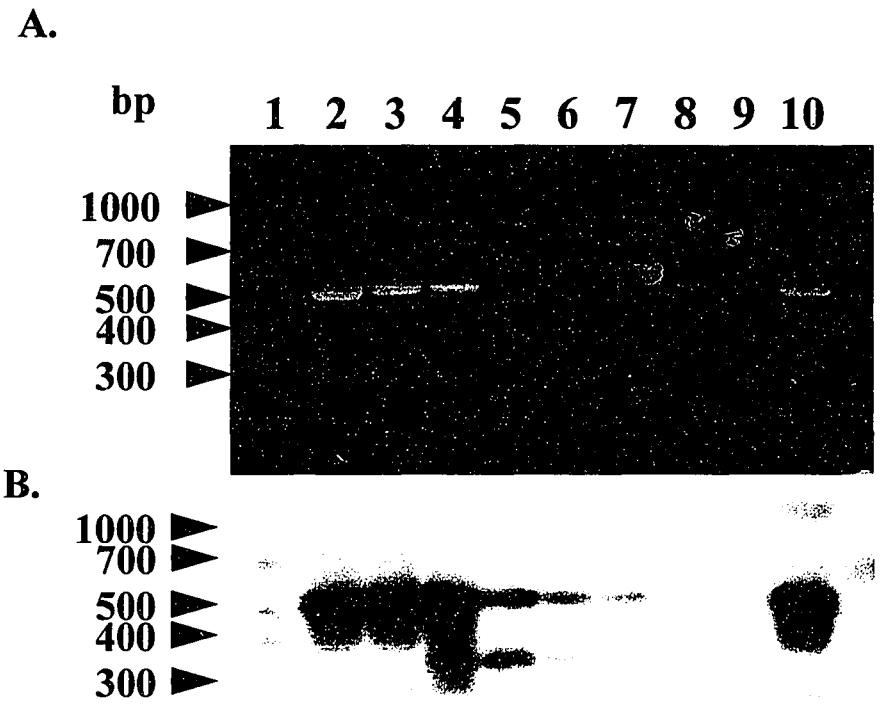
Lane 1, Marker; Lane 2, *Salmonella typhimurium*; Lane 3, *S. heidelburg;* Lane 4, *S. newport*; Lane 5, *S. welteureden*; Lane 6, *Escherichia coli*; Lane 7, *Shigella flexneri*; Lane 8, *Staphylococcus aureus*; Lane 9, *Proteus vulgaris*; Lane 10, *Bacillus cereus*.

Figure 1. Specificity of *Salmonella* Primers (BRSALa and BRSALb)

material (Tsai et al., 1993). Next the purified DNA was submitted to PCR using primer BRSALa and BRSALb. As seen in Figure 2 (plate A), PCR products were visualized on an ethidium bromide-stained gel at levels as low as 1.2 x 10^2 CFU/ ml (Lane 6). Southern blot hybridization (plate B), with an internal probe, confirmed these products and demonstrated an increase in sensitivity to 12 CFU/ ml (Lane 7). No PCR products were seen in unseeded sewage (Lane 9) and no *Salmonella* spp. were culturally isolated from this sample. This would indicate that even among the diverse microbial populations found in sewage there was no cross-reactivity with these primers. It is interesting to note that the Southern blot results demonstrated the presence of an additional band in Lane 4, 5 and 6 (Figure 2) . This may be a reflection of truncated PCR products and may be artifacts of the PCR process since this band was not detected in unseeded sewage (Lane 9). There is also the possibility of environmentally-derived entities capable of degrading some of the PCR products.

C. Sensitivity of Nested PCR Primers in Ocean Water

As demonstrated in seeded sewage experiments, PCR amplification and hybridization with an internal probe was able to detect as few as 12 CFU/ml of *S. typhimurium*. In order to achieve this sensitivity, hybridization with an internal probe is required. Unfortunately, hybridization takes at least 24 hrs to perform and increases the time required to detect *Salmonalla* spp. in environmental waters. In order to decrease the time required for detection, a second set of nested primers



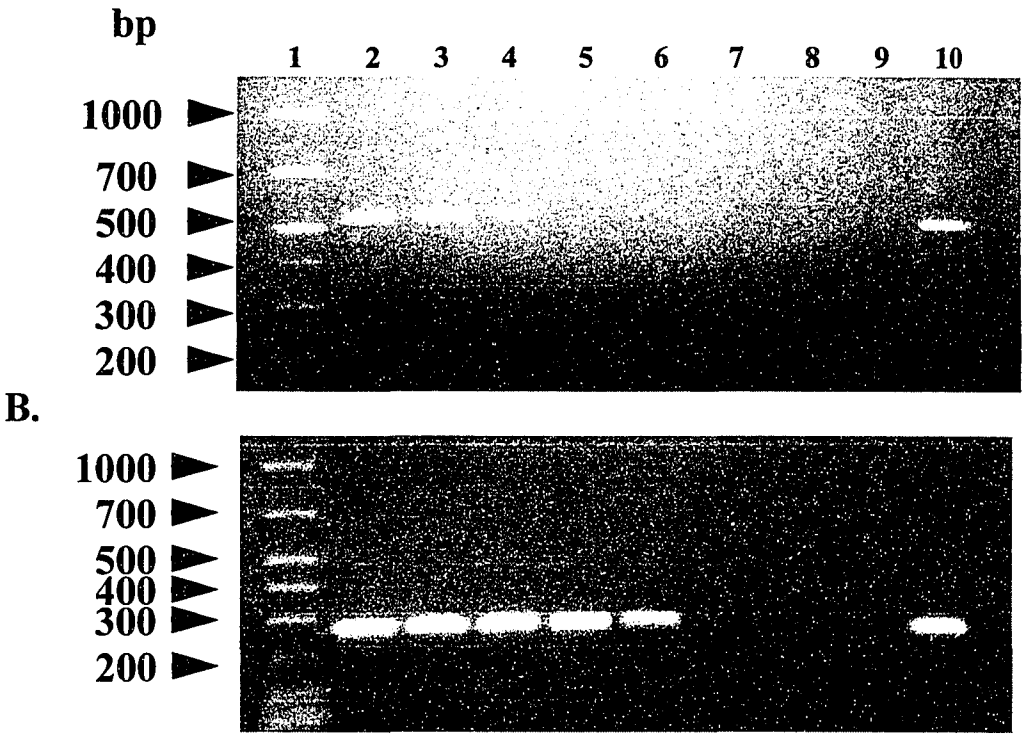
Lane 1, Marker; Lane 2, 1.2 x 10^6 CFU; Lane 3, 1.2 x 10^5 CFU; Lane 4, 1.2 x 10^4 CFU; Lane 5, 1.2 x 10^3 CFU; Lane 6, 1.2 x 10^2 CFU; Lane 7, 1.2 x 10^1 CFU; Lane 8, 1.2 CFU; Lane 9, unseeded sewage; Lane 10, Positive control (1.2 x 10^6CFU). (A) PCR products of an ethidium bromide-stained 2 % SeaKem agarose gel. (B) Autoradiogram of panel A produced by using a chemiluminescent internal probe.

Figure 2. Detection of *Salmonella*-specific Gene Sequence in Primary Treated Sewage

were chosen which targeted sequences inside the 526 bp product generated by primers BRSALa and BRSALb. By performing nested PCR after the first round of amplification, hybridization with an internal probe could be avoided without a loss in sensitivity. In order to demonstrate the feasibility of using nested PCR on environmental waters, a mid-log phase culture of *S. typhimurium* was serially diluted and each dilution was seeded into ocean water. Total DNA from seeded ocean water samples was then extracted and submitted to PCR amplification using primers BRSALa and BRSLAb. Immediately following the first round of PCR a 2 μl portion of each sample was submitted to a second round of PCR with nested primers SALIa and SAlIb. As seen in Figure 3, after the first round of PCR a sensitivity of 1.4×10^2 CFU/ml was visualized on an ethidium bromide-stained gel. After amplification with the set of nested primers the sensitivity was increased to as few as 1.4 CFU/ml. These results indicate that by using nested PCR the sensitivity of this assay could be increased to 1.4 CFU while greatly reducing the time previously required to detect PCR products. This avoids the time consuming process of confirming with an internal probe and allows for detection in less than one day.

D. Detection of *Salmonella* spp. in Environmental Waters

In order to determine the feasibility of using PCR to detect *Salmonella* spp. in environmental waters, samples were collected from sewage, ocean and stream waters and submitted to PCR detection and cultural isolation. Because of the



Lane 1, Marker; Lane 2, 1.4 x 10^4 CFU; Lane 3, 1.4 x 10^3 CFU; Lane 4, 1.4 x 10^2 CFU; Lane 5, 1.4 x 10^1 CFU; Lane 6, 1.4 CFU; Lane 7, 0.14 CFU; Lane 8, 0.014 CFU; Lane 9, Unseeded ocean water; Lane10, Positive control (1.4 x 10^6 CFU). (A) PCR products of 1st primer set BRSALa & BRSALb; (B) PCR products of nested primer set SALIa & SALIb.

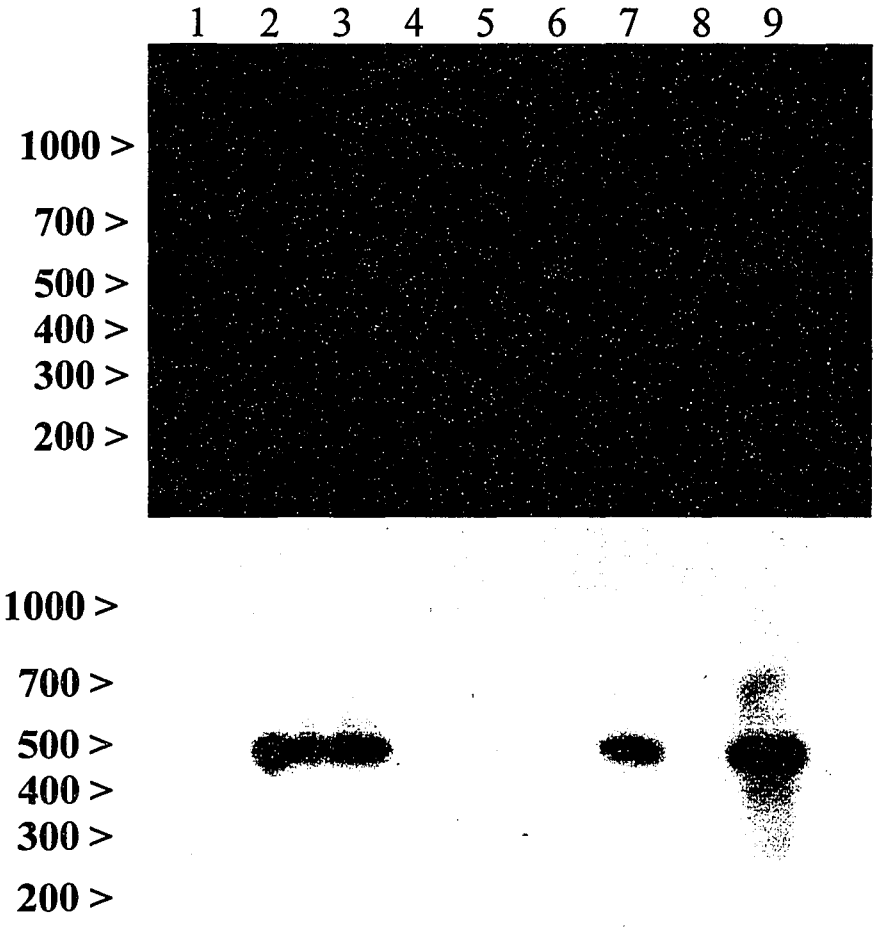
Figure 3. Detection of *Salmonella*-specific Gene Sequence in Ocean Water Using Nested PCR

intermittent and low concentration of pathogens in environmental waters, large volumes (5 to 20 liters) of these waters were sampled and submitted to vortex flow filtration (VFF). VFF concentrated samples were split into two equal volumes with half being utilized for PCR amplification and the other half for cultural isolation. As seen in Figure 4, three samples demonstrated the presence of *Salmonella* specific gene sequences (plate A, Lanes 2, 3 & 7). Southern blot hybridization with an internal probe demonstrated hybridization for Lanes 2, 3 & 7 confirming the amplification of the 526 bp product seen in plate A (Figure 4). No other bands were seen with the remaining samples. In addition to PCR detection, *Salmonella* was also recovered by culture method in two of the 6 samples submitted to cultural isolation (Table 9).

Table 9. Culture and PCR Results for Field Samples

Sample Type	Volume Sampled	*Salmonella* spp. MPN/liter	PCR Product
Manoa Stream	20 liters	2.5×10^1	+
Primary effluent A	5 liters	1.2×10^3	+
Primary effluent B	5 liters	ND[a]	_
Ocean water A	20 liters	ND[a]	_
Ocean water B	20 liters	ND[a]	_
Ocean water C	20 liters	ND[a]	+

[a] Not detected



Lane 1, marker; Lane 2, Manoa Stream (20 liters); Lane 3, primary effluent A (5 liters); Lane 4, primary effluent B (5 liters); Lane 5, ocean water A (20liters); Lane 6, ocean water B (20 liters); Lane 7, ocean water C (20 liters); Lane 8, negative control; Lane 9, positive control.

Figure 4. Detection of *Salmonella* spp. in Environmental Waters

As seen in Table 9, Manoa Stream and primary effluent A demonstrated a *Salmonella* spp. MPN of 2.5 x 10^1/ liter and 1.3 x 10^3/ liter, respectively. These results indicate that PCR was able to detect *Salmonella* spp. in all samples showing the presence of culturally viable *Salmonella* spp. No *Salmonella* was culturally isolated from the remaining 4 samples, although *Salmonella*-specific gene sequences were detected in ocean water C. This may reflect the lower sensitivity of the culture method or high concentrations of viable but nonculturable and dead *Salmonella* spp. which are not detected by culture method. In summary, a total of 6 samples were submitted to detection of *Salmonella* spp. with PCR and standard culture method. Of the 6 samples tested, 3 (50%) demonstrated the presence of Salmonella-specific gene sequences while 2 (33 %) demonstrated culturally viable *Salmonella* spp. Of the three samples showing *Salmonella* spp. gene sequences, 2 were confirmed as also having culturally viable *Salmonella* spp.

Chapter 4

Survival of *Escherichia coli* and *Salmonella typhimurium* in Ocean Water

I. Introduction

The use of *Escherichia coli* as a water quality indicator is based on its ability to represent fecal contamination and the possible presence of pathogenic bacteria in recreational waters. Currently, public health decisions concerning the safety of recreational waters are based on the presence and concentration of *E. coli* in recreational waters. The survival of indicators and pathogens in marine environments is of critical importance in order to determine the possible health risks associated with contacting sewage-contaminated recreational waters. The survival and decline of enteric bacteria in marine waters has been attributed to a variety of interacting chemical, physical and biological factors. These factors include sunlight (Fujioka et al., 1981; Barcina, 1988), temperature (McCambridge and McMeekin, 1980; Rhodes and Kator, 1988; Stuart et al., 1994), predation (Mitchell et al., 1967; McCambridge and McMeekin, 1980), and nutrient availability (Carlucci and Pramer,1960; Jannasch, 1968). Studies relating these factors and their influences on indicator bacteria is of importance to public health officials in order to assess the validity and suitability of indicators to predict the sanitary quality of recreational waters.

Previous studies comparing the survival of *E. coli* and pathogen *Salmonella* spp. in estuarine and coastal waters have offered conflicting results. Studies by Beard

and Meadowcraft (1935) demonstrated that *E. coli* survived longer than *Salmonella typhosa* in sterile seawater. In contrast, other studies using sterile seawater have reported prolonged survival of *Salmonella typhimurium* compared to that of *E. coli* (Vasconcelos et al., 1976) In research conducted by McCambridge and McMeekin (1980), populations of both *E. coli* and *S. typhimurium* remained virtually intact after ten days of exposure to sterile estuarine water in vitro. The discrepancies seen among various studies can be attributed to a number of factors and may include laboratory manipulations (Rhodes et al., 1983) and isolate history (Granai and Sjogren, 1981) which can affect cell viablity, particularly when various selective and non-selective media are used (Bissonnette et al., 1975; Rhodes et al., 1983). If a bacterium is able to form colonies on a nonselective growth medium but not on a medium which contains selective agents to which the bacterium is normally resistant, it is termed injured (James et al., 1994). Numerous studies have termed these injured bacteria "sublethally stressed" (Smith et al, 1994; Rhodes and Kator, 1988). Currently selective media like mTEC are utilized to isolate *E. coli* from recreational waters (*Standard Methods*, 1992). Because of mTEC's selective nature, sublethally stressed *E. coli* may not be detected and this would lead to an underestimation of the actual number of *E. coli* present in marine waters.

In addition to sublethally stressed bacteria, as defined by selective and nonselective media, there is also another classification of stressed bacteria that are unable to demonstrate growth on either selective or non selective media but are still

considered viable and capable of causing disease. This group of stressed bacteria is termed viable but nonculturable and is of public health significance since *Salmonella* spp. have been shown to enter a viable but nonculturable state in marine waters (Colwell, et al., 1983).

To date the vast majority of studies relating the factors responsible for a decline of *E. coli* and *Salmonella* spp. in marine waters have been conducted with sterile seawater and do not address the impact of naturally occurring marine populations (autochthonous microbiota) and their impact on *E. coli* and *Salmonella* spp. populations. This is of vital concern since native populations may compete with enteric bacteria for nutrients (Carlucci and Pramer, 1960) and also serve as predators and antagonists (McCambridge and McMeekin, 1980).

When studying the survival of enteric bacteria in marine waters, a number of experimental approaches have been cited in the literature which include closed vessel systems and vessels that are in direct contact with the tested waters. Both systems have advantages and disadvantages. Closed vessel systems, or more commonly referred to as "laboratory microcosms", are relatively easy to set up in the laboratory where temperature and light can be regulated. In addition it is easy to collect and process samples. There are, however, disadvantages to using these systems since these systems have been criticized for creating a "bottle effect" and the termination of solute exchange with the environment (Ferguson et al., 1984; McFeters and Terzieva, 1991). Vessels having direct contact with testing waters avoid many of the problems

associated with the a lack of solute exchange seen in closed vessel systems. This type of systems utilizes porous membranes with small pore sizes (0.22 μm) that allow for solute exchange with the environment while retaining the test organism within the vessel. This allows the organisms to be in direct contact with the solutes transversing the membrane from the environment and better represent actual field conditions. One of the more popular systems, membrane diffusion chambers, utilizes plexiglass vessels fitted with resealable sampling ports and o-rings that retain 0.22 μm pore-size membranes (McFeters and Stuart, 1972). The diffusion chamber can be suspended in the test water and samples can be collected at periodic intervals. Unfortunately diffusion chambers require great care in handling, since membranes are very fragile. In addition, care must be taken to seal chambers in order to avoid leakage around the membranes (Rhodes and Kator, 1988).

Recently the development of the polymerase chain reaction (PCR) method has offered an attractive alternative to the problematic cultural isolation of indicator bacteria and waterborne pathogens. Since PCR methods target gene sequences, time consuming cultural isolation can be avoided and results can be obtained in a fraction of the time required by cultural methods. In addition, PCR has the potential of detecting sublethally stressed and viable but non-culturable pathogens. PCR has proven to be a sensitive and specific technique capable of detecting as few as one bacterial cell per 100 ml of water (Bej, et al. 1991) and has been utilized to detect a variety of waterborne pathogens including *Legionella* spp.(Palmer et al.,

1995), *Shigella* spp. (Bej et al., 1991), *Campylobacter* spp. (Oyofo and Rollins, 1993) and enteric viruses (Morteza et al. 1993). In order for PCR technology to be applicable for the detection of indicators and waterborne pathogens we must know the persistence of target gene sequences and its relationship with cultural viablity. This is of public health significance since only viable pathogens have the potential of causing disease.

II. Study Objective

To date a number a studies have utilized membrane diffusion chambers to analyze the cultural survival characteristics of *E. coli* and *Salmonella* spp. in estuarine and coastal marine waters (Vasconcelos and Swartz, 1976; Dawe and Penrose, 1978; Anderson et al., 1983; Rhodes and Kator, 1988; Smith et al., 1994). For the most part, however, the majority of these studies have focused on the survival characteristics in waters from temperate climates. The purpose of this study is to use traditional culture method and the new PCR method to determine the fate (culturablity, viability, and structural integrity) of indicator bacteria (*E. coli*) and the pathogen *Salmonella typhimurium* suspended in tropical marine waters.

III. Experimental Design

In this study, human derived isolates of *E. coli* and *S. typhimurium* were exposed to marine waters in the presence and absence of autochthonous microbiota

for a period of 18 days. Samples were collected at regularly spaced intervals and submitted to acridine orange direct counts (AODC) and cultural isolation using both nonselective and selective media. In addition, PCR was performed targeting *E. coli* and *Salmonella* spp. gene sequences in order to determine the persistence of their gene sequences (structural integrity of cell) in marine waters. Finally, the relationship between the persistence of culturally viable cells and PCR-targeted gene sequences was established.

IV. Materials and Methods

A. Bacterial Stains and Growth Conditions in Duffusion Chambers

Escherichia coli was isolated from human feces using mTEC agar and characteristic colonies were confirmed by API20 E strips (Ayerst Laboratoires Inc., Plainview, NY). Human *Salmonella typhimurium* was obtained from the Hawaii Department of Health, Honolulu, Hawaii. *Escherichia coli* and *Salmonella typhimurium* cultures were inoculated into separate 100 ml tryptone-lactose-yeast extract broth (Smith et al., 1994) in 250 ml Erlenmeyer flasks. The flasks were incubated on a shaker (150 rpm) at 37°C. Cultures were grown to early stationary phase (20 h) as monitored by A_{260}. Cells were pelleted at 3,000 X *g* for 10 min. and washed twice with filter-sterilized (1 and 0.22 μm pore size filters) ocean water obtained from Queen's Beach, adjacent to the Honolulu Aquarium. Cells were then resuspended in filtered or unfiltered ocean water at a concentration of 10^6 cells per

ml and injected slowly into 100 ml diffusion chambers (McFeters, et al., 1972). Chambers were shaken and time zero samples were collected, and the chambers were simultaneously suspended at a depth of 10 inches inside a 110 gallon fiberglass tank at the Honolulu Aquarium for a duration of 18 days. Fresh seawater was constantly pumped into this tank which contained no metal fittings. The water turn over time was 1.5 hours. Temperature and salinity were 24.5°C and 34.0 ppt respectively and remained constant throughout the experiment. Subsamples for all assays (2.0 ml) were removed with sterile 5 ml syringe and transported to the lab immediately.

Table 10. Water quality Characteristics of Ocean Water Utilized in Diffusion Studies

Parameter	Value
Temperature (°C)	24.5
Salinity (ppt)	34.0
pH	7.7
Dissolved Oxgen (mg/l)	7.2
Nitrites plus nitrates (mg/l)	0.13
Total Phosphorus (mg/l)	0.022

B. Plate Counts and Assessment of Injury

Viable concentrations of *E. coli* and *Salmonella* were measured by methods which included a repair detection procedure to recover injured bacteria as described

by Hackney et al. (1979) and Rhodes et al. (1988). Dilutions from of all samples were membrane filtered or spread-plated in duplicate onto selective and nonselective media. Viable counts (CFU/ml) of *E. coli* were determined by spread-plating on tryptic soy agar (Difco Laboratories, Detroit, Mich.) and by membrane filtration using mTEC agar (*Standard Methods*, 18th ed., 1992). Both tryptic soy agar plates and mTEC agar plates were incubated at 35°C for 2 hrs followed by 44.5 °C for 22± 2h. Viable counts (CFU/ml) of *S. typhimurium* were determined by spread-plating as described by Rhodes and Kator (1988) on tryptic soy agar and incubated at 37°C for 24 hr. In addition samples were spread-plated on tryptic soy agar and incubated for 2 h at 37°C and then overlaid with 10 ml brilliant green sulfa agar (Difco Laboratories, Detroit, Mich.) and incubated for an additional 22 hrs. at 37°C.

C. Determination of Sublethal Stress.

A quantitative index of sublethal stress was obtained by comparing the concentration of bacterial colonies recovered using nonselective procedures described above with those obtained using selective media. The percentage of *E. coli* and *S. typhimurium* populations exhibiting sublethal stress was calculated by the following equation: $[(CFU_{nonselcetive} - CFU_{selective}/CFU_{nonselective}) \times 100]$ as described by Shingh and McFeters (1990).

D. Acridine Orange Direct Count Staining

To obtain total cell counts (dead & live cells), samples collected from membrane diffusion chambers were also submitted to acridine orange direct counts (AODC) by filtering a sample through a 0.22 μm pore-size, preblackened polycarbonate filtration membrane (Poretics, Livermore , CA) and staining with 0.01% acridine orange (Polyscience Inc., Warrington, PA.) in 2 mM tris buffer (pH 7.0) for 3 minutes. The acridine orange solution was removed via filtration and the filter was air-dried for 15 s. The membrane was then placed on a clean slide pre-coated with 1 to 2 drops of immersion oil. Another drop of immersion oil was added on top of the membrane and a cover slip was applied. Cells were counted using a Nikon Labophot fluorescent microscope fitted with a B2 filter set (Nikon Inc., NJ.) At least 20 random fields were surveyed and a total of at least 400 cells were counted in order to calculate the total number of cells in a sample.

E. DNA Extraction and Purification

A 1-ml portion of the samples obtained from membrane diffusion chambers was filtered through a 13 mm diameter 0.45 μm poresize HVLP filter (Millipore Inc., Bedford, MA.). The filters were then added to 1.0 ml of 5 % (w/v) chelex® 100 (Biorad) in sterile deionized water and mixed thoroughly. This solution was then boiled for 15 min to release DNA from cells and incubated at room temperature for twenty minutes and then transferred to an centricon-100 (Amicon

Inc., Beverly, MA.) concentrator and the sample concentrated to a final volume of 30 µl as per manufacturer's instructions.

F. DNA Primers and PCR Amplification

Primer BRSALa (5'-CTG GAT GAG ATG GAA GAA TG- 3') and BRSALb (5'-ACG GTT GTT TAG CCT GAT AC-3') were chosen using Olgio 4.0 computer software (National Biosciences, Inc.) from a previously published *Salmonella* restriction fragment sequence (Tsen, et al., 1991). These primers were utilized to detect *Salmonella*-specific gene sequences and are described in detail in Chapter 3. PCR detection of *E. coli* was performed using primers UAL-754 (5'-AAA CGG AAG AAA GCA G-3') and UAR-900 (5'- ACG CGT GGT TAC AGT CTT GCG-3') , which have previously been described by Bej. et al. (1991). These primers were used to amplify a 147-bp coding region of the *uid*A gene. All primers were produced with an automatic DNA synthesizer (ABI 381A; Applied Biosytems Internatinal, Foster City Calif.). PCR reaction mixture contained 1 x PCR buffer (10 mM Tris-HCL [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM (each) deoxynucleoside triphosphate, 0.3 µM (each) primer, and 0.5 µl Taq DNA polymerase per 100 µl (Perkin-Elmer Cetus, Norwalk, Conn.). The PCR temperature cycling profile for the detection of *Salmonella* spp. (Primers BRSALa & BRSALb) consisteted of initial denaturation at 94°C for 5 min followed by 35 cycles each consisting of 120 sec. at 94°C, 20s at 57.5°C, and 60s at 72°C.

Synthesis was completed at 72°C for 7 minutes. For the detection of *E. coli* a two temperature profile described by Bej.et al. (1991) was used and consisted of an initial denaturation at 94°C for 3 min followed by 25 cycles with a 94°C denaturation step for 60 sec and primer annealing and extension at 50°C for 60 sec. PCR products were analyzed by gel electrophoresis on a 2 % SeaKem GTG agarose (FMC BioProducts, Rockland, Maine) gel stained with ethidium bromide (0.5 μg/ml) and were identified using a model 6-63 tranilluminator (Ultra-Violet Products, San Gabriel, Calif.).

G. DNA Hybridization

PCR products generated from Salmonella-specific primers were confirmed with an internal probe, TS21 (5'-TAC ATC GTA AAG CAC CAT CGC AAA T-3'), which was chosen from a previously described *Salmonella* spp.-specific sequence (Tsen et al. 1991). PCR products generated from *E. coli* -specific primers were confirmed with an internal probe UAP-1 (5'-TGC CGG GAT CCA TCG CAG CGT AAT G-3') as described by Tsai et al. (1993). The internal probes were 3' labeled with digoxigenin-11-ddUTP using a Genius 5 nonradioactive DNA labeling kit (Boehringer Mannheim, Indianapolis, Ind.) PCR products were transferred onto Hybond-N+ positively charged nylon membranes (Amersham Arlington, Heights, Ill.) using a Minifold II slot blotter (Scheicher & Schuell, Kenne, N. H.) DNA was fixed to the nylon membranes by UV irradiation at 254

nm for 2 min using a CL-1000 ultraviolet crosslinker (UVP, San Gabriel, Calif.). Salmonella internal probe hybridization was performed at 55°C in presence of a labeled oligonucleotide internal probe (2 pmol/ml). The hybridized filters were washed twice with a high-salt solution (2 x SSC in 0.1% sodium dodecyl sulfate) at room temperature and then twice with a low-salt solution (0.1 x SSC in 0.1% sodium dodecyl sulfate) at 50°C. For the *E. coli* internal probe hybridization was performed at 60°C in the presence of labeled oligonucleotide internal probe (2 pmol/ml). The hybridized filters were washed twice with a high-salt solution (2 x SSC in 0.1% sodium dodecyl sulfate) at room temperature and then twice with a low-salt solution (0.1 x SSC in 0.1% sodium dodceyl sulfate) at 50°C. A Genius 3 nucleic acid detection kit (Boehringer Mannheim, Indianapolis, IN) was used to prepare the hybridized filters for chemiluminescent detection. The hybridization signals were visualized on X-OMAT film (Eastman Kodak, Rochester, N. Y.) using autoradiography.

V. Results and Discussion

A. Survival of *E. coli* and *S. typhimurium* in Filtered Ocean Water

The premise behind the use of water quality indicators like *E. coli*, is its presence in environmental waters is indicative of human wastes and the possible presence of enteric pathogens. This premise dictates that *E. coli* should survive as long or longer than enteric pathogens, like *Salmonella* spp., in environmental waters.

Currently the selective medium, mTEC, is utilized to recover *E. coli* from marine waters and is used by public health official to predict the possible presence of pathogens like *Salmonella* spp. Previous research, however, has demonstrated that when *E. coli* is exposed to sea water it enters a physiological stressed state (sublethal stress) which cannot be detected with selective media (Dawe et al., 1978; Anderson, et al., 1983; Rhodes and Kator, et al., 1988; Smith et al., 1994). This stressed state may play a role in the recovery of *E. coli* from tropical marine waters and may influence the ability of *E. coli* to predict the presence of *Salmonella* spp. in marine waters. Because of the problems associated with the cultural recovery of *E. coli* and *Salmonella* spp. from marine waters, PCR was utilized to determine persistence of *E. coli* and *S. typhimurium* gene sequences (structural integrity). In order to better understand the survival characteristics of *E. coli* and *Salmonella* spp. in tropical marine waters, a series of survival studies were conducted. These experiments involved placing pure cultures of *E. coli* and *S. typhimurium* in separate membrane diffusion chambers which were then suspended in seawater for 18 days.

The viable concentration, structural integrity, and total cell concentrations of *E. coli* and *S. typhimurium* suspended in filtered sea water was followed over 18 days based on colony formation (CFU/ml) on a selective medium [mTEC agar for *E. coli* and TSA overlayed with brilliant green agar (TSAB) for *S. typhimurium*], nonselective medium (TSA), acridine orange total cell counts (AODC) and by PCR targeting *E coli* and *S. typhimurium* gene sequences.

The culture results (Figure 5 and 6) show that during the first two days in filtered seawater there were relatively stable *E. coli* and *S. typhimurium* populations recovered by nonselective and selective media. After two days in filtered sea water, however, there was a relatively rapid decline in the number of culture-viable *E. coli* and *S. typhimurium* recovered on selective and nonselective media (Figure 5 and 6). The number of culture-viable *E. coli* recovered on nonselective (TSA) declined from 4.1×10^6 CFU/ml seen at day 2, to 5.0×10^3 CFU/ml by day 6 (Figure 5). After day 6 the number of culture-viable *E. coli* recovered on nonselective medium (TSA) appeared to decline slightly (5.0×10^3 CFU/ml at day 6 to 1.9×10^3 CFU/ml at day 8) and level off at concentrations between 1.2×10^2 CFU/ml and 5.0×10^1 CFU/ ml between day 12 and 18. The number of culture viable *E. coli* recovered on the selective medium (mTEC) declined from 8.3×10^5 CFU/ml at day 2 to 8.0×10^1 CFU/ml by day 6 and no CFU could be detected by selective medium after day 6 (Figure 5). As seen in Figure 6, there was a similar decline in the number of culture-viable *S. typhimurium* recoved by selective (TSAB) and nonselective media (TSA) during the 18 day exposure period. The number of culture-viable *S. typhimurium* recovered on nonselective medium (TSA) declined from 7.0×10^5 CFU/ ml on day 2 to 1.2×10^3 CFU/ml by day 6 while on the selective medium (TSAB) numbers declined from 4.8×10^5 CFU/ml on day 2 to 4.0×10^1 CFU/ml by day 6 and no culture-viable *S. typhimurium* was recovered by selective medium (TSAB) after 6 days of exposure (Figure 6). Although, no culture-viable *E. coli* and *S. typhimurium*

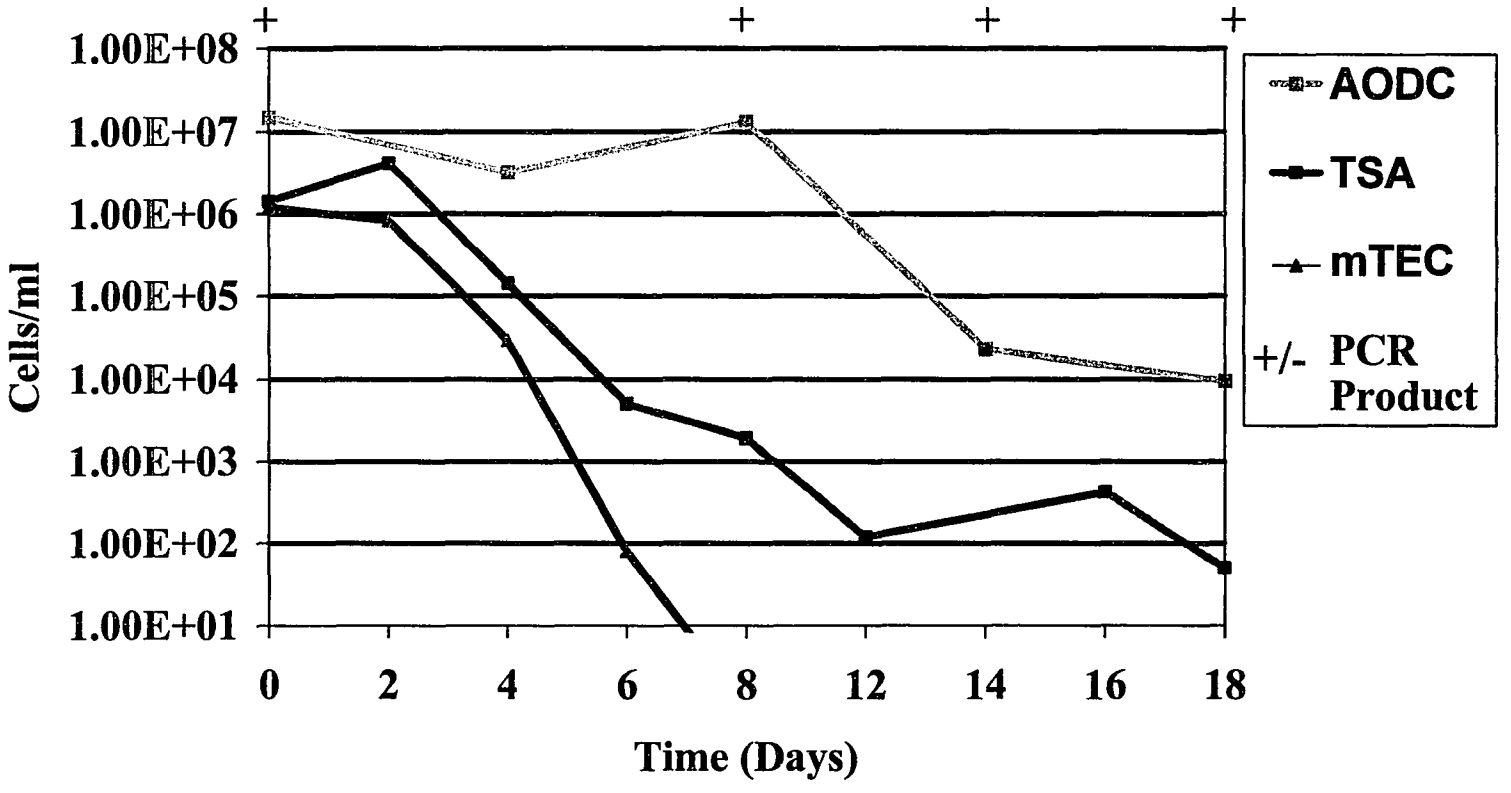


Figure 5. Plate Counts, AODC Counts and PCR Results for *E. coli* in Filtered Ocean Water

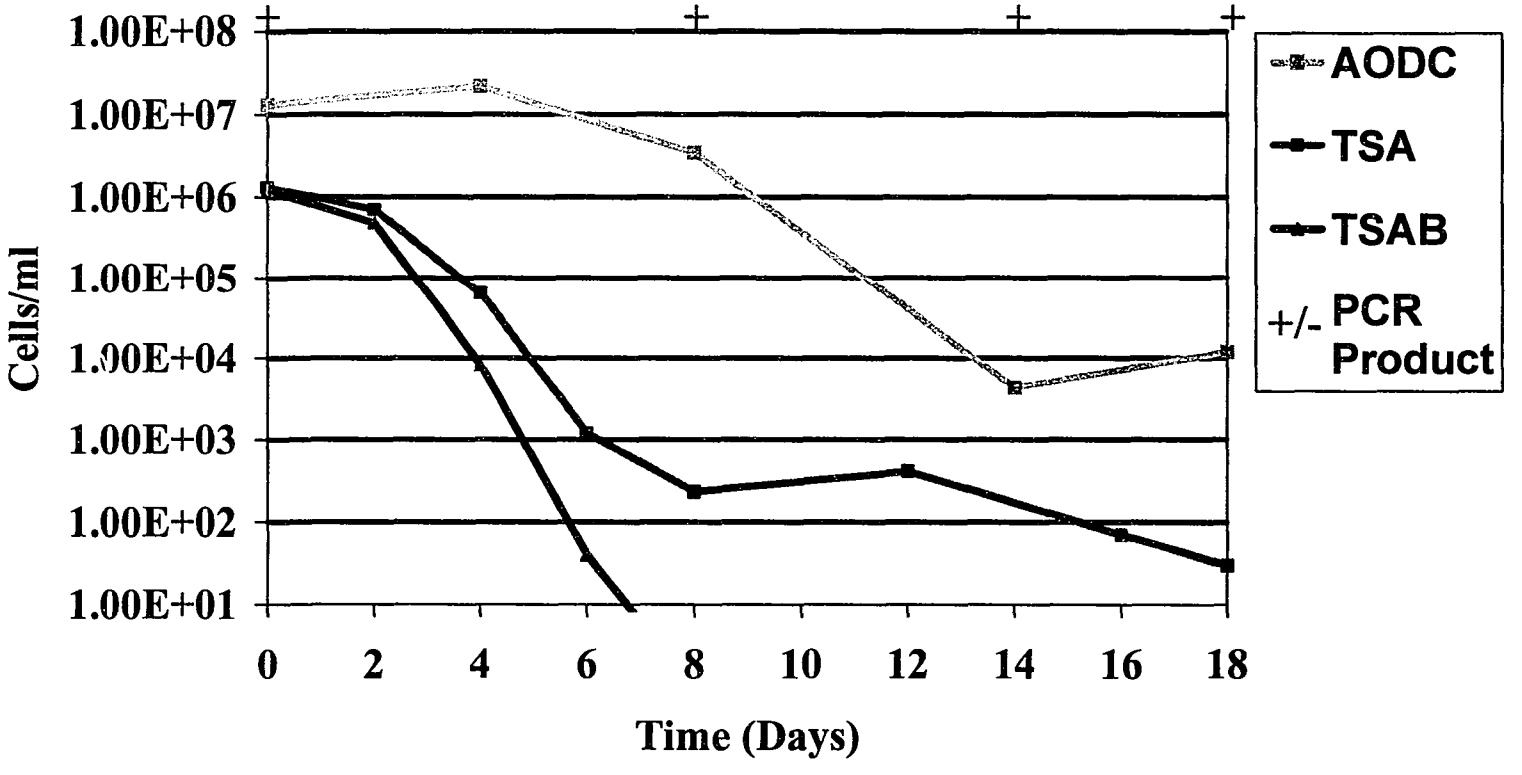


Figure 6. Plate Counts, AODC Counts, and PCR Results for *S typhimurium* in Filtered Ocean Water

were recovered on selective media after 6 days of exposure (Figure 5 & 6) both bacteria could still be recovered on nonselective medium (TSA) after 18 days of exposure. Other researchers have observed a similar, but less rapid, decline for culture-viable *E. coli* and *S. typhimurium* exposed to filtered polar (-1.4 °C) and temperate (14.5°C) marine waters (Smith et. al., 1994; Vasconcelos and Swartz, 1976). This may be a reflection of the higher ocean water temperature (25.5 °C) in this study, since previous studies have shown that, as water temperatures increase, there is a more rapid decline in culture viable *E. coli* and *S. typhimurium* (Mancini, 1978; McCambridge and McMeekin, 1980).

Occurring concomitantly with the decline of culture-viable *E. coli* and *S. typhimurium* was an increase in *E. coli* and *S. typhimurium* populations exhibiting sublethal stress. This is evident by the differences among *E. coli* and *S. typhimurium* counts recovered by selective and nonselective media [(CFUnonselective- CFU selective/ CFU nonselective) x 100]. As seen in Table 11 there was an increase in the populations of *E. coli* and *S. typhimurium* exhibiting sublethal stress as exposure times increased. At time zero 14.3 % of the *E .coli* population and 7.8% of the *S. typhimurium* population exhibited sublethal stress and as exposure time increased the percentage of sublethal stressed *E. coli* and *S. typhimurium* populations increased to 98.4 % and 96.7 % after six days of exposure, respectively. These results are similar to survival studies conducted by Smith et al. (1994) in polar marine waters. In these studies 96 % of *E. coli* and 87 % of *S. typhimurium* populations were sublethally

stressed after 4 days of exposure to polar waters (-1.8 °C). In other studies conducted in temperate marine waters, Dawe and Penrose (1978) reported a sublethal stressed *E. coli* population of 98 % after four days of exposure (12 °C). These studies and the research reported in these experiments, demonstrate an increase in sublethal stressed populations of *E. coli* and *S. typhiumurium* exposed to filtered ocean water.

Table 11. Sublethally Stressed Cells in Filtered Ocean Water as Measured by Percent Culturability, Using Nonselective and Selective Methods

Exposure Time (days)	% Sublethal Injury[a]	
	E. coli	*S. typhimurium*
0	14.3	7.8
2	79.8	31.4
4	79.3	87.0
6	98.4	96.7
8	$\geq$ 99.5[b]	$\geq$ 95.7[b]
12	$\geq$ 91.7[b]	$\geq$ 97.6[b]
16	ND[c]	ND[c]
18	ND[c]	ND[c]

[a] % Sublethal injury, $[(CFU_{nonselective}-CFU_{selective}/ CFU_{nonselective}) X 100]$
[b] Selective CFU below detection limit (10 CFU), 10 CFU used in calculation
[c] Not done

The growthof *E. coli* on mTEC from marine waters is commonly used by water quality monitoring agencies to predict the presence of pathogens in recreational waters. In this study no *E. coli* could be recovered by selective medium (mTEC) after

6 days of exposure while sublethally stressed populations of *S. typhimurium* could be recovered during the entire 18 day exposure period. This evidence questions the ability of the medium mTEC to predict the presence of sublethally stressed *S. typhimurium* after prolonged exposure (>6 days) to tropical marine waters.

The total cells counts (live and dead), as demonstrated by acridine orange staining, demonstrated relatively stable total *E. coli* and *S. typhimurium* cell populations until day 8 (Figures 5 and 6). After 8 days of exposure the total cell counts for both *E. coli* and *S. typhimurium* declined rapidly. Total cells counts for *E. coli* declined from 1.3×10^7 cells/ml at day 8 to 2.3×10^4 cells/ml by day 14. Total cell counts for *S. typhimurium* declined from 3.4×10^6 cells/ml at day 8 to 4.3×10^3 cells/ml by day 14. These results are in contrast with other studies (Grimes et al., 1986) reporting relatively constant levels of total cells counts for *E. coli* and *S. enteritidis* exposed to filtered ocean water in closed vessels. There are a number of possible reasons for these discrepancies which may include; 1) the presence of bacteriophage (Mitchell, 1968), 2) the presence of autochthonous microbial toxins (Elliot and Colwell, 1985), and 3) the differences among closed vessel and diffusion chamber experiments (McFeters and Stuart, 1983).

In order to determine the persistence of *E. coli* and *S. typhimurium* gene sequences (structural integrity of cell) during exposure to filtered ocean water, PCR was performed targeting *E. coli* and *S. typhimurium* gene sequences. This was accomplished by filtering 1 ml samples from each diffusion chamber through a 0.45

μm pore size filter. DNA from bacterial cells retained by the filter was extracted and submitted to PCR. In this study, *E. coli* and *S. typhimurium* gene sequences could be detected by PCR during the entire 18 day exposure (Figure 5 and 6) and would indicate that PCR was able to detect structurally intact DNA. This would be expected since culture viable *E. coli* and *S. typhimurium* could be recovered on nonselective medium (TSA) throughout the 18 day exposure period (Figures 5 and 6).

Together these results demonstrate the viable concentration, persistence of structurally intact *E. coli* and *S. typhimurium* DNA, and total cell concentrations of *E. coli* and *S. typhimurium* exposed to filtered ocean water. In order to gain an understanding of sublethal stress pure cultures of *E. coli* and *S. typhimurium* were required during these survival experiments. For the most part, these results represent the impact of solutes capable of traversing diffusion chamber membranes (0.22 μm) and do not address the impact of direct contact with marine organisms.

B. Survival of *E. coli* and *S typhimurium* in Unfiltered Ocean Water

Naturally occurring microbial population (autochthonous microbiota) can have an impact on the survival of *E. coli* and *S. typhimurium* in environmental waters (Mitchell et al., 1967; Enzinger and Cooper, 1976;; McCambridge and McMeekin, 1980; Rhodes and Kator, 1988). In order to gain an understanding of the impact of autochthonous microbiota on the survival of *E. coli* and *S. typhimurium* in tropical ocean water, diffusion chambers were injected with both autochthonous microbiota

(unfiltered ocean water) and either *E. coli* or *S. typhimurium.* These chambers were then suspended in ocean water for a period of 18 days. Since these chambers contained mixed populations, only selective media could be used to isolate culturally-viable *E. coli* (mTEC medium) and *S. typhimurium* (TSAB medium). The viable concentration, structural integrity, and total cell concentrations of *E. coli* and *S. typhimurium* suspended in unfiltered sea water were followed over 18 days based on colony formation (CFU/ml) on a selective medium [mTEC agar for *E. coli* and TSA overlayed with brilliant green agar (TSAB) for *S. typhimurium*], acridine orange total cell counts (AODC) and by PCR targeting *E. coli* and *S. typhimurium* gene sequences in structurally intact cells.

The culture results (Figure 7 and 8) show that during the first two days in unfiltered seawater there was a relatively stable culture viable *E. coli* population (1.4 x 10^6 CFU/ ml at time 0 and 8.0 x 10^5 CFU/ml at day 2) while the number of culture viable *S. typhimurium* declined slightly (3.2 x 10^6 CFU/ml at day 1 and 4.5 x 10^5 CFU/ml). Between day 2 and day 8, however, there was a relatively sharp decline in in the number of culture viable *E. coli* and *S. typhimurium*. Culture viable *E. coli* declined from 8.0 x 10^5 CFU/ml at day 2 to 2.0x 10^1 CFU/ml at day 6 and no culture viable *E. coli* could be recovered on day 8 (Figure 7). *S. typhimurium* displayed a similar survival pattern with a rapid decline in the number of culture viable cells from day 2 (4.5 x 10^5 CFU/ml) to day 6 (9.0 x 10^1 CFU/ml) and an absence of culture-viable *S. typhimurium* at day 8. These results are very similar to the results obtained

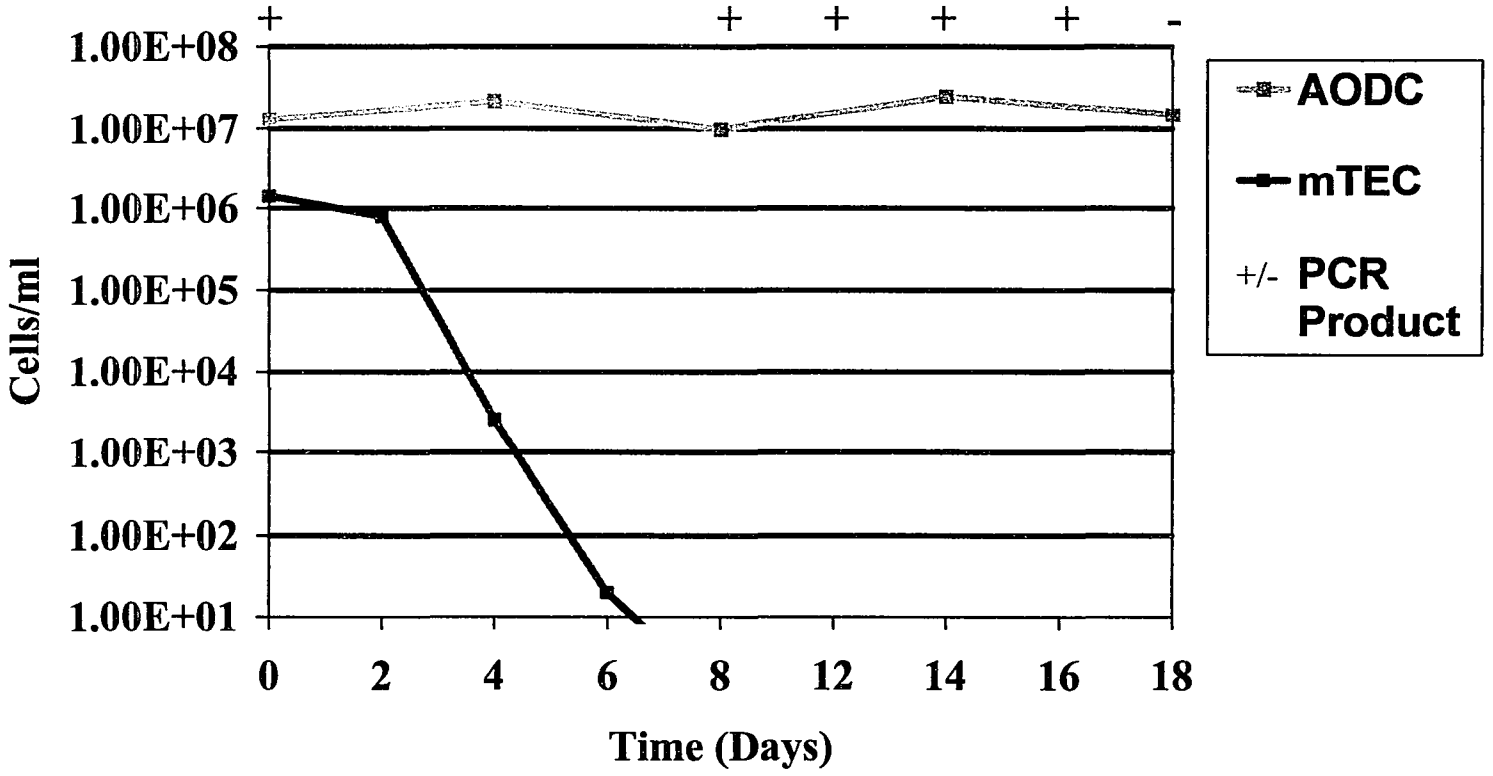


Figure 7. Plate Counts, AODC Counts and PCR Results for *E. coli* in Unfiltered Ocean Water

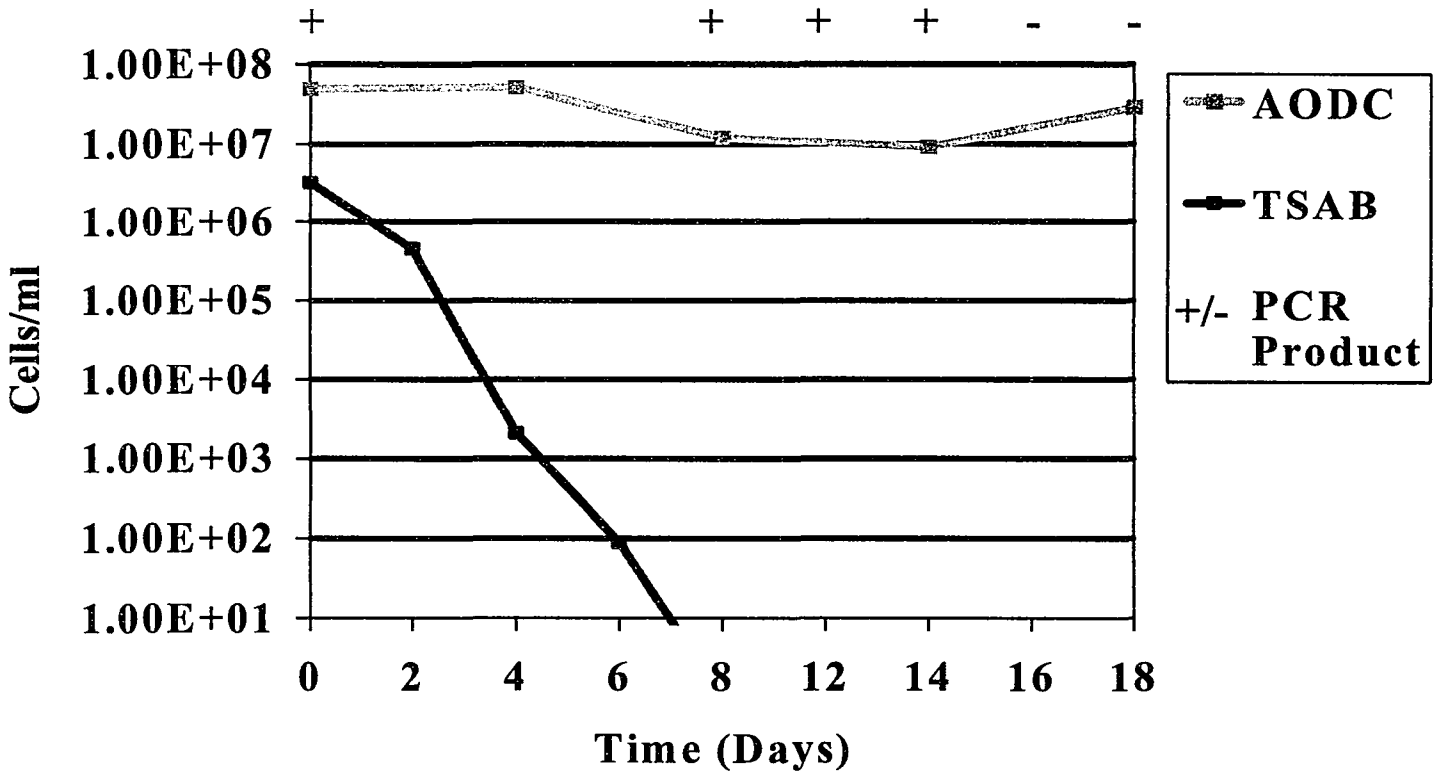


Figure 8. Plate Counts, AODC Counts and PCR Results for *S. typhimurium* in Unfiltered Ocean Water

in filtered ocean water survival experiments (Figure 5 and 6) where there was a similar decline from day 2 to day 8 for the number of culture viable *E. coli* and *S. typhimurium* recovered on selective media. These results would indicate that during the first 6 days of exposure, autochthonous microbiota did not appear to have an impact on the number of culture viable *E. coli* and *S. typhimurium* recovered by selective media.

The total cell counts (live and dead), as demonstrated by acridine orange staining, were relatively stable throughout the 18 day exposure period for both diffusion chambers (Figures 7 and 8). The discrepancies between these results and the ones obtained in filtered ocean water experiments are most likely a reflection of the contributions made by autochthonous microbiota. It is however, difficult to interpret the meaning of the these result since these diffusion chambers contained a diverse microbial population. It would appear however, the total cell population in these chambers remained relatively constant during the 18 day exposure period even though there was a decline in culture viable *E. coli* and *S. typhimurium* (Figures 7 & 8) similar to the decline observed in filtered ocean water (Figures 5 and 6).

In order to determine the persistence of *E. coli* and *S. typhimurium* gene sequences (structural integrity of cell) during exposure to unfiltered ocean water, samples from each diffusion chamber were collected at regular intervals and filtered through a 0.45 μm filter. Total DNA was extracted from the filter and submitted to PCR amplification targeting *E. coli* and *S. typhimurium* gene sequences. As seen in

Figure 9, *E. coli* and *S. typhimurium* PCR products were detected until days 16 and 14, respectively. No PCR products were detected at day 18 for *E. coli* and no PCR products were detected at day 16 for *S. typhimurium*. The signal intensity for *E. coli* PCR product detection, showed the greatest intensity at time zero and day 8. The intensity of these signals appears to diminish in intensity after day 8 and until the loss of signal at day 18. PCR results for *S. typhimurium* demonstrate a similar decline in signal intensity with highest intensities at time zero and day 8. From day 8 until the loss of signal at day 16 there was a decline in signal intensity. These results indicate a decline in the number of structurally intact PCR-targeted *E. coli and S. typhimurium* gene sequences starting at day 12 and continuing until no signal could be detected. This would imply a loss of cellular structural integrity for both *E. coli* and *S. typhimurium* during these experiments. These results are very different from those obtained in filtered ocean water survival experiments where PCR products were detected throughout the 18 day exposure period. It would appear that the presence of autochthonous microbiota has a dramatic impact on the decline of cellular integrity for both *E. coli* and *S. typhimurium* after 8 days of exposure. Since culture results for *E. coli* and *S. typhimurium*, as demonstrated by growth on selective media, were similar for both filtered and unfiltered ocean water experiments (Figures 5,6,7 & 8), these results would indicate the influence of autochthonous microbiota on the decline of the number of structurally intact *E. coli* and *S. typhimurium* gene sequences has its greatest influence after 6 to 8 days of exposure in unfiltered ocean water. Together

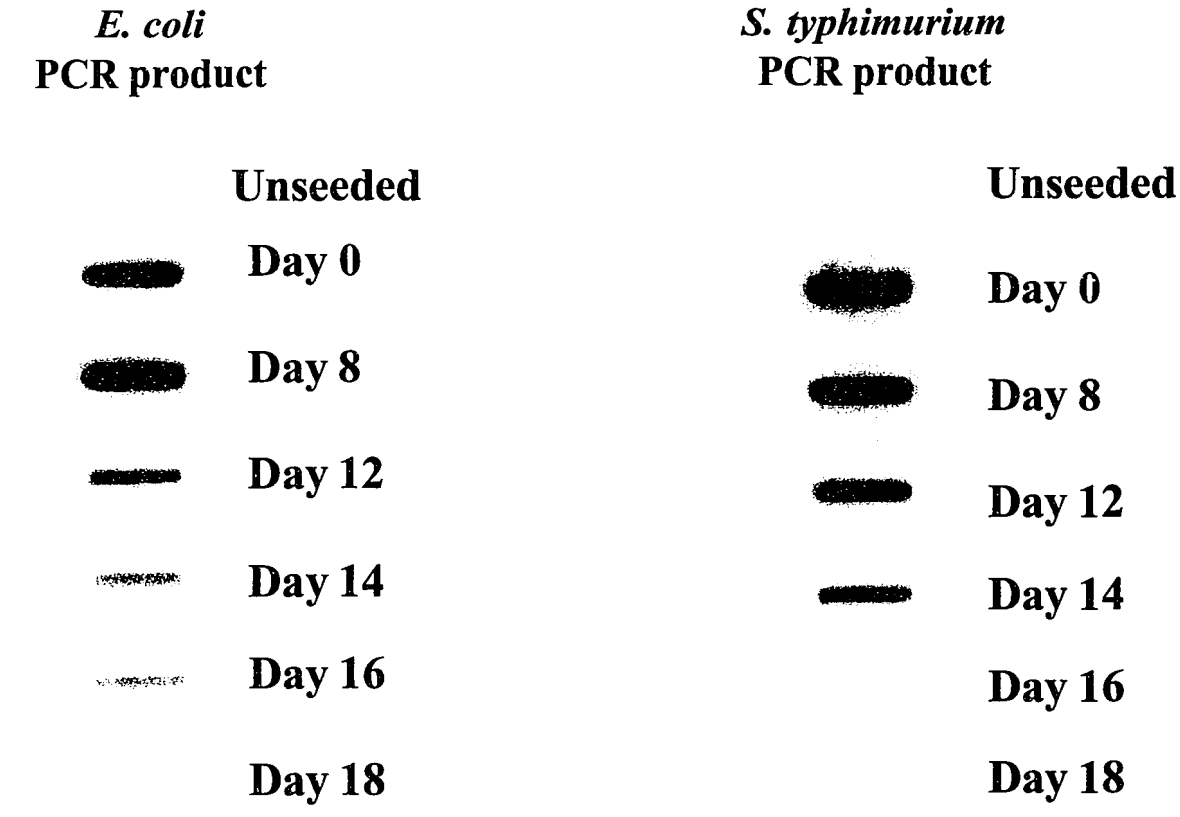


Figure 9. PCR Detection of *Salmonella* spp. and *E. coli* Gene Sequences After Exposure to Unfiltered Seawater

these results indicate a loss of structurally intact *E. coli* and *S. typhimurium* gene sequences which would suggest the absence of sublethally stressed and viable but nonculturable *E. coli* and *S. typhimurium* populations. These results indicate that autochthonous microbiota play a role in the loss of structurally intact *E. coli* and *S. typhimurium* in marine waters and appear to influence the persistence of sublethally stressed and viable but nonculturable populations. A number of agents may be responsible for the decline of detectable *E. coli* and *S. typhimurium* gene sequences. These agents include bacteriophage (Mitchell, 1968), lytic marine bacteria (Mitchell, 1967), predation (Enzinger and Cooper, 1976; McCambridge and McMeekin, 1979) and antagonistic chemicals produced by autochthonous microbiota (Pike et al., 1970; Brown et al., 1977).

Chapter 5

Development of a PCR Detection System for the "*Bacteroides fragilis* Group"

I. Introduction

Currently the water quality indicators *Escherichia coli* and enterococci are utilized to determine the sanitary quality of recreational waters. These indicators are normal inhabitants of the intestinal tract of warm-blooded animals and their presence in recreational waters is considered indicative of fecal contamination. In addition, their concentrations in water correlates with the incidence of diarrheal diseases among swimmers (Cabelli, 1983). The United States Environmental Protection Agency (USEPA) research supporting the use of *E. coli* and enterococci as a water quality indicator was conducted in temperate climates where environmental sources of these indicator bacteria are insignificant. However, in tropical climates such as Hawaii, Puerto Rico and Guam, *E. coli* and enterococci appear to be naturally present in high concentrations in fresh waters and soil. A number of studies have shown that *E. coli* is capable of surviving and even replicating in tropical environments. In Puerto Rico, *E. coli* was able to survive and replicate in rain forest streams (Carrillo et al.,1985;Lopez-Torres et al., 1987; Valdes-Collazo et al.,1987). Other studies by Hardina and Fujioka (1988) indicated that fecal coliform, *E. coli* and fecal streptococci are naturally occurring in Hawaii's streams and soils. Together this evidence questions the use of *E. coli* as a water quality indicator for tropical

climates and demonstrates the need for alternative means to determine the sanitary quality of recreational waters.

A number of alternative indicators have been proposed which include *Clostridium perfringens* (Bisson, 1980; Fujioka, 1985), bacteriophage (Kott et al., 1981) and *Bacteroides fragilis* (Allsop and Strickler, 1985). Although these alternative indicators have a number of advantages over the existing water quality indicators, they all require cultural isolation which takes at least twenty four hours to render results. This is of concern to public health officials who are charged with assuring public safety and the closing of recreational waters contaminated with human feces. The time required to cultivate indicators can impede our ability to respond to events which may immediately impact human health. During the time required to cultivate indicators many individuals may be exposed to contaminated recreational waters. Because of the limitations associated with the cultural isolation of indicators, we must seek a more rapid means of detecting water quality indicators.

The use of *Bacteroides* spp. as water quality indicators have a number of advantages over existing water quality indicators. *Bacteroides* spp. are nonsporulating, obligate anaerobic Gram-negative bacteria and are the predominant genus of the human intestinal tract with levels 100 to 1000 fold higher than *E. coli* (Oliveri, 1980). Four species are dominant in the human intestinal tract, *B. vulgatus, B. thetaiotaomicron. B. fragilis* , and *B. distasonis*. Collectively these

four species are referred to as the "*Bacteroides fragilis* group" (BFG). BFG are an important potential indicator of fecal pollution since their habitat is restricted to the feces of humans and warm-blooded animals (Allsop and Strikler, 1985). In addition *Bacteroides* spp. are not found in high numbers in a variety of warm blooded animals (i.e. 10^5-10^{10}-fold lower levels) making this group more human specfic (Allsop and Strickler, 1985.) Cultural isolation of *Bacteroides* spp. has been demonstrated by Allsop and Stickler (1984) but to date this method has not gained widespread use. This is most likely a reflection of the fact that BFG are strict anaerobes and do not survive long in environmental waters. Studies by Fikdal et al. (1985) showed that *Bacteroides fragilis* does not survive as long as *E. coli* in environmental waters. This evidence indicates that cultural isolation may not be an adequate means of detecting BFG in environmental waters.

The polymerase chain reaction (PCR) has recently become a powerful tool to detect water quality indicators like *E. coli* and a variety of waterborne pathogens (Bej et al., 1991; Palmer et al., 1995; Oyofo and Rollins, 1993; Morteza et al. 1993). Unlike cultural methods, PCR does not require cultivation of the target organism and results are much more rapid. PCR has a number of attractive advantages over conventional cultural methods since it does not require cultivation and is extremely sensitive (Bej et al., 1991). Although strict anaerobes like BFG do not survive well in environmental water their DNA may persist in the environment and could serve as good candidate for PCR detection.

II. Study Objective

The objective of this study was to develop and evaluate a new PCR method for the "*Bacteroides fragilis* group" as a reliable and sensitive means to determine whether environmental water is contaminated with human feces or sewage. This new PCR method will be compared to existing PCR-based detection systems for *E. coli.*

III. Experimental Design

A pair of BFG specific primers were chosen from a previously described *Bacteroides fragilis* 16s RNA sequence (Weisburg et al., 1985). In order to determine the specificity of these primers, all of the members of the *Bacteroides fragilis* group as well as a variety of other Gram-negative and Gram-positive bacteria were submitted to PCR amplification using these primers. The sensitivity of these primers was determined by performing PCR on a series of diluted primary-treated sewage samples. In addition, PCR detection of the *E. coli* gene *uid* was performed on these same dilutions series and the sensitivity between these systems compared. Cultural isolation of *E. coli* was performed in order to determine the number of culturally viable *E. coli* in primary treated sewage. Next, the survival of *B. thetaiotaomicron* were determined by seeding both unfiltered ocean water and sterile phosphate-buffered water with *B. thetaiotaomicron.* Both PCR and culture methods were used to track the cultural survival of *B. thetaiotaomicron* and persistence of BFG gene sequences. Finally, this new PCR methods was field tested on a variety of

environmental waters and compared to the PCR detection of *E. coli* using previously described primers (Bej., 1991).

IV. Material and Methods

A. Bacterial Strains and Culture Media

Bacterial isolates representing the "*Bacteroides fragilis* group" (*B. fragilis, B. vulgatus, B. thetaiotaomicron* and *B. distasonis*) and a variety of other Gram-negative and Gram-positive isolates were obtained from the American Type Culture Collection, Rockville, Md. *Bacteroides* spp. were grown in reinforced clostridial medium (Oxoid,) under anaerobic conditions (90% N, 10% CO2) at 37°C. Non-Bacteroides species were grown in brain heart infusion broth (Difco Laboratories, Detroit, Michigan) at 37°C for 24 hrs. For survival experiments *B. thetaiotaomicron* was grown in reinforced clostridial medium to late log phase growth. *B. thetaiotaomicron* was culturally isolated using membrane filtration and WCGP agar as described by Allsop and Stickler (1984). *Escherichia coli* was isolated from human feces using mTEC agar and characteristic colonies were confirmed by API20 E strips. Human derived *E. coli* was grown in brain heart infusion broth until late log phase. For sewage and field samples, *E.coli* was detected by membrane filtration using mTEC agar in accordance with *Standard Methods* (1992).

B. Sample Locations and Preparation.

A variety of environmental waters were sampled (both marine and fresh waters). Manoa and Makiki Streams, are located on the leeward side of the island of Oahu and are fed by rainwater. Samples were collected at locations above human contact (Manoa A and Makiki). In addition, one sample was collected from Manoa Stream at a site under the influence of urban storm water drainage (Manoa B). Manoa Stream discharges into Ala Wai Canal which serves as an urban drainage system for the Waikiki area of of Honolulu. In addition, samples were collected from Kaelepulu stream and Kawainui Canal located on the windward side of Oahu. These waterways receive stream run-off and also serve as urban drainage for the City of Kailua. Ala Moana beach is a popular bathing beach and is located in the City of Honolulu. Primary sewage samples were collected from the Sand Island treatment facility located on the island of Oahu. For sewage sensitivity testing a series of ten-fold dilutions (10^{-2} to 10^{-7}) were created using primary treated sewage and phosphate buffered saline (pH 7.2) as the diluent. For survival experiments 5 mls of a late log culture of *B. thetaiotaomicron* were pelleted at 3,000 X *g* for 10 min. and washed twice with PBS and resuspended in PBS or ocean water at a final concentration of 10^9 cells per ml. This suspensions was then added to 1000 ml ocean water or 1000 ml PBS to achieve a final concentration of approximately 10^6 cells/ ml. For sewage sensitivity experiments a series of ten-fold dilutions (10^{-2} to

10^{-7}) were created using primary treated sewage or phosphate buffered saline as the diluent.

B. DNA Extraction.

For specificity testing, DNA was extracted from exponential phase cultures by lysis with 0.5 % sodium dodecyl sulfate treatment as described in *Short Protocols in Molecular Biology 2nd ed.* (Wiley and Sons, 1992). Following alkaline lysis, 0.007 M NaCl-1% hexadecyltrimethyl ammonium bromide was used to complex polysaccharides. Proteins and other impurities were removed by chloroform-isoamyl alcohol (24:1), and DNA was further purified by phenol-chloroform-isoamyl alcohol (24:24:2) extractions. DNA was then precipitated by 2.5 volumes of isopropyl alcohol and pelleted by centrifugation at 12,000 x *g* for 15 min. The DNA pellets were washed once with cold 70 % alcohol and dried under vacuum. (Difco Laboratories, Detroit, Michigan) for 24 hrs at 37°C. For field samples total DNA was extracted by filtering 50 ml of the respective water samples through a 0.45 μm pore-size HVLP filter (Millipore). The filter was than placed in 500 μl of sterile water containing 5% chelex (w/v), mixed thoroughly and placed in a boiling water bath for 15 min. Samples were allowed to cool to room temperature and then were concentrated using a centricon 100 (Amicon) concentrator to a final volume of 30 μl.

E. DNA Primers and PCR Amplification

Primer BF410 (5'-GTG AAG GAT GAA GGC TCT AT-3') and BF800(5'-CGT TTA CTG TGT GGA CTA CC-3') were chosen using Olgio 4.0 computer softeware (National Biosciences, Inc.) from a prevoiusly published *Bacteroides fragilis* 16s RNA sequence (Weiburg, et al., 1985). These primers generated a 410 bp PCR product. PCR detection of *E. coli* was performed using primers UAL-754 (5'-AAA CGG AAG AAA GCA G-3') and UAR-900 (5'- ACG CGT GGT TAC AGT CTT GCG-3') , which have previously been described by Bej. et al. (1991). They were used to amplify a 147-bp coding region of the *uid*A gene in *E. coli*. All primers were produced with an automatic DNA synthesizer (ABI 381A; Applied Biosytems International, Foster City, Calif.) PCR reaction mixture contained 1 x PCR buffer (10 mM Tris-HCL [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM (each) deoxynucleoside triphosphate, 0.3 μM (each) primer, and 0.5 μl Taq DNA polymerase per 100 μl (Perkin-Elmer Cetus, Norwalk, Conn.). The PCR temperature cycling profile for the detection of *Bacteroides fragilis* group (Primers BF410 & BF800) was initially denatured at 94°C for 5 min and then subjected to 30 cycles each consisting of 120 sec. at 94°C, 30s at 53 °C, and 60s at 72°C; then synthesis was completed at 72°C for 7 minutes. For the detection of *E. coli* a two step temperature profile, described by Bej.et al. (1991), was used and consisted of an initial denaturation at 94°C for 3 min followed by 25 cycles consisting of a 94°C denaturation step for 60 sec and primer annealing and

extension at 50°C for 60 sec. PCR products were analyzed by gel electrophoresis on a 2 % SeaKem GTG agarose gel (FMC BioProducts, Rockland, Maine) stained with ethidium bromide (0.5 μg/ml) and were identified using a model 6-63 transilluminator (Ultra-Violet Products, San Gabriel, Calif.).

F. DNA Hybridization

PCR products generated from *Bacteroides* spp. primers were confirmed using an internal probe, BFI 5'-GGA TTT ATT GGG TTT AAA GGG-3'. PCR products generated from *E. coli* -specific primers were confirmed with an internal probe UAP-1 (5'-TGC CGG GAT CCA TCG CAG CGT AAT G-3') as described by Tsai et al. (1993). The internal probes were 3' labeled with digoxigenin-11-ddUTP using a Genius 5 nonradioactive DNA labeling kit (Boehringer Mannheim, Indianapolis, Ind.). PCR products were transferred onto Hybond-N+ positively charged nylon membranes (Amersham Arlington, Heights, Ill.) using a PosiBlot pressure blotter (Stratagene, La Jolla, Calif.) for the Southern analysis. DNA was fixed to the nylon membranes by UV irradiation at 254 nm for 2 min using a CL-1000 ultraviolet crosslinker (UVP, San Gabriel, Calif.) Hybridization was perfromed at 55°C in presence of a labeled oligonucleotide internal probe (2 pmol/ml). The hybridized filters were washed twice with a high-salt solution (2 x SSC in 0.1% sodium dodecyl sulfate) at 50°C. A Genius 3 nucleic acid detection kit (Boehringer Mannheim) was used to prepare the hybridized filters for

chemiluminescent detection. The hybridization signals were visualized on X-OMAT film (Eastman Kodak, Rochester, N. Y.) using autoradiography (Sambrook et al.).

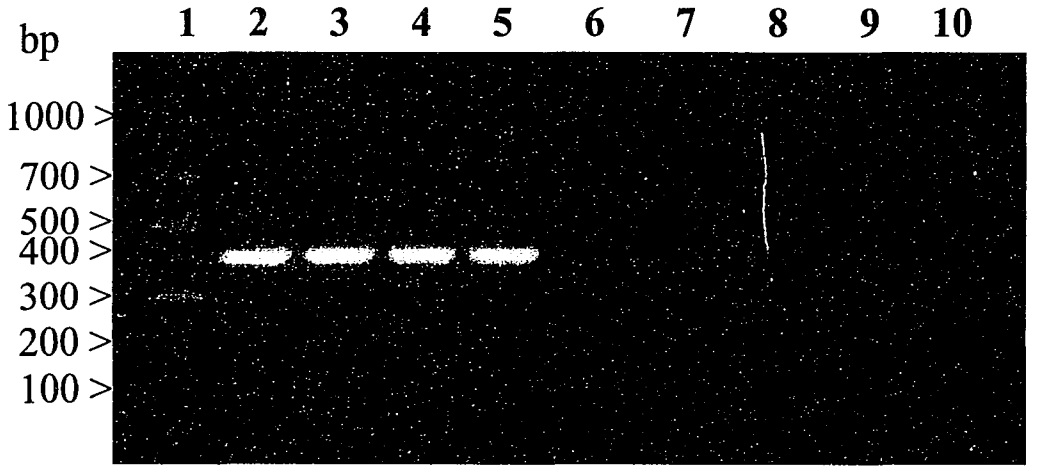
V. Results and Discussion

A. Specificfty of BFG Primers

In order for PCR to demonstrate a high degree of specificity, primers and target sequences must be conserved among BFG and not share similarities with other Gram-negative and Gram-positive bacteria. Primers BF410 and BF800 were initially tested to show they amplified the anticipated target sequence with all four members of the *Bacteroides fragilis* group (BFG) which includes *B. fragilis, B. vulgatus, B. thetaiotaomicron* and *B. distasonis* (Table 12). In order to determine the specificity of primers BF410 and BF800, 27 different enteric bacteria (e.g. *E. coli, Shigella* spp. and *Enterobacter* spp.) were utilized to test the specificity of these primers (Table 12). As an example, Figure 10 shows the PCR amplification results for the members of the *Bacteroides fragilis* group and other non-Bacteroides isolates. As seen in Table 12 and Figure 10 all four members of the *Bacteroides fragilis* group tested, generated a 410 bp PCR product while no products were detected for any of the non Bacteroides isolates. These results indicate that the primers BF410 and BF800 consistently generated a 410 bp product for all of the members of the *Bacteroides fragilis* group and the sequence targeted by these primers appears to be conserved for all of these members.

Table 12. Isolates Tested with Primer Set BF410 and BF800

Organism	Source	410 bp PCR Product
Bacteroides fragilis	ATCC 25285	+
Bacteroides vulgatus	ATCC 8482	+
Bacteroides thetaiotaomicron	ATCC 29741	+
Bacteroides distasonis	ATCC 8503	+
Salmonella typhimurium	ATCC 14028	-
Escherichia coli	ATCC 25922	-
Escherichia coli	ATCC 35401	-
Escherichia coli	ATCC 43886	-
Escherichia coli	ATCC 43889	-
Escherichia coli	ATCC 43890	-
Escherichia coli	ATCC 43894	-
Escherichia coli	ATCC 43895	-
Escherichia coli	ATCC 43896	-
Enterobacter aerogenes	ATCC 13048	-
Citrobacter freundii	ATCC 8090	-
Klebsiella pneumoniae	ATCC 13883	-
Shigella flexneri	ATCC 12022	-
Shigella sonnei	ATCC 25931	-
Bacillus cereus	ATCC 14579	-
Bacillus subtilis	ATCC 6051	-
Pseudomonas aeruginosa	ATCC 27853	-
Staphylococcus aureus	ATCC 25923	-
Streptococcus faecalis	ATCC 29212	-
Acinetobacter calcoaceticus	ATCC 19606	-
Serratia marcescens	ATCC 8100	-
Streptococcus pyogenes	ATCC 19615	-
Enterobacter cloacae	ATCC 23355	-
Proteus vulgaris	ATCC 13315	-
Staphylococcus epidermidis	ATCC 12228	-



Lane 1, marker; Lane 2, *Bacteroides fragilis*; Lane 3, *B. vulgatus;* Lane 4, *B. distasonis*; Lane 5, *B. thetaiotaomicron*; Lane 6, *E. coli*; Lane 7, *Salmonella typhimurium*; Lane 8, *Staphylococcus aureus*; Lane 9, *Proteus vulgaris*; Lane 10, *Bacillus cereus*.

Figure 10. Specificity of *Bacteroides* 16s RNA Primers

B. Sensitivity of PCR Primers in Serially Diluted Sewage

Since the levels of BFG in the human intestinal tract are at least 100 to 1000 fold higher than the levels of *E. coli* they should be found at higher level in sewage than *E. coli* (Post et al. 1967). This would imply that BFG gene sequences should also be higher in concentration than *E. coli* gene sequences. In this experiment primary-treated sewage was serially diluted with PBS. These dilutions were submitted to PCR amplification using primers BF410 and BF800 targeting BFG. In addition a set of *E. coli* specific primers were utilized to detect *E. coli* gene sequences and culturally viable *E. coli* were detected using mTEC agar. As seen in Figure 11, BFG PCR products could be detected by hybridization with an internal probe at a 10^{-6} dilution and *E. coli* could be detected at a 10^{-4} dilution. The concentration of *E. coli* in the sewage used for this experiment had a starting concentration 5.8×10^5 CFU/ml. Based on these results PCR detection of *E. coli* had a sensitivity of 58 CFU/ ml. These results are similar to studies by Tsai et al. (1993) that showed a similar sensitivity using these same primers to detect *E. coli* in seeded sludge (80 CFU/g of sludge). These results indicate that BFG gene sequences appear to be in higher concentrations than *E. coli* sequences in sewage and that the PCR test for BFG is a more sensitive test for the presence of sewage in a sample. These results

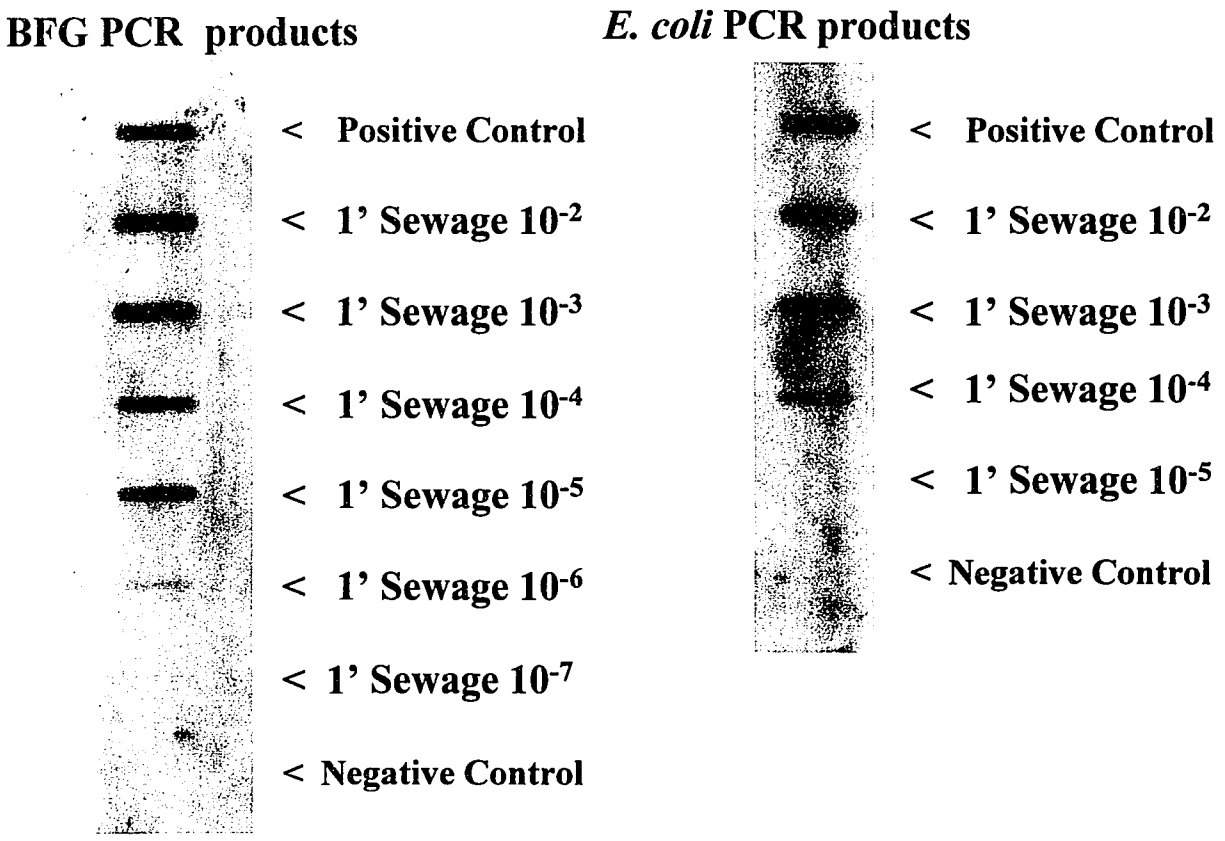


Figure 11. Senstivity of BFG primers and *E. coli* Primers in Serially Diluted Primary-Treated Sewage

are consistent with cultural data indicating that BFG levels are at least 100 to 1000 fold higher than *E. coli* in human feces (Allsop and Stickler, 1985). In summary, PCR detection of BFG appears to be a sensitive means of detecting low levels of sewage and is more sensitive than PCR detection of *E. coli*.

C. Survival of *Bacteroides thetaiotaomicron* in Ocean Water and PBS

As demonstrated by the serially diluted sewage experiment, PCR amplification using primers BF410 and BF800 is able to detect low levels of sewage in a sample. To be a useful marker of sewage, the survival and structural integrity of *Bacteroides* spp. in environmneatl waters should be known. To obtain this information an early stationary phase culture of *B. thetaiotaomicron* was seeded into ocean water and sterile phosphate buffer. Samples were collected at periodic intervals and submitted to cultural isolation. As seen in Table 13 there was a rapid decline in the number of cultural viable *B. thetaiotaomicron*. At time zero there was 1.4×10^6 CFU in ocean water and 2.6×10^6 CFU in sterile PBS. After four hour of exposure to these waters the number of culturally viable *B. thetaiotaomicron* in ocean water and PBS fell to 3.2×10^2 and 2.5×10^2, respectively. After 8 hour of exposure no *B. thetaiotaomicron* was detected by culture in ocean water or PBS. Thus, evidence was obtained that he anaerobic *B. thetaiotaomicron* can survive for 4 hrs but not 8 hrs in environmental waters.

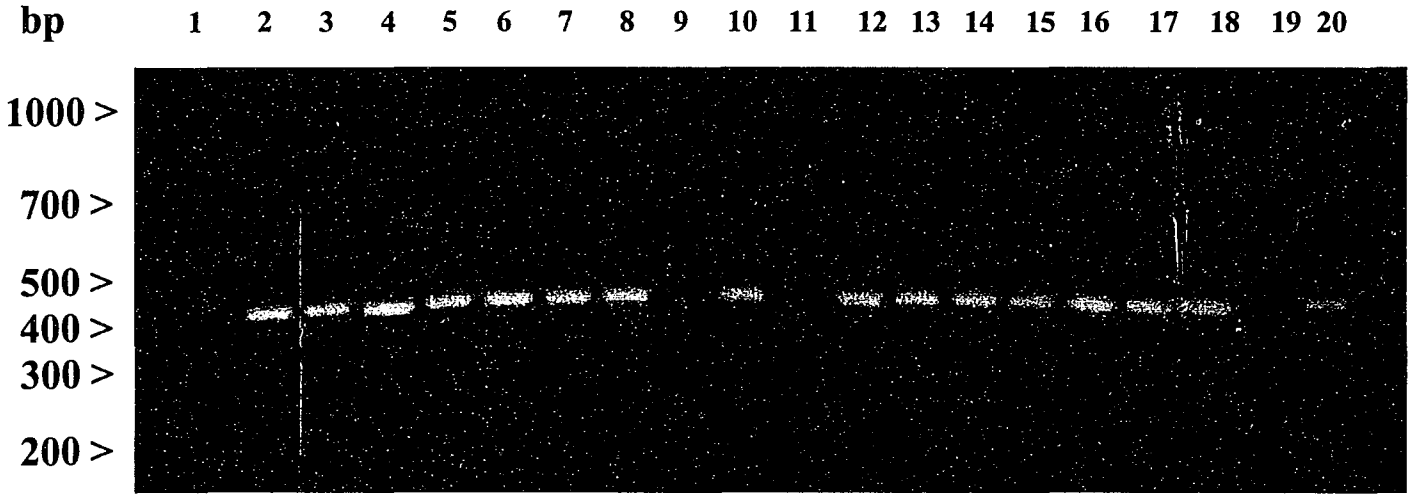
In order to determine persistence of BFG gene sequences in ocean water, samples were collected at regular intervals throughout the survival study and submitted to PCR detection targeting the 16s RNA gene of *Bacteroides fragilis*. This was accomplished by filtering samples through a 0.45 μm pore-size filter. DNA was extracted from these filters and submitted to PCR. As seen in Table 13 and Figure 12, PCR products were detected for the entire eight day exposure period. This would indicate that although cultural viablity was lost after 4 hrs of exposure, PCR-targeted gene sequences persisted for at least 7 days.

Table 13 Survival Study of *B. thetaiotaomicron*:
Plate Counts and PCR Products

	Ocean Water		Sterile PBS[a]	
Time	CFU/ml	PCR Product	CFU/ ml	PCR Product
0	1.4×10^6	+	2.6×10^6	+
4 hrs	3.2×10^2	+	2.5×10^2	+
8 hrs	< 1	+	< 1	+
22 hrs	< 1	+	< 1	+
48 hrs	ND[b]	+	ND[b]	+
72 hrs	ND[b]	+	ND[b]	+
4 days	ND[b]	+	ND[b]	+
5 days	ND[b]	+	ND[b]	+
6 days	ND[b]	+	ND[b]	+
7 days	ND[b]	+	ND[b]	+
8 days	ND[b]	+	ND[b]	+

[a] Phosphate buffered saline
[b] Not done

These results are consistant with previous research by Fiksdal et al. (1985) who demonstrated that *B. fragilis* cells remained intact after eight days of exposure



Lane 1, marker; Lane 2 ocean water time O; Lane 3, ocean water day 1; Lane 4, ocean water day 2; Lane 5, ocean water day 3; Lane 6, ocean water day 5; Lane 7, ocean water day 6; Lane 8, ocean water day 8; Lane 9, unseeded ocean water, Lane 10, positive control; Lane 11, marker; Lane 12 phosphate buffer time 0; Lane 13, phosphate buffer day 1; Lane 14, phosphate buffer day 2; Lane 15, phosphate buffer day 3; Lane 16, phosphate buffer day 5; Lane 17, phosphate buffer day 6; Lane 18 phosphate buffer day 8; Lane 19, unseeded phosphate buffer; Lane 20, positive control.

Figure 12. Persistence of *B. thetaiotaomicron* PCR-targeted Gene Sequences in Ocean Water and Phosphate Buffer

to environmental waters. These results indicate that although culture viability is lost after 4 hour of exposure to PBS and ocean water, gene sequences persist for at least 7 days. This would indicate that in ocean water PCR detection of BFG could serve as a marker for sewage for at least seven days. This is significant, since many pathogenic viruses and protozoans are capable of surviving for long periods of time in ocean water (Elliot and Colwell, 1985).

E. Detection of BFG and *E. coli* in Environmental Waters

In tropical climates, *E. coli* appears to be naturally occurring in tropical fresh waters and therefore may not be indicative of human wastes. Since the PCR method for BFG was shown to be a more sensitive test of sewage than *E. coli*, the presence or absence of BFG genes in environmental waters would indicate that these waters are or are not contaminated with sewage. To test this hypothesis, various environmental water samples were tested by PCR for *E. coli* and BFG gene sequences as well as for cultural concentration of *E. coli*. As seen in Table 14, *E. coli* was isolated from all 9 sources and ranged from 4.3×10^6 CFU/100ml in primary treated sewage to 12 CFU at Ala Moana beach Park. PCR-products for *uid* amplification were detected for 8 of the 9 samples (Figure 13). One sample, Ala Maona beach Park, did not show the presence of the *uid* gene sequence. This may be a reflection of the low numbers of *E. coli* (12 CFU/100ml) detected by culture in this sample. Since 50 ml of this sample was used for PCR there would be a theoretical 6 CFU which is close to the detection

limit (1-2 cells of *E. coli*) described by Bej. et al. (1991) using these primers. As seen in Table 14 and Figure 13, BFG gene sequences were detected in one (primary sewage) of the 9 samples tested. In the remaining eight samples no BFG PCR products were detected. In summary these findings are consistent with previous studies by Hardina and Fujioka (1988) who found high levels of *E. coli* in Manoa Stream in the absence of any known sewage contamination. These results indicate that PCR-based detection of BFG may be a more accurate means of detecting the presence or absence of fecal material in tropical waters.

Table 14. *E. coli* and BFG PCR Products Detected in Environmental Waters

Location	*E. coli* Culture CFU/100 ml	*uid* PCR Product	BFG PCR Product
1'Treated Sewage	4.3×10^6	+	+
Ala Wai Canal	2.1×10^3	+	-
Manoa Stream	2.8×10^3	+	-
Makiki Stream	8.2×10^2	+	-
Kaelepulu Stream	4.6×10^2	+	-
Kawainui Canal	6.8×10^2	+	-
Ala Moana Beach	12	-	-
Lower Manoa Stream	1.8×10^3	+	-

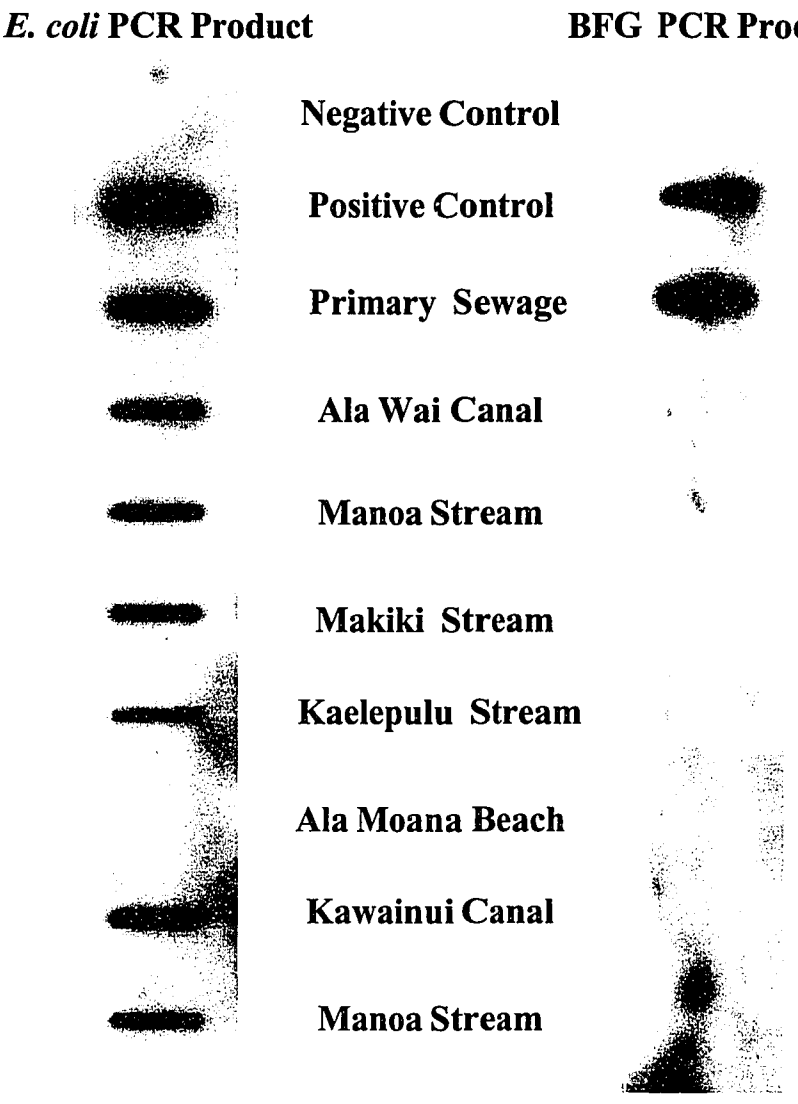


Figure 13. PCR Detection of *E. coli* and BFG in Environmental Waters

Chapter 6

Summary and Conclusions

I. Development of a PCR Detection System for *Salmonella* spp.

The cultural isolation of *Salmonella* spp. from environmental waters is a tedious and laborious endeavor often requiring days to render results. In this study the cultural isolation of *Salmonella* spp. took in excess of 5 days to render results. A new PCR-based detection system was developed which decreases the time for detection to less than one day. A set of PCR primers targeting a highly conserved genetic sequence common to the genus *Salmonella* was developed which did not cross react with a variety of other Gram-negative and Gram-positive bacteria. PCR amplification using primers, BRSALa and BRSALb, and confirmatory hybridization with an internal probe were able to detect as few as 12 CFU/ml in treated sewage. By using a second set of nested primers (SALIa and SAIIb) the sensitivity of this assay was increased to 1.4 CFU/ml in seeded ocean water experiments. The nested primer set allowed for an increase in sensitivity and avoided the time consuming process of confirming with an internal probe. This allowed for the detection of *Salmonella* spp. in less than one day. Field samples collected from sewage, ocean water, and fresh water were able to detect *Salmonella* specific sequences. Of the three samples collected showing *Salmonella*-specific sequences, two were confirmed by cultural isolation of *Salmonella* spp. These results demonstrate the

sensitivity and specificity for PCR detection of the waterborne pathogen *Salmonella*. In addition these methods are very rapid and can be performed in less than one day, greatly reducing the detection time needed by cultural methods. In the future this same technology may be applied to the detection of variety of other pathogens and can greatly reduce the time required to detect waterborne pathogens in a variety of environmental waters.

II. Survival of *E. coli* and *S. typhimurium* in Ocean Water

The purpose of this study was to use traditional culture method and the new PCR method to determine the fate (culturability, viability, and structural integrity) of indicator bacteria (*E. coli*) and the pathogen *Salmonella typhimurium* suspended in tropical marine waters. The viable concentration, structural integrity, and total cell concentrations of *E. coli* and *S. typhimurium* suspended in filtered and unfiltered sea water were followed over 18 days based on colony formation (CFU/ml) on a selective medium [mTEC agar for *E. coli* and TSA overlayed with brilliant green agar (TSAB) for *S. typhimurium*], nonselective medium (TSA), acridine orange total cell counts (AODC) and by PCR targeting *E coli* and *S. typhimurium* gene sequences.

In filtered ocean water during the first 6 days of exposure the number of culture viable *E. coli* and *S. typhimurium* recovered on selective media declined by four log units while the number of *E. coli* and *S. typhimurium* recovered on nonselective media declined by 3 log units. After six days of exposure to filtered

ocean water no culture-viable *E. coli* and *S. typhimurium* were recovered by selective media while low numbers (<103 CFU/ml) of *E. coli* and *S. typhimurium* persisted on nonselective media for the remaining 12 days of exposure. These results indicated an increase in the percentage of culture viable *E. coli and S. typhimurium* populations exhibiting sublethal stress as exposure times increased. At time zero 14.3 % of the *E. coli* population exhibited sublethal stress while 7.8 % of the *S. typhimurium* population exhibited sublethal stress. After six days of exposure to filtered ocean water the percentage of *E. coli* and *S. typhimurium* populations exhibiting sublethal stress increased to 98.4 % and 96.7 %, respectively. This evidence questions the ability of the medium mTEC to predict the presence of sublethally stressed *S. typhimurium* after prolong exposure (>6 days) to tropical marine waters. Further studies will need to be conducted in order to determine the infectious dose of sublethally stressed *S. typhimurium* needed to express disease in people. Previous research, however, has indicated that viable but nonculturable *Salmonella* spp. are capable of causing disease (Colwell et al, 1985) and this may indicate that sublethally stressed *Salmonella* spp. are also capable of causing disease.

In unfiltered ocean water survival experiments, there was a similar decline in the number of culture-viable *E. coli* and *S. typhimurium* recovered by selective media during the first 8 days of exposure. The number of culture-viable *E. coli* and *S. typhimurium* recovered on selective media declined by four log units during the first 6 days of exposure and no culture viable *E. coli* and *S. typhimurium* were recovered

after day 6. These results are very similar to filtered-ocean-water survival experiments and would indicate autochthonous microbiota did not have an impact on the recovery of culture-viable *E. coli* and *S. typhimurium* on selective media during the first 6 days of exposure.

PCR results, however, indicated the presence of autochthonous microbiota did have an impact on the recovery of structurally intact *E. coli* and *S. typhimurium* gene sequences after 8 days of exposure to unfiltered ocean water. In filtered ocean water PCR was able to detect *E. coli* and *S. typhimurium* gene sequences during the entire 18 day exposure period while in unfiltered ocean water no *E. coli* and *S. typhimurium* PCR products were detected after 16 and 14 days respectively. In addition, the PCR detection signals for *E. coli* and *S. typhimurium* appeared to diminish in intensity starting at day 12 and continuing until the loss of PCR signals. This would indicate a decline in the number of intact *E. coli* and *S. typhimurium* gene sequences that were available for PCR amplification. The loss of *E. coli* and *S. typhimurium* PCR products indicate that that structurally intact cells could not be detected after 16 and 14 days, respectively. These results indicate that the presence of autochthonous microbiota appear to influence the structural integrity of *E. coli* and *S. typhimurium* gene sequences after 8 days of exposure. In addition, *E. coli* gene sequences were able to persist at least two days longer than *S. typhimurium* gene sequences. This would support the use of PCR to detect the water quality indicator *E. coli*, since its gene sequences appears to persist longer than the pathogen *S. typhimurium* in ocean water.

III. Development of a PCR Detection System for BFG

Because of the ubiquitous nature of *E. coli* in tropical climates, alternative water quality indicators are needed. Previous research has proposed the use of the *Bacteroides fragilis* group as a water quality indicator. Unfortunately, this group does not survive for long periods of time in environmental waters and therefore cultural isolation is not an adequate means of detecting this group in environmental waters. In this study, a new PCR-based detection was developed which decreases the time for detection and does not require cultural isolation. A set of PCR primers targeting the 16s RNA gene of *B. fragilis* were chosen. These primers generated the anticipated 410 bp PCR product for all 4 members of the *Bacteroides fragilis* group and did not cross react with any of the 27 nonBacteroides isolates. In order to determine the sensitivity of these primers, primary treated sewage was diluted and submitted to PCR amplification using primers BF410 and BF800 and a set of primers targeting the *uid* gene of *E. coli*. BFG PCR products could be detected at a 10^{-6} dilution as compared to 10^{-4} dilution for *E. coli*. These results indicate that primers BF410 and BF800 are able to detect the presence of sewage at levels at least 100 fold lower than the *uid* primers. Samples collected from a variety of environments with no known sewage impact were positive for the presence of *E. coli* while negative for BFG sequences. This would indicate that PCR detection of BFG is a more accurate means of detecting the presence or absence of sewage in tropical waters.

IV. Speculations and Comments

This study was the first of its kind to utilize PCR to detect indicators and pathogens in Hawaii's recreational waters. The overall goal of the research was to develop and evaluate the usefulness of PCR-based methods to address the existing problems and needs in water quality. It would appear PCR is a powerful tool for detecting water quality indicators and pathogens, and has a promising future in water quality microbiology. Although these methods show great promise, a number of issues must be resolved before PCR can make the transition from a research tool to a water quality monitoring tool. These issues include; 1) recovery of PCR-targeted gene sequences from different environmental waters, 2) rapid and simple methods to detect PCR products, 3) methods to quantitate PCR products 4) detection of cellular viablity, and 4) the public health significance of detecting PCR-targeted gene sequences.

In this study PCR-targeted gene sequences were efficiently recovered from a variety of environmental waters. This involved the use of vortex flow filtration (VFF) and membrane filtration techniques to concentrated PCR-targeted gene sequences from environmental waters. These methods varied however, depending on the type of waters analyzed. Only 5 liters of sewage could be concentrated by vortex flow filtration while 20 liters of ocean water could be concentrated. This is a reflection of the particulate matter found in sewage which prohibited the concentration of larger volumes. Membrane filtration was capable of concentrating PCR-targeted gene sequences from small volumes of water (<100 ml) but these volumes varied

depending on the type of water tested. In order for this technology to find its way into water quality monitoring labs, new methods are needed which should be able to concentrate gene sequences from large volumes of water containing high particulate matter.

In this research, hybridization with an internal probe was needed to detect PCR products. Unfortunately, hybridization with an internal probe is a time consuming process often requiring 24 hours to complete. To decrease the time required for detection, nested PCR techniques were developed so hybridization with an internal probe could be avoided. Nested PCR, was able to attain a high degree of sensitivity while greatly reducing the time required to detect PCR products. However, nested PCR requires gel electrophoresis in order to detect PCR products. Gel electrophoresis requires some technical training as well as the purchase of expensive equipment. This is a limitation if this method is going to be used in water quality monitoring laboratories. New detection techniques need to be developed which do not require gel electrophoresis to detect PCR products. These techniques will need to be rapid and simple to perform.

Although PCR is capable of detecting as few as 1 CFU in environmental waters it cannot determine the number of pathogens in a given sample. In this research, culture methods were used in conjunction with PCR methods to gain an understanding of the sensitivity. Unfortunately without culture methods it is difficult

to know the number of cells in a sample. Methods need to be developed which enable quantitation of PCR products.

One of the major limitation of using PCR for water quality monitoring is its inability to distinguish between live and dead cells. This is a concern since only live cells are capable of causing disease. This problem may be solved by targeting mRNA which has a very short half life in the environment and may indicate recent cellular viablity. Unfortunately, to date, it has been very difficult to recover mRNA from environmental waters. New methods need to be developed which efficiently recover mRNA from environmental waters.

Ultimately we will need to determine the public health significance of using PCR to detect water quality indicators and pathogens. Previously, epidemiological studies have been utilized to demonstrate the relationship between the levels of indicator bacteria in recreational waters and the expression of illness among swimmers. Epidemiological studies need to be conducted using PCR techniques in order to establish the relationship between PCR detected indicators and the expression of illness among swimmers.

Appendix

Table 15. Plates Counts for *S. typhimurium* and *E. coli* in Filtered Seawater

Bacteria/medium	DAY (CFU/ml)							
	0	2	4	6	8	12	16	18
S. typhimurium (TSA)	1.3×10^6	7.0×10^5	6.4×10^4	1.2×10^3	2.3×10^2	4.1×10^2	$7.0\ 10^1$	3×10^1
S. typhimurium (TSAB)	1.2×10^6	4.8×10^5	8.3×10^3	4.0×10^1	$< 10^1$	$<10^1$	ND	ND
E. coli (TSA)	1.4×10^6	4.1×10^6	1.4×10^5	5.0×10^3	1.9×10^3	1.2×10^2	4.2×10^2	5.0×10^1
E. coli (mTec)	1.2×10^6	8.3×10^5	2.9×10^4	8.0×10^1	$< 10^1$	$< 10^1$	ND	ND

Tables 16. Plate Counts for *S. typhimurium* and *E. coli* in Unfiltered Seawater

	DAY (CFU/ml)							
Bacteria/medium	0	2	4	6	8	12	16	18
Salmonella (TSAB)	3.2×10^6	4.5×10^5	2.1×10^3	9.0×10^1	$< 10^1$	$< 10^1$	$<10^1$	ND[a]
E. coli (mTec)	1.4×10^6	8.0×10^5	2.5×10^3	2.0×10^1	$<10^1$	$< 10^1$	$< 10^1$	ND

Table 17. Acridine Orange Direct Counts for *E. coli* and *S. typhimurium*

Bacteria/ water type	AODC (cells/ml)				
	0	4	8	14	18
S. typhimurium (unfitered ocean water)	4.9×10^7	5.1×10^7	1.2×10^7	9.1×10^6	2.9×10^7
S. typhimurium (filtered ocean water)	1.3×10^7	2.2×10^7	3.4×10^6	4.3×10^3	1.2×10^4
E. coli (unfiltered oceean water)	1.3×10^7	2.2×10^7	9.8×10^6	2.5×10^7	1.5×10^7
E. coli (filtered ocean water)	1.5×10^7	3.2×10^6	1.3×10^7	2.3×10^4	9.4×10^3

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